Functional Analysis of Barley MLA-triggered Disease Resistance to the Powdery Mildew Pathogen

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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Köln, May 2004

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Tag der mündlichen Prüfung:	05. July 2004

Abbreviations

AD	activation domain
Adh	alcohol dehydrogenase
AGT	appressorial germ tube
APP	appressorium
ATP	adenosine 5-triphosphate
Avr	avirulence
BAC	bacteria artificial chromosome
BD	binding domain
Bgh	*Blumeria graminis* f sp *hordei*
bp	base pair
CA	carbonic anhydrase
CC	coiled-coil
CFU	colony forming unit
CHORD	cysteine- and histidine- rich domain
CS	CHORD and SGT1 motif
CSN	COP9 signalosome
CT	carboxy-terminal non-LRR region
cv.	cultivar
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytidinetriphosphate
DEPC	diethylpolycarbonate
dGTP	deoxyguanosinetriphosphate
DMF	dimethylformamide
DMSO	dimythysulfoxide
DNA	deoxyribonuleic acid
DNase	deoxyribonuclease

dNTP	deoxynucleosidetriphosphate
dsRNAi	double-stranded RNA interference
DTT	dithiothrietol
dTTP	dioxythimydinetriphosphate
ECL	enhanced chemiluminecence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
g	gram
Gal	galactose
GAL1	galactokinase
GUS	β-glucuronidase
h	hour
HR	hypersensitive response
HRP	horseradish peroxidase
HSP90	heat shock protein 90
IgG	Immunoglobulin gamma chain
IT	infection phenotype
kb	kilobase (s)
kDa	kilodalton (s)
LacZ	β-galactosidase
L	litre
LiAc	lithium acetate
LRR	leucine-rich repeat
min	Minute(s)
Mla	mildew-resistance locus A
mmol	millimolar
mRNA	messenger ribonucleic acid
NB	nucleotide binding site
ng	nanogram

ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pg	picogram
PGT	primary germ tube
pmol	picomolar
Raf	raffinose
Rar1	required for *Mla12* resistance
RGH	resistance gene homolog
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SAR	systemic acquired resistance
SCF	SKP1/CULLIN/F-box protein
SD	synthetic dropout (media)
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	second(s)
Sgt1	suppressor of G-two allele of skp1
SKP1	suppressor of kinetochore protein
TIR	*Drosophila* Toll and human interleukin-1 receptor
TPR	tetratricopeptide repeat
TRIS	Tris-(hydroxymethyl)-aminomethane
U	unit
O/N	over night
V	Volt
vir	virulence
VIGS	virus induced gene silencing
%(v/v)	volume-percent
%(w/v)	weight-percent

WT	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid

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1. General Introduction

1.1. Barley powdery mildew disease

1.1.1. The host plant

The host plant barley, *Hordeum vulgare* L. emend. Bowden, belongs to the grass family *Gramineae*, tribe *Hordeae* (von Bothmer and Jacobsen, 1985). Barley is grown in many parts of the world, mostly in temperate regions. It was suggested that the progenitors of barley originated from Asia in the Israel-Jordan area; the world centre for genetic diversity is Ethiopia (von Bothmer and Jacobsen, 1985; Badr et al., 2000; Salamini et al., 2002). Cultivated barley has either a winter or a spring growth habit (winter and spring barley, respectively). Barley is a diploid, self-pollinator with seven pairs of chromosomes and has been extensively studied both genetically and cytologically (Jogensen, 1994; Ramage, 1985). Like other crops, barley often suffers from various diseases. One common fungal pathogen is the powdery mildew disease whose spread is promoted by the relatively long vegetation period and cool and humid climate in the northern hemisphere. It is estimated that the powdery mildew disease can cause ~10% of yield losses in cooler climates like in Europe in the absence of fungicides.

1.1.2. The pathogen

Powdery mildew disease in grass species is caused by *Blumeria graminis* (= *Erysiphe graminis*). This fungus is strictly adapted into *formae speciales* colonizing individual genera of the grass family, e.g. *Blumeria graminis* f sp *hordei* (*Bgh*) is the causal agent of powdery mildew disease on barley. *Bgh* successfully reproduces on wild and cultivated species of

Hordeum but fails to colonize other closely related cereals, such as wheat, rye, or oats (Mathre,1982). The molecular basis of this narrow host range of *Bgh* is not understood. The ascomycete *Bgh* is an obligate biotrophic pathogen on barley, *i.e*. the fungus can reproduce only on living host tissue. During its life cycle, the haploid form prevails except for a short diploid phase after mating that includes the formation of cleistothecia and sexual reproduction leading to ascospore formation. The common form of asexual reproduction involves the formation of conidiophores that produce haploid spores, called conidiospores. These spores are dispersed by wind and will initiate a new infection cycle upon landing on a leaf blade or leaf sheath of neighbouring plants. Airborne spores can migrate hundreds of kilometres (Jørgensen, 1994; Thordal-Christensen et al., 2000).

Bgh attacks all aerial parts of the plant and infects only the epidermal cell layer. Growth of fungal mycelium on the leaf surface leads to the powdery appearance. The optimal temperature for development of *Bgh* is 20 ^{o}C.

1.1.3. Fungal development and disease symptoms

Under favourable conditions, the conidia will progress through a germination phase including the formation of a primary and appressorial germ tube (PGT and AGT, respectively). The AGT will swell at the end to form a mature appressorium (APP). An appressorial infection peg is produced beneath the APP to penetrate the plant cell wall and to make contact with the plasma membrane of a leaf epidermal cell. Successful penetration leads to the formation of a haustorium (a specialized feeding organ of *Bgh*) by invagination of the plant plasma membrane. Subsequently elongating secondary hyphae are formed on the leaf surface. Fungal growth at the leaf surface typically results in the formation of a single powdery mildew colony and eventually the formation of a new generation of conidia. The whole asexual life cycle takes approximately 5-6 days (Boyd et al, 1995; Thordal-Christensen et al., 2000). The *Bgh* haustorium is the sole fungal structure that

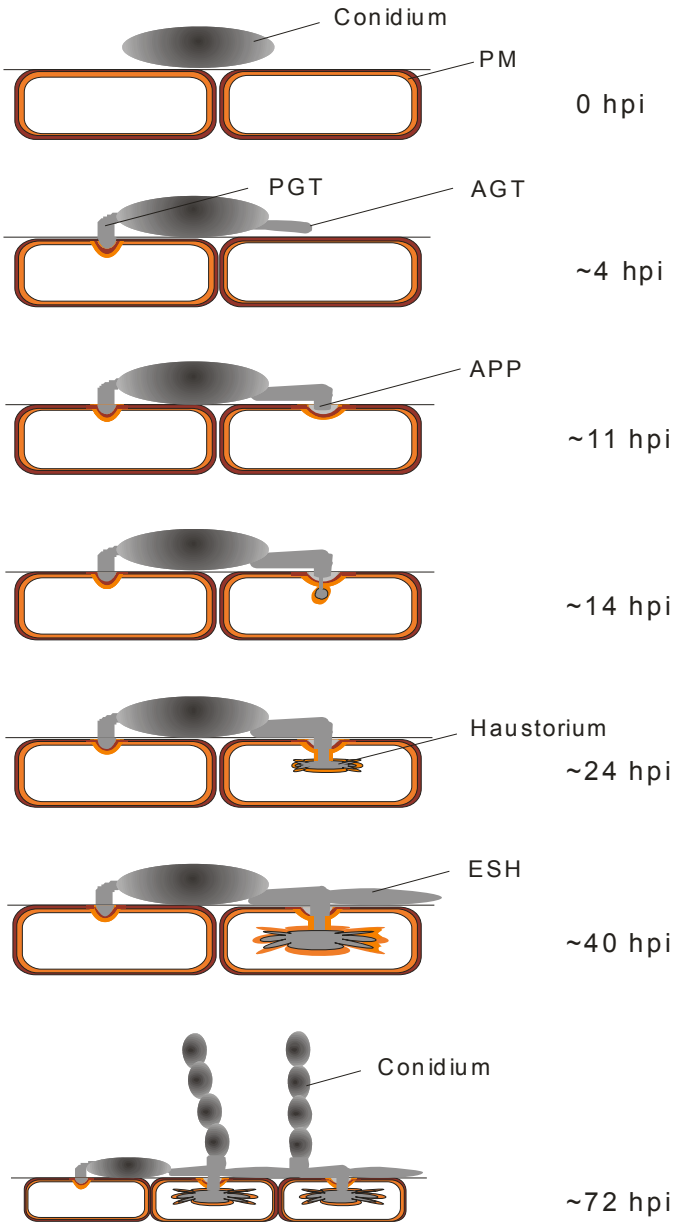

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Figure 1. Diagram of the development of *Blumeria graminis* f sp *hordei* on barley leaf epidermis.

AGT, appressorial germ tube; APP, appressorium; ESH, elongating secondary hyphae; hpi, hours post infection; PGT, primary germ tube; PM, plasma membrane; Modified from Body et al,1995; Thordal-Christensien et al, 2000.

is in intimate physical contact with a host membrane (called extra-haustorial membrane) and produces finger-like appendages when mature. The haustorium complex (haustorium, extra-haustorial membrane, and extra-hautorial matrix) is crucial for nutrient retrieval and mycelial growth on the leaf surface (Fig.1; Schulze-Lefert and Vogel, 2000).

In interactions that lead to disease (frequently called compatible interactions) or to immune responses (called incompatible interactions), it is not unusual to observe at interactions sites on the same leaf different stages of fungal development. However, the relative frequencies of the various developmental stages differ and this often allows macroscopic discrimination of five infection types (ITs), ranging from IT0, IT1, IT2 and IT3 (frequently seen in incompatible interactions) to IT4 (compatible interaction) (Boyd et al, 1995; Thordal-Christensen et al., 2000). IT0 denotes an immune response with no visible fungal growth and no immune response symptoms visible by the naked eye. IT1 to IT3 denote infection types with increasing amounts of fungal mycelium without sporulation and an increasing area of plant cells at infection sites that die as part of the resistance response ('necrotic flecks'). IT4 corresponds to profuse colony growth including sporulation and lack of recognizable immune responses at infection sites.

1.2. Plant race-specific resistance genes

Plants evolved different mechanisms to defend themselves against microbial pathogens. A widespread form of plant immunity is race-specific resistance that is governed by specific interactions involving gene pairs in plant and pathogen ('gene-for-gene' interaction; Flor, 1971). In these cases, a disease resistance response is triggered in the presence of matching pathogen *Avr* (avirulence) and plant disease resistance (*R*) genes (Flor, 1971). At the species level, natural polymorphisms at *R* and *Avr* loci make it possible to discriminate numerous plant lines and pathogen isolates, respectively. A loss or alteration of either *R* or *Avr* gene leads to disease. The

isolation of *R* and *Avr* genes has been critical for understanding the underlying molecular mechanisms of race-specific immunity in plants. Many *R* genes from monocots and dicots have been cloned during the last 10 years, encoding R proteins to bacterial, viral, fungal, oomycete, nematode and insect pathogens (Dangl and Jones, 2001; Hammomd-Kosack and Parker, 2003; Table 1 for an overview of isolated plant R genes). Products of known *Avr* genes encode highly diverse effector molecules that are released during pathogenesis (Bonas and Lahaye, 2002).

Deduced R proteins from a number of plant species to different pathogen classes (insects, fungi, bacteria, viruses, oomycetes, nematodes) revealed striking sequence similarities and a limited number of modular structural features. This strongly suggests the existence of common molecular recognition mechanisms in plants to microbial pathogens. Most *R* genes encode proteins containing variable numbers of sequence-diversified Leucine-rich repeats (LRRs), a protein domain that is known to participate in protein-protein interactions (Jones and Jones, 1996; Kobe and Deisenhofer, 1995; Kajava, 1998). LRR containing R proteins can be broadly divided into two classes, one with intracellular and the other with extracellular LRRs (eLRRs; see below). The largest class of known *R* genes encodes predicted intracellular proteins. These share a central nucleotide-binding (NB) site and C-terminal LRRs. The NB site includes kinase 1a (also called P-loop), kinase 2 and 3a motifs (Traut, 1994) and is part of an extended domain, designated NB-ARC, which includes additional sequence motifs present in animal cell death effectors such as APAF-1 and CED4 (NB-ARC is an acronym for a nucleotide-binding adaptor shared by APAF-1, most known intracellular NB-LRR plant R proteins, and CED-4; van der Biezen & Jones, 1998; Dangl and Jones, 2001). Members of this class can be further divided in two subclasses containing either N-terminal sequences predicted to form a coiled-coil (CC) structure (CC-NB-LRR subfamily) or sequences that are related to the cytoplasmic domain of the *Drosophila* Toll and human interleukin-1 receptor

Table1. The major classes of isolated plant resistance genes[a]

Class	R gene	Plant	Pathogen (*Avr* gene or product)	Predicted structure of R protein	Race-specific	References
1-a	*RPS2*	Arabidopsis	*P. syringae* p.v. *tomato(avrRpt2)*	CC-NB-LRR	Yes	Bent et al., 1994
	RPS5	Arabidopsis	*P. syringae* p.v. *tomato (avrPphB)*	CC-NB-LRR	Yes	Warren et al., 1998
	RPM1	Arabidopsis	*P. syringae* p.v. *maculicula (avrRpm1;avrB)*	CC-NB-LRR	Yes	Grant et al., 1995
	RPP8	Arabidopsis	*Peronospora parasitica (avrRpp8)*	CC-NB-LRR	Yes	McDowell et al., 1998
	HRT	Arabidopsis	Turnip crinkle virus (Coat protein)	CC-NB-LRR	Yes	Cooley et al., 2000
	Prf	Tomato	*P. syringae* p.v. *tomato (avrPto)*	CC-NB-LRR	Yes	Salmeron et al., 1994
	Mi-1	Tomato	*Meloidogyne incognita* (*?* nematode);	CC-NB-LRR	Yes	Milligan et al., 1998
			Marcosiphum euphorbiae (*?* aphid)	CC-NB-LRR	Yes	Rossi et al., 1998
	I2	Tomato	*Fusarium oxysporum*	CC-NB-LRR	Yes	Simons et al., 1998
	Rx1	Potato	Potato virus X (Coat protein)	CC-NB-LRR	Yes	Bendahmane et al., 1999
	Rx2	Potato	Potato virux X (Coat protein)	CC-NB-LRR	Yes	Bendahmane et al., 2000
	Gpa2	Potato	*Globodera pallida*	CC-NB-LRR	Yes	Van der Voort et al., 1999
	R1	Potato	*Phytophthora infestans* (race1)	CC-NB-LRR	Yes	Ballvora et al., 2002
	Dm3	Lettuce	*Bremia lactuca*	CC-NB-LRR	Yes	Meyers et al., 1998
	Bs2	Pepper	*X. campestris* p.v. *vesicatoria (avrBs2)*	CC-NB-LRR	Yes	Tai et al., 1999
	Xa1	Rice	*X. oryzae* p.v. *oryzae*	CC-NB-LRR	Yes	Yoshimura et al., 1998
	Pib	Rice	*Magnaporthe grisea*	CC-NB-LRR	Yes	Wang et al., 1999
	Pi-ta	Rice	*Magnaporthe grisea (avrPita)*	CC-NB-LRD	Yes	Bryan et al., 2000
	Cre3	Wheat	*Heterodera avenae*	CC-NB-LRR	Yes	Lagudah et al., 1997
	Rp1-D	Maize	*Puccinia sorghi*	CC-NB-LRR	Yes	Collins et al., 1999
	Mla1	Barley	*Blumeria graminis* f.sp. *hordei (avrMla1)*	CC-NB-LRR	Yes	Zhou et al., 2001
	Mla6	Barley	*Blumeria graminis* f.sp. *hordei (avrMla6)*	CC-NB-LRR	Yes	Halterman et al., 2001

1-b	*N*	Tobacco	Mosaic virus (Replicase)	TIR-NB-LRR	Yes	Whitham et al., 1996
	RPS4	Arabidopsis	*P. syringae* p.v. *tomato (avrRps4)*	TIR-NB-LRR	Yes	Gassmann et al., 1999
	RPP1,10,14	Arabidopsis	*Peronospora parasitica*	TIR-NB-LRR	Yes	Botella et al., 1998
	RPP4,5	Arabidopsis	*Peronospora parasitica*	TIR-NB-LRR	Yes	Van der Biezen et al.,2002
	L6, L1-12	Flax	*Melampsora lini (AL6)*	TIR-NB-LRR	Yes	Lawrence et al., 1995
	M	Flax	*Melampsora lini (AM)*	TIR-NB-LRR	Yes	Anderson et al., 1997
2	*RRS-1*	Arabidopsis	*Ralstonia solanacearum* (race1)	TIR-NB-LRR-NLS-WRKY	Yes	Deslandes et al., 2002
3	*Cf-9*	Tomato	*Cladosporium fulvum (avr9)*	eLRR-TM-sCT	Yes	Jones et al., 1994
	Cf-4	Tomato	*Cladosporium fulvum (avr4)*	eLRR-TM-sCT	Yes	Thomas et al., 1997
	Cf-2	Tomato	*Cladosporium fulvum (avr2)*	eLRR-TM-sCT	Yes	Dixon et al., 1996
	Cf-5	Tomato	*Cladosporium fulvum (avr5)*	eLRR-TM-sCT	Yes	Dixon et al., 1998
	Hcr9-4E	Tomato	*Cladosporium fulvum (avr4E)*	eLRR-TM-sCT	Yes	Takken et al., 1999
	Hs1^{pro-1}	Sugar beet	*Heterodera schachtii*	eLRR-TM-sCT	Yes	Cai et al., 1997
4	*Ve1*	Tomato	*Verticillium albo-atrum*	CC-eLRR-TM-ECS	Yes	Kawchuk et al., 2001
	Ve2	Tomato	*Verticillium albo-atrum*	eLRR-TM-PEST-ECS	Yes	Kawchuk et al., 2001
5	*Xa21*	Rice	*X. oryzae* p.v. *oryzae* (all races)	eLRR-TM-kinase	Yes	Song et al., 1995
	FLS2	Arabidopsis	Multiple bacteria (flagellin)	eLRR-TM-kinase	No	Gómez-Gómez et al., 2000
6	*Hm1*	Maize	*Cochliobolus carbonum* (race1)	Detoxifying enzyme HC toxin reductase	Yes	Johal and Briggs, 1992

7	*mlo*	Barley	*Blumeria graminis* f.sp. *hordei*	7 TM protein	No	Bueschges et al., 1997
8	*Rpg1*	Barley	*Puccinia graminis* f.sp. *tritici*	Receptor kinase-like protein with 2 tandem kinase domains	No	Brueggeman et al., 2002
9	*RPW8.1* *RPW8.2*	Arabidopsis	Multiple powdery mildew species	Small, probable membrane protein with CC domain	No	Xiao et al., 2001

[a] Compiled from van't Slot KAE and Knogge W, 2002; Hammond-Kosack and Parker, 2003;
CC, Coil-Coiled domain; ECS, endocytosis signal; LRD, leucine-rich domain; LRR, Leucine-rich repeat; PEST, Pro-Glu-Ser-Thr; sCT, single cytoplasmic tail; TIR, *Drosophila* Toll and the mammalian interleukin-1 receptor; TM, transmembrane

(TIR-NB-LRR subfamily). Most NB-LRR type R proteins consist of these protein modules except few containing additional domains. For example, *Arabidopsis RRS1-R*, confers resistance against the bacterium *Ralstonia solanacearum* and possesses an additional C-terminal WRKY domain (Deslandes et al., 2002). Some NB-LRR R proteins contain an additional C-terminal non-LRR region (CT region) lacking homology to known protein domains (Dodds et al, 2001; Shen et al, 2003).

A second eLRR containing R protein class is membrane-anchored by a single transmembrane helix. Structural variations are also found within members of this class. For example, the rice *Xa21* product has an additional intracellular Ser/Thr kinase module, whereas the tomato *Cf* gene products lack any significant intracellular domains (reviewed by Ellis et al., 2000). Two more recently isolated *R* genes from tomato, *Ve1* and *Ve2*, encode eLRR type proteins with a cytoplasmic domain possessing sequences that in mammalian receptors stimulate their endocytosis and degradation (the ECS domain; Kawchuk et al., 2001).

The modular structural organization of plant R proteins might be significant with regard to distinct functions possibly fulfilled by an individual domain as well as co-operations among different domains. In plants, the LRR domain of membrane-anchored Cf proteins has been shown to have a role in recognition specificity (Van der Hoorn et al., 2001; Wulff et al., 2001). This was shown by domain swap experiments between sequence-related Cf proteins recognizing different pathogen-derived effectors. Similar data have been reported so far only for intracellular TIR-NB-LRR proteins encoded by alleles of the flax rust *R* locus *L* and were shown for the first time in this work for a CC-NB-LRR-CT type R protein (Ellis et al., 1999; Luck et al., 2000; Shen et al., 2003).

Unlike highly variable LRR sequences, the NB-ARC domain is largely conserved among NB-LRR type R proteins. Recently, biochemical analysis

and site directed mutagenesis of residues implicated in nucleotide binding/hydrolysis (kinase1a, 2 and 3a motifs) of the NB-ARC domain of tomato R proteins I-2 and Mi-2 provided experimental evidence for ATP binding but not of other nucleotide triphosphates (Tameling et al, 2002). Thin layer chromatography revealed that both I-2 and Mi-1 exerted ATPase activity, suggesting that the NB-ARC domain is a functional nucleotide binding pocket capable of binding and hydrolyzing ATP (Tameling et al., 2002). Because most characterized R-triggered plant immune responses are tightly linked to a localized cell death response at sites of attempted pathogen infection (frequently termed hypersensitive response; HR) and NB-ARC adaptor containing *Caenorhabditis elegans* CED-4 and its human homolog APAF-1 mediate programmed cell death during development (apoptosis), it has been hypothesized that the NB-ARC domain in plants may have similar functions in death signalling as in animals (van der Biezen and Jones, 1998a).

Interestingly, separate expression of the CC–NB and LRR parts of the potato R protein Rx to potato virus X (PVX) resulted in intramolecular physical interactions *in planta* (as did the CC domain with the NB–LRR part) and both interactions were disrupted in the presence of the PVX effector (Moffett et al., 2002). However, the interaction between the CC and NB–LRR parts was dependent on a wild-type P-loop motif in the NB-ARC domain, whereas the interaction between CC–NB and LRR was not (Moffett et al., 2002). It was concluded that activation of Rx involves sequential disruption of at least two intramolecular interactions (Moffett et al, 2002). In analogy to APAF-1 function, it has been hypothesized that the activation of R proteins may involve Avr-dependent release of the NB-ARC domain from inhibition by the C-terminal LRRs , followed by multimerization of a complex that recruits additional proteins to the amino-terminal domain for further signalling events (Dangl and Jones, 2001). It is conceivable that the different N-terminal structures of cytoplasmic NB-LRR protein, TIR or CC domains, respectively links to one of at least two distinct signalling pathways specified by different

components (Aarts et al, 1998).

1.3. The *Mla* locus and *Mla* resistance genes

1.3.1. Molecular characterization of the *Mla* locus and *Mla* genes

In barley, *R* genes to *Bgh* have been mapped to 10 loci: ***Mla, Mlat, MlGa, Mlk, Mlnn, Mlra, Mlp*** on chromosome 5 (1H; barley chromosome 5 is also denoted as 1H according to its homoeologous relationships with chromosomes of other *Triticeae* species; Barley Genetics Newsletter V27); ***Mlg*** on chromosome 4 (4H); ***MlLa*** on chromosome 2 (2H) and ***Mlh*** on chromosome 6 (6H). Out of a total of approximately 85 identified resistance specificities (Jørgensen 1994; Görg et al., 1993; Büschges et al., 1997; Giese et al., 1993), approximately 30 are encoded at the *Mla* locus on the short arm of chromosome 5. These resistance specificities have been defined using a large set of differential barley accessions and powdery mildew isolates that produce gene-for-gene type interactions (Giese, 1981; Giese et al 1981; Wise and Ellingboe, 1983, 1985; Jahoor and Fischbeck, 1993; Jorgensen 1994). Most of the *Mla* resistance specificities have been introduced into barley cultivars from the wild relative *Hordeum spontaneum*. This suggests that much of the recent coevolution between barley and *Bgh* was concentrated at a single *R* locus, *Mla*, in the host. Due to its highly polymorphic nature, the *Mla* locus is an excellent model to study 'gene-for-gene' specific recognition events of effectors encoded by a biotrophic fungal pathogen.

Various molecular marker techniques were used to genetically map the complex *Mla* locus. The locus was genetically and physically delimited within an interval of approximately 240 kb on chromosome 5 ({Wei, 1999 #27}). DNA markers tightly linked to *Mla* were used to identify BAC contigs from cultivar Morex spanning the *Mla* cluster. A contiguous DNA sequence of the interval in Morex revealed 32 predicted genes of which eight encode CC-NB-LRR resistance gene homologs (*RGHs*; {Wei, 2002 #45}). The *RGHs* belong to

three dissimilar families sharing less than 43% amino acid sequence similarity between families (Wei et al., 1999; 2002). Since Morex lacks a known *Mla* resistance specificity, the first two identified *Mla* powdery mildew *R* genes, *Mla1* and *Mla6*, were isolated from other barley accessions (Halterman et al., 2001; Zhou et al., 2001). The deduced proteins share 91% identical residues and show each highest overall similarity to the deduced Morex *RGH1bcd* family member (83% and 79% identity to MLA1 and MLA6, respectively) (Halterman et al., 2001; Wei et al., 2002). Recently two further specificities, *Mla12* and *Mla13*, have also been isolated (Halterman et al., 2003; part of the present work). All *Mla R* specificities isolated to date share a common exon/intron structure and encode CC-NB-LRR type proteins that possess an extra C-terminal non-LRR (CT) region (CC-NB-LRR-CT structure). The extent of sequence similarity between deduced MLA R proteins is remarkable: ~97% sequence identity in the CC-NB domains and ~87% in the LRR-CT region (Halterman et al., 2003; Shen et al, 2003).

1.3.2. *Mla*-mediated infection types

A common feature of most but not all characterized *R* gene-mediated resistance responses is a rapid and localized host cell death (HR) at attempted infection sites that is thought to shut off nutrient supply to microbial pathogens (Shirasu and Schulze-Lefert, 2000). Although MLA R proteins to *Bgh* are highly sequence-related, immune responses triggered by different *Mla R* specificities result in diverse infection phenotypes (Boyd et al., 1995). This was shown by quantitatively assessing *Bgh* growth stages and the timing of HR onset at single interaction sites in a set of near-isogenic barley lines containing different *Mla R* specificities. To exclude genetic background variation of different *Bgh* isolates, Boyd et al. used a single isolate expressing multiple *AvrMla* genes. *Mla1* and *Mla6* resistance terminates fungal growth at an early stage (essentially no secondary hyphae formation on the leaf surface) and triggers a rapid HR that is mainly confined to attacked leaf epidermal cells. In contrast, *Mla3* and *Mla7* mediate growth cessation at a

later developmental stage, permitting growth of some elongating hyphae. This is linked with a delayed onset of HR including both epidermal and subtending mesophyll cells (Boyd et al., 1995). Consistent with a rapid *Mla1*-mediated resistance response, Koga et al. reported fungal growth cessation coincident with haustorium maturation and onset of an epidermal HR within 24 hours after spore inoculation (Koga et al., 1990). Race-specific immunity triggered by an barley *R* gene at another *R* locus to *Bgh*, *Mlg*, was shown to terminate *Bgh* growth even earlier, i.e. concomitant with the process of cell wall penetration before onset of haustorium differentiation (Görg et al., 1993). Many factors could contribute to the phenotypic variation of *R* gene-triggered resistance responses to *Bgh* (see discussion). *Mlg* gene dosage experiments (*Mlg Mlg*, *Mlg mlg*, *mlg mlg* genotypes) in a near-isogenic background as well as greatly different infection phenotypes reported in homozygous and heterozygous *Mla12* lines (Görg et al., 1993; Torp and Jørgensen, 1986) suggest that R protein levels could be rate-limiting for the onset and/or speed of resistance responses (if *R* gene dosage is directly linked to R protein levels).

1.4. *Rar1* and *Sgt1*--- two genes required for disease resistance

1.4.1. The *Rar1* gene

A mutant screening of suppressors of *Mla12* function identified barley *Rar1* (Required for *Mla12* resistance-1) (Torp and Jørgensen, 1986; Jørgensen, 1996). The susceptible *rar1* mutants are unable to mount an HR response and also show a significant reduction in the incidence of whole-cell H_2O_2 accumulation (Freialdenhoven et al, 1994; Shirasu et al, 1999). Genetic studies have shown that wild-type *Rar1* is required for many, but not all, *Mla R* specificities to *Bgh* (Jørgensen, 1996). In addition, several powdery mildew *R* loci on other barley chromosomes require *Rar1* for efficient resistance (Jørgensen, 1988; Freialdenhoven et al, 1994). Similar mutational studies or virus induced gene silencing (VIGS) experiments revealed that *Rar1* homologues in *Arabidopsis* and *Nicotiana benthamiana* play a conserved role

in the function of a subset of NB-LRR R proteins that confer resistance to different pathogens, e.g. oomycete, bacteria, fungus and virus (Liu et al., 2002a; Muskett et al., 2002; Tornero et al., 2002). This revealed that RAR1 activity is essential for the function of both structural R subtypes, TIR–NB–LRR and CC–NB–LRR proteins.

Barley *Rar1* gene was isolated by a map-based cloning approach (Freialdenhoven et al, 1994; Lahaye, 1998a,b; Shirasu,et al 1999a). The deduced intracellular 25.5-kD RAR1 protein contains a pair of tandemly duplicated 60 amino acid sequence-related domains, designated CHORD-I and CHORD-II (cysteine- and histidine-rich domains), each possibly adopting a novel zinc-finger structure (Shirasu, et al., 1999). DNA sequence data of *RAR1* homologues and systematic database searches revealed examples with similar arrangement of CHORD domains from a broad range of phyla in addition to plant species, except in yeast (reviewed in Shirasu and Schulze-Lefert, 2003). Although the two CHORD domains show overall sequence similarity, distinctive sequence features of each domain are conserved across proteins from different species, suggesting non-identical functions performed by each CHORD (reviewed in Collins et al, 2003). In plants, an extra stretch of ~20 highly conserved amino acids, termed the CCCH motif, is located between the CHORD domains, while metazoan RAR1 homologs contain an extra C-terminal extension adjacent to CHORD-II, designated the CS motif (CHORD and SGT1 motif).

1.4.2. The *Sgt1* gene

Kitagawa et al. (1999) originally identified SGT1 as essential component for cell cycle progression at G1/S and G2/M transitions in yeast. Isolation and characterization of temperature sensitive mutant *sgt1* alleles revealed that yeast SGT1 physically associates with SKP1 in at least two complexes: the CBF3 (centromere binding factor 3) kinetochore complex and the SCF (SKP1/CULLIN/F-box protein) ubiquitin ligase complex (Kitagawa et

al., 1999). SCF complexes play a broad role in regulating the stability/activity of many proteins in diverse physiological processes, recruit specific substrates and catalyze their ubiquitination, thereby often marking the substrates for degradation by the proteasome (Hochstrasser, 2000). The sgt1-3 mutant protein abolishes the interaction with SKP1 and leads to compromised CBF3 complex assembly, while ubiquitination of SCF target proteins remained unaltered in this yeast mutant (Kitagawa et al 1999). In contrast, the sgt1-5 mutant protein leads to compromised SCF function but retains its ability to interact with SKP1 and retains also CBF3 function. This strongly suggests allele-specific perturbations of distinct SGT1 functions and indicates that the physical association between SGT1 and SKP1 is not critical for SCF activity.

Yeast two-hybrid screenings for interacting partners of *Arabidopsis* RAR1 identified two proteins with significant sequence similarity to yeast SGT1, designated as *AtSgt1a* and *AtSgt1b* (Azevedo et al, 2002). This protein-protein interaction is conserved since both AtSGT1a and AtSGT1b were found to interact also with barley RAR1. All known SGT1 proteins in species from different phyla contain the CS motif that metazoan RAR1 homologs also possess at the C-terminal end (Shirasu et al., 1999; Kitagawa et al., 1999). This finding is indicative of an ancient CS domain fusion event. Such fusion events often indicate a functional link between two proteins mediated by direct protein-protein interactions (Rosetta stone hypothesis; Marcotte et al*.,* 1999). Co-immunoprecipitation experiments, using barley leaf protein extracts from non-inoculated plants, corroborated a physical interaction between SGT1 and RAR1. Furthermore, dsRNAi gene silencing of *Sgt1* showed that *Mla6* but not *Mla1* requires *Sgt1* for full resistance to *Bgh* and co-silencing of *SGT1* and *RAR1* resulted in an additive level of susceptibility, again indicative of co-operation between these proteins in *Mla* gene-mediated resistance (Azevedo et al, 2002). These dsRNAi gene

silencing experiments in barley provided genetic evidence for a critical role of barley *Sgt1* as novel factor in *Mla*-mediated race-specific resistance.

In *Arabidopsis*, *sgt1b* mutants were identified in a forward genetic screen for plants defective in resistance mediated by the *R* gene, *RPP5*, to an isolate of the oomycete pathogen *Peronospora parasitica* (Austin et al., 2002). *Sgt1b* mutants, like *Rar1* mutants, exhibit significantly disabled HR and reduced whole cell H_2O_2 accumulation at most infection sites, allowing efficient colonisation of *P. parasitica*. Interestingly, although a delayed plant cell death response was observed in both *sgt1b* and *rar1* single mutants followed by appearance of necrotic plant cells trailing the pathogen at later stages of infection (trailing necrosis), the double *sgt1b*/*rar1* mutant has additive disease susceptibility and no plant cell death response was observed. The conclusion from these data is that *SGT1b* and *RAR1* co-operate in *RPP5*-mediated resistance, consistent with the results obtained from experiments in barley (Azevedo et al, 2002; Austin et al., 2002; reviewed in Muskett and Parker, 2003).

More genetic evidence supports a more general role of *Sgt1* in *R*-gene triggered disease resistance in plants. Using a virus-induced gene silencing approach in *Nicotiana benthamiana*, silencing of the two copies of *SGT1* in this plant compromised the functions of *Rx* conferring resistance to potato virus X (PVX) and tobacco N to the tobacco mosaic virus (TMV) (Peart et al, 2002; Liu et al., 2002b). Interestingly, *SGT1* was also found involved in non-host resistances against certain types of pathogens (Peart et al, 2002). Non-host resistance is a class of disease resistance in plant species that are outside the host range of a pathogen species (Heath 2000). Taken together, *SGT1* serves critical roles in *R*-gene mediated disease resistance to different pathogen classes as well as in certain non-host responses; like *RAR1*, *SGT1* is required for resistance triggered by R proteins from both TIR-NB-LRR and

CC-NB-LRR subclasses, indicating its common role in plant disease resistance.

1.5. Current models of pathogen recognition in plant disease resistance

1.5.1. Direct physical R protein-AVR effector interaction

A commonly accepted theory regarding pathogen-host plant interactions is the “gene-for-gene” hypothesis, put forward by Flor more than 50 years ago when he worked with flax and the flax rust fungus (Flor, 1971). Central to this theory is that disease resistance in plants commonly requires two complementary genes: an avirulence (*Avr*) gene in the pathogen and a matching, resistance (*R*) gene in the host. One out of several possible biochemical interpretations of this hypothesis is a receptor-ligand model in which plants activate defence mechanisms upon R-protein-mediated recognition of pathogen-derived Avr products (Hammond-Kosack and Jones, 1997). Most plant R proteins contain either an extra- or intracellular LRR domain that is thought to participate in protein-protein interactions (Kobe and Deisenhofer, 1994; Kajava 1998). Importantly, sequence comparisons of both NB-LRR or membrane-anchored type R proteins shows that the predicted solvent-exposed residues in the LRRs are hypervariable and subject to diversifying selection (Botella et al., 1998; McDowell et al., 1998; Meyers et al., 1998; Halterman et al., 2001). This is interpreted as evidence that R proteins have the capacity to directly recognize pathogen effectors. However, extensive studies carried out for many *Avr-R* gene pairs, has shown only two examples supporting such a direct interaction (the rice blast resistance protein *Pi-ta* and *Avr-Pita* from *Magnaporthe grisea*, and *RRS1-R/PopP2* of *Arabidopsis* and *Ralstonia solanacearum* (Jia et al., 2000; Deslandes et al., 2003). Thus, it seems possible that at least some R proteins mediate indirect pathogen recognition by a process involving additional host proteins.

1.5.2. The Guard Hypothesis

The 'guard hypothesis' postulates that R proteins function in the surveillance of a host protein or a complex (the ‘guardee’) that is targeted by *AVR* products for modifications favoring pathogen growth. Detection of the modifications by the R protein triggers the resistance response (Dangl and Jones, 2001). Initial evidence for this model was found in disease resistance triggered in tomato plants to the tomato speck pathogen *P. syringae* containing *AvrPto*. The resistance response was shown to require two host proteins, the NB-LRR protein *Prf* and the *Pto* protein kinase; while *Pto* was found to interact physically with *AvrPto* (Scofield et al., 1996; Tang et al., 1996) *Prf* does not. *Pto* is considered to be the virulence target of *AvrPto*, which is guarded by the R protein, *Prf* (Van der Biezen and J.D.G. Jones, 1998b).

More evidence is emerging to support the indirect recognition model. The study of *Arabidopsis-Pseudomonas* interactions identified RIN4 (RPM1-interacting protein) as a common ‘guardee’ targeted by two sequence unrelated effectors, AvrRpm1 or AvrB (Mackey et al, 2002). RIN4 was first identified in yeast two-hybrid screens to interact with AvrB, and was subsequently found to interact also with the NB-LRR type protein RPM1 conferring resistance against *Pseudomonas syringae* expressing AvrRPM1 and AvrB. RIN4 was shown to co-immunoprecipitate with AvrB, AvrRpm1, and the NB-LRR protein RPM1 *in vivo*. RIN4 is essential for RPM1-dependent defences, as the reduction of RIN4 protein levels inhibits the restriction of pathogen growth and the HR in response to bacteria that express *AvrRpm1* or A*vrB*. Phosphorylation of RIN4 was induced by AvrRpm1 and AvrB, independent of the presence of RPM1. It was proposed that RIN4 positively regulates *RPM1*-mediated resistance.

More evidence for the role of RIN4 as a guardee of another NB-LRR protein, RPS2, was recently described (Mackey et al., 2003; Axtell and Staskawicz, 2003). In the same pathosystem another R-Avr gene pair

(*AvrRpt2*-*RPS2*) was used to explore the relationships involving RIN4. RPS2 was shown to physically interact with RIN4. Furthermore, it was found that AvrRpt2 induces RIN4 disappearance. Over-expression of *RIN4* blocks the detection of AvrRpt2 by RPS2, while loss-of-function *rin4* mutations are lethal in RPS2 plants but have no phenotype in *rps2* mutant plants independent of pathogens. These data provide evidence for interference between two R protein functions and suggest that RIN4 is a negative regulator of RPS2 function. RPS2 appears to detect the disappearance of RIN4 mediated by AvrRPT2 and triggers cell death-associated resistance when RIN4 levels drop below a threshold. Despite these advances it remains unclear which biochemical role RIN4 serves during *Pseudomonas* pathogenesis and how the LRRs of RPS2 and RPM1 participate in pathogen recognition. Moreover, the present data do not exclude the possibility of transient direct interactions between R and AVR proteins that might occur subsequent to an initial binding of the bacterial effectors to the guardee, e.g. through conformational changes of RIN4 containing heterocomplexes.

The *Arabidopsis* genome contains approximately 128 and the rice genome an estimated number of 600 NB-LRR type genes (The *Arabidopsis* Initiative, 2000; Dangl and Jones 2001; Goff et al., 2002). Although many *R* genes are highly polymorphic in natural populations of a species, are often organized in *R* gene clusters, and evolve faster than the rest of the genome, no experimental evidence exists for a dedicated machinery that facilitates the generation of new *R* gene specificities. Therefore, it remains a fundamental question whether a plant species encodes a sufficient repertoire of R proteins to directly recognize the collective repertoire of effectors generated by all pathogenic microorganisms. Unlike the receptor-ligand model, indirect recognition of effector activities by R proteins may necessitate the presence of a smaller number of *R* genes since one would expect a limited number of effector targets (guardees) in the host that can be manipulated to the advantage of pathogens.

2. Material and Methods

2.1. Materials

2.1.1 Antibiotics

Ampicillin (1000x): 100 mg/ml in H_2O

Kanamycin (1000x): 50 mg/ml in H_2O

Stock solution stored at –20 ^oC

2.1.2 Antibodies

Listed below are optimum dilutions for each antibody used in the present study. The secondary antibodies are all Horseradish Peroxidase (HRP) labelled.

Antibodies and dilutions			
Primary	Dilution	Secondary	Dilution
HA	5,000	Rat IgG	5,000
Myc	1,000	Rabbit IgG	10,000
LexA	500	mouse IgG	10,000

2.1.3 Bacterial strains

2.1.3.1 *E coli* strains

DH5α:

Genotype: supE44 DlacU169 hsdR17, recA1, endA1, gyrA96, thi-1, relA1, F^-

DH10B:

Genotype**:** F^-, mcrAΔ(mrr-hsdRMS-mcrBC)Φ80dlacX74, deoR, recA1, endA1, araD139, (ara,leu)7607, galU, galK, λ $^-$rspl, nupG

2.1.4 Yeast strains

EGY48(*8Op-LacZ*): Yeast strain EGY48 transformed with the autonomously replicating *p8op-lacZ* plasmid.

Genotype: *MATa, ura3, his3, trp1, LexAop (x6)-LEU2*

YM4271

Genotype: *MATa, ura3- 52, his3- 200, lys2-801, ade2-101, ade5, his3, trp1, trp1-901, leu2-3, 112, tyr1-501, gal4Δ, gal80Δ, ade5 : : hisG*

AH109

Genotype: *MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, HIS3, ADE2, lacZ, trp1, leu2, gal4Δ, gal80Δ, LYS2 : : GAL1$_{UAS}$-GAL1$_{TATA}$- HIS3, MEL1 GAL2 $_{UAS}$ -GAL2 $_{TATA}$ -ADE2, URA3::MEL1$_{UAS}$-MEL1 $_{TATA}$ -lacZ*

Y190

Genotype: *MATa, ura3- 52, his3- 200, ade2- 101, lys2-801, trp1, leu2, trp1- 901, leu2- 3, 112, gal4Δ, gal80Δ, cyhr2, cyhr2, LYS2 : : GAL1 $_{UAS}$-HIS3 $_{TATA}$-HIS3, MEL1, URA3 : : GAL1$_{UAS}$-GAL1 $_{TATA}$-lacZ*

2.1.5 Fungal strains

The known avirulence/virulence gene profiles of the two *Bgh* isolates is listed below (*Avr*-Avirulence, *vir*-virulence)

Isolate A6:

Avr: AvrMla3, AvrMla6, AvrMla9, AvrMla10, AvrMla12, AvrMla13, AvrMlg, AvrMl(CP), AvrMlH, AvrMlK1, AvrMlLa, AvrMl(Ab)

vir: virMla1, virMla22

Isolate K1:

Avr: AvrMla1, AvrMla3, AvrMla7, AvrMla22, AvrMlLa, AvrMl(Ab)

vir: virMla6, virMla9, virMla10, virMla11, virMla12, virMlg, virMl(CP), virMlH, virM1K, virMlra

Bgh strains were maintained on live barley plants or detached leaves. A6 was maintained on P01, a near-isogenic line from cv. Pallas containing *Mla1*; K1 was maintained on I10, a near-isogenic line of Ingrid containing *Mla12*. Plants or detached leaves were kept at 18 ^{o}C, 60% relative humidity, and 16 h light/8 h darkness after inoculation with *Bgh* conidia spores.

2.1.6 Plant materials

All barley seedlings were grown at 20 ^{o}C and 16 h light/8 h darkness in a protected environment.

Golden Promise: a barley cv. containing no *Mla* genes
Sultan-5: a chromosome-doubled haploid barley cv. containing *Mla12*
I10: a near-isogenic line in Ingrid background containing *Mla12*

Near-isogenic lines in Pallas background:
P01: containing *Mla1*
P03: containing *Mla6* and *Mla14*
P10: containing *Mla12*

Mutant lines generated by chemical mutagenesis from Sultan-5 seeds:
M66: *Mla12* Mutant
M86: *Mla12* Mutant
M22: originally designated as the *rar2* mutant; it is actually a *Mla12* Mutant (Chapter 2.2, 2.3).
M100: *rar1*-2 mutant allele
(Torp and Jorgensen, 1986)

Ingrid (*mlo3 Rar1*): generated by seven backcrosses with cv. Ingrid
Ingrid (*mlo29 rar1*-2): double mutant, originally isolated from a re-mutagenized *rar1-2* M_2 population, this line was used to test *Rar1* dependency of MLA chimeras in this study.

2.1.7 Vectors

pGEM-T	Promega
pTOPO	Invitrogen, Heidelberg
pENTR 4	Enter vector, GATEWAY® compatible, Invitrogen, Heidelberg
pDONR 201	Invitrogen, Heidelberg
pDEST 32 (BD)	Invitrogen, Heidelberg
pBluescript (S/K)+	Stratagene, Heidelberg
pUbi-GFP-Nos	Maize-ubiquitin1-promoter :: GFP :: Nos-polyA-signal, (Shirasu et al., 1999)
p8op-LacZ	reporter vector in LexA system, *LacZ* under control *lexAop*(x8), CLONTECH
pLexA	bait vector in LexA system , LexA(1-202) DNA-BD, CLONTECH
pB42AD	prey vector with acidic activator B42, CLONTECH
pAS2-1(M)	bait vector in GAL4 system containing GAL4(1-147) DNA binding domain, modified as GATEWAY® compatible, containing attB sites, CLONTECH
pACT2	prey vector from GAL4 system containing GAL4(768-881) activation domain, CLONTECH
pQSHvRar1-myc	generated in the present study by modifying pLexA vector, leaving out the LexA DNA binding domain and the Adh promoter driving the expression of RAR1-myc tagged variant
pRS315-GAL	yeast expression vector containing the Gal promoter with Leu^+ autotrophy selection, for the expression of MLA-HA tagged variants in the present study.

2.1.8 Oligonucleotides

Listed below are primers used in the present study and were synthesized by Introvigen or Promega

Primers	Primer sequence 5' → 3'
Exon-5as	AATCGTCATCATGAGCACCTT
M66-s	CTGAGATAGGAAAACGGCAGTTT
Mla12BsrDIs1	ACATTGCATCAGATGTGCTCTG
Mla12DNas2	GCTTCCATTGCCTCCCCAACCCT
Mla1EcoRIas1	AAGCGGCCGCGAATTCTAATACTACTAGGACTC
MlaBbSIs	TGGGAATAGCATGTCTTCACAG
MlaBsrDIas1	TGATGCAATGTGAGTCGCTCTGG
MlaBsrDIs1	CTGATCCAGAGCGACTCACATTGC
MlaPstIs1	CTTCTGCAGACTGAGTCATCGGCACCTTGC
MlaAgeIas1	TGGCACCGGTGACAATATCCAT
NotIas	GCAAGACCGGCAACAGGATTCAA
P10as	TCGCAGTGCAGAGAGTTGGCT
P10s	AGCCAACTCTCTGCACTGCGA
P12as	TCAAACAATATCTGCGTGGCA
P5as	CAAGATCCAACACCTCCAAAAACT
P5s	AGTTTTTGGAGGTGTTGGATCTT
sh007	CCGATCAAGCTTGGATCCTGATGGATATTGTCACCGGTGCCATTT
sh008	CGCATGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTAGTTCT CCTCCTCGTCCTCACACAA
sh009	CGCATGCGGCCGCTCAGTTCTCCTCCTCGTCCTCA
sh010	CCGATCAAGCTTGGATCCTGATGGATATTGTCACCGGTGCCATTTCCA
sh011	CGCATGCGGCCGCTTAAGCGTAATCTGGAACATCGTATGGGTAGT TCTCCTCCTCGCCCTCACACAA
sh012	CGCATGCGGCCGCTCAGTTCTCCTCCTCGCCCTCA
sh013	CGCATGCGGCCGCTCACTCTGTCGCTTCAGCATA

Primers	Primer sequence 5' → 3'
sh014	CCGATCAAGCTTGGATCCTGATGCATAAGCATGGGATAGCTCGCATGC
sh015	GGCCGCTCACCTTGAAAGAGATGGCATGA
sh016	CCGATCAAGCTTGGATCCTGATGGGGAATAGCATGTCTTCACA
sh017	CGCATGCGGCCGCTCACAATATCTGCGTGGCAGA
sh018	CCGATCAAGCTTGGATCCTGATGCAACGGCTGCTAGTCAT
sh019	CGCATGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTACTCT GTCGCTTCAG CATA
sh020	CGCATGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTACCTT GAAAGAGATGGCATGA
sh021	CGCATGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTACAATA TCTGCGTGGCAGA
sh022	CCGATCAAGCTTGGATCCTGATGAGCCAACTCTCTGCACTGCGA
sh023	CGCATGCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTATCGC AGTGCAGAGAGTTGGC
sh030	GGTCCAGAACCATAACATGTACA
sh031	GTATGTCGTGTACATGTTATGGT
sh032	GGTCCAGAACCATATCAGCTACA
sh033	GTATGTCGTGTAGCTGATATGGT
sh034	CCTCGTCATTGTTCTCGTTCCCTT
sh035	GGTCAGGTCGTTGTCGCACGTATT
sh036	CCTGACCTACAGGAAAGAGTT
sh037	CGTAAA GCGGCCGCTCAATCAACCTGTACGAGGAA
sh038	GCAACGGTCCGAACCTCATAACAACT
sh039	GAAAGCAACCTGACCTACAGGAAAGAG
sh040	GCATGACGCCGAAAACCATTCTT
sh041	GAGACAGCATAGAATAAGTG
sh042	CGTAAAGCGGCCGCTCACCAGAGCTTGTCTTGGCTGT
sh043	CGTAAAGCGGCCGCTCAGGCAGCGTTCATGCTCTCAAG
sh044	CCGATCAAGCTTGGATCCTGATGCACAAGGGTGTCAAGAA
sh045	CCAGCCTCTTGCTGAGTGGAGATG

2.1.9 Enzymes

2.1.9.1 Restriction enzymes

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

2.1.9.2 Nucleic acid modifying enzymes

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

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

2.1.10 Chemicals

Laboratory grade chemicals and reagents were purchased from Roth (Karlsruhe), Serva (Heidelberg), Boehringer (Mannheim), Merck (Darmstadt), Beckman (München), GIBCO BRL (Neu Isenburg) and Sigma (Deisenhofen) unless otherwise stated. Filter paper was obtained from Whatman. Chemicals for yeast culture, transformation were obtained from Sigma or Merck unless otherwise stated.

2.1.11 Media

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

LB (Lauria Bertani) Broth

tryptone peptone	1%
yeast extract	0.5%
NaCl	0.5%

Agar plates

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

SOC-Medium (100 ml)

Bacto -tryptone	2.0g
Bacto -yeast extract	0.5g
1M NaCl	1ml
1M KCl	0.25ml
2M Mg^{2+} stock, filter-sterilized	1ml
2M glucose, filter-sterilized	1ml

Add tryptone, yeast extract, NaCl and KCl to 97ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Just before each use, add 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20mM.

SD medium (1 L, 2% glucose or dextrose, pH to 5.8 if necessary)

Yeast Nitrogen Base	6.7 g
Agar(for plate only)	20 g
Drop-out solution (10X)	100 ml
40% glucose	50 ml
H_2O	850 ml

Allow medium to cool to ~ 55°C before adding 3-AT, cycloheximide, additional adenine, or X-gal. If add the sugar solution before autoclaving, autoclave at 121°C for only 15 min.

YPD (1 L, 2% glucose or dextrose, pH to 6.5 if necessary)

Peptone	20 g
Yeast extract	10 g
Agar (for plate only)	20 g
40% glucose stock	50 ml
H_2O	950ml

YPAD

Add to 1L of YPD 15ml of 0.2% Adenine hemisulfate (final concentration 0.003%)

Galactose/Raffinose SD/X-gal plates (1L)

Prepare SD medium use 725 ml of H2O and do not add carbon source and not adjust the pH. Autoclave and cool to 55 ^{o}C, then add:

40% Galactose	50 ml
40% Raffinose	25 ml
BU salts (10x)	100 ml
20 mg/L X-gal	4 ml

2.1.12 Buffers and solutions

2.1.12.1 General buffers and solutions

Sodium acetate, 3 M

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

Dissolve sodium acetate trihydrate in 800 ml H_2O, adjust pH to 4.8, 5.0, or 5.2 (as desired) with 3 M acetic acid, add H_2O to 1 L. Filter sterilize.

TE (Tris/EDTA) buffer

10 mM Tris/HCl (pH 8,0, 7,4 or 7,5)
1 mM EDTA (pH 8,0) in dH_2O

Tris/HCl (1 M)

Tris-Base	121 g
dH_2O	1000ml

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

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

Na_2EDTA	186,1 g
H_2O	1000 ml

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

Sodium phosphate buffer (0.1 M)

Solution A: 27.6 g $NaH_2PO_4 \cdot H_2O$ per L (0.2 M final) in water.
Solution B: 53.65 g $Na_2HPO_4 \cdot 7H_2O$ per L (0.2 M) in water.

Mix the different volumes of solutions A and B to 100ml for desired pH, then dilute with water to 200 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

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

SDS	20 g
H_2O	100 ml

Slightly heat may be necessary to fully dissolve the powder

IPTG stock (0.1M)

1.2 g IPTG add water to 50 ml final volume, Filter-sterilize and store at 4 ^{o}C.

Ethidium bromide stock (10 mg/ml)

ethidium bromide	0.2 g
H_2O	20 ml

Stored at 4 ^{o}C in dark or in a foil-wrapped bottle. Do not sterilize.

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

Tris base	24.2 g
glacial acetic acid	5.71 ml
$Na_2EDTA{\cdot}2H_2O$	3.72 g

Add H_2O to 1 L

TBE (Tris/borate/EDTA) buffer (10x)

Tris base	108 g
boric acid	55 g
H_2O	960 ml
0.5 M EDTA (pH 8.0)	40 ml

Carbon sources for yeast cultures

40% glucose or Dextrose

40% Galactose.

40% Raffinose

Filter sterilized or autoclaved Store at 4°C

10X BU Salts for yeast (1 L, H2O)

70 g $Na_2HPO_4 \cdot 7H_2O$

30 g $NaH_2 PO_4$

Adjust to pH 7, then autoclave and store at room temperature.

X-gal (20 mg/ml in DMF)

Dissolve 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in N,N-dimethylformamide.

Stored in the dark at –20°C.

2.1.12.2 DNA buffers

DNA Gel loading buffer (6x)

bromphenol blue	0.25%(w/v)
xylene cyanol FF	0.25%(w/v)
sucrose	40%(w/v)
or Ficoll 400	15%(w/v)
or glycerol	30%(v/v)

Store at 4°C (room temperature if Ficoll is used). Sucrose, Ficoll 400, and glycerol are interchangeable in this recipe.

DNA extraction buffer

100 mM Tris-HCl pH 8.5, 100 mM NaCl, 50 mM EDTA pH 8.0, 2% SDS and 0.1 mg/ml proteinase K (added at the time of use)

2.1.12.3 Western buffers

10x running buffer (1L)

Tris-HCl	30.2g
Glycine	188g
H_2O	800ml
SDS 10%	100ml
H_2O	

2x loading buffer (40ml)

water	5ml
Tris pH 6.8 (1M)	5ml
SDS (10%)	20ml
glycerol	10ml
Bromphenol blue	0.01g

Prior to use, add DTT (20µl DTT (1M) to 80µl loading buffer)

Transfer buffer (1L)

$NaPO_4$ pH 7 1M	15ml
SDS 10%	5ml
Methanol	200ml
H_2O	add up to 1L

Pre-cool transfer buffer on ice

PBS (phosphate buffered saline solution) 10x (1L)

Na_2HPO_4	115g
NaH_2PO_4	29.6g
NaCl	58.4g
H_2O	add up to 1L
(pH 7.5)	

PBS-T

Add Tween-20 (1/1000 v/v) to 1x PBS solution

Blocking milk solution

5% (w/v) skim milk powder made with PBS-T solution

2.2 Methods

2.2.1 Nucleic acid manipulations

2.2.1.1 Polymerase chain reaction (PCR) amplification

PCR amplification Puffer, 10x

200mM Tris/HCl (pH 8.4)

500mM KCl

25mM MgCl2

Stock solution is sterilized by autoclaving

Plasmid or genomic PCR (*Taq* polymerase)

Reaction mix

Reagent	Amount per reaction
Template DNA (genomic or plasmid)	20-50 ng
PCR amplification buffer (10x)	1/10 of reaction volume
dNTP mix (dATP, dGTP, dCTP, dTTP)	0.2 mM each
upstream primer (10µM)	0.5 µM
downstream primer (10µM)	0.5 µM
homemade Taq DNA polymerase	2.5 U
Nuclease free water	variable

Thermal profile

Stage	Temperature (°C)	Time	No. of cycles
Initial denaturation	94	2-3 minutes	
Denaturation	94	15-30 seconds	
Annealing	50-65 °C	20-60 seconds	25-35 x
Extension	72	1-2 min	
Final extension	72	7 min	

Yeast colonies PCR:

Essentially follow the plasmid or genomic PCR protocol except that 2 µl of the clear lysate from yeast colony lysised in 25µl of 20 mM NaOH was used as template and the cycle number was increased to 40x.

PCR with other polymerase, e.g., *Pfu, Pfx Pwo*, or Expand High Fidelity System were performed according to the manufacturer's protocol.

2.2.1.2 Restriction endonuclease digestion of DNA

All restriction digests were carried using the manufacturers recommended conditions. Typically, reactions were carried out in 1.5 ml eppendorfs using 1-2 Units of restriction enzyme per 10-20µl reaction. All digests were carried out at the appropriate temperature in incubators with proper temperature for a minimum of 30 minutes. Eppendorfs occasionally were replaced with sterile 250µl PCR tubes and digests might be carried out in a thermal cycler with a heated lid.

2.2.2 DNA analysis

2.2.2.1. Plasmid DNA isolations

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

Barley cDNA library DNA was isolated combining the alkaline lysis method and CsCl gradient ultra-centrifugation method. Isolation of library plasmid DNA from *E.coli* stock was performed according to normal max prep method upto the clarification of bacterial lysates. Afterwards, the lysates were directly precipitated with isopropanol instead of using cartridge or column with silica membrane for binding

DNA. The precipitated DNA was resuspended in TE and further purified using CsCl gradient ultra-centrifugation method (Sambrook, et al., 1989). Purified library DNA were tested for concentration and diluted in TE at ~1µg/µl and stored at –20 °C as aliquots.

2.2.2.2. Plant genomic DNA isolation

The Nucleon PhxtoPure resin system (Amersham LIFE SCIENCE) was used for DNA isolation from barley leaf materials according to the manufacturer's protocol with small modifications.

2.2.2.3. Isolation of DNA fragment from Agrose-gel

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

2.2.2.4. DNA sequencing

DNA sequences were determined by the Automatische DNA-Isolierung und Sequenzierung (ADIS-Unit) in MPIZ on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377 and 3700 sequencers using Big Dye-terminator chemistry (Sanger et al.1997*)*.

PCR products were purified with the Nucleospin Extract-Kit (MACHEREY-NAGEL) or Qiagen Extract Kit, ensuring sufficient amount at appropriate concentration to be directly sequenced. When large scale of PCR products needed to be purified for sequencing, the Milllipore Montage™ $PCR_{\mu 96}$ filter plate were used, or purified by the ADIS-Unit by Sephadex method.

2.2.2.5. DNA sequence analysis

Sequencing data were analysed mainly using Clone Manager 6, version 6.00 and alignment made using Align Plus 4, version 4.10 from Scientific & Educational Software. Alternatively using the GCG-

Programm (Version 10.0) from Genetics-Computer-Group, Inc., University of Wisconsin, Madison, or ClustalW (http://www.ebi.ac.uk/clustalw/).

2.2.2.6. Database searching

DNA sequence data was directly used for database searching using NCBI Blast (http://www.ncbi.nlm.nih.gov/BLAST/), or translated into polypeptide for motif similarity searching. Other databases were used, including Phytopathogenic Fungi and Oomycete EST Database (Version1.4) (http://cogeme.ex.ac.uk/), TAIR (http://www.Arabidopsis.org/), TIGR (http://www.tigr.org), IPK Barley ESTs Database (http://pgrc.ipk-gatersleben.de/), and so on.

2.2.3. RNA analysis

2.2.3.1. Isolation of total RNA from plant tissues

Plant materials were finely ground in liquid nitrogen and resuspended in the total RNA extraction buffer and incubated at 37°C for 1 hour. Following three phenol/chloroform extractions, RNA was precipitated with 1 volume 8 M LiCl prepared in DEPC (Diethylpolycarbonate) water, washed with 70% ethanol and resuspended in DEPC treated water.

Alternatively, harvested plant material, previously maintained at -80°C was transferred to a pre-chilled, autoclaved mortar, then ground in the presence of liquid nitrogen to a fine powder. Approximately 0.5ml of tissue was transferred to an RNase-free 2ml centrifuge tube, before 1ml of Tri reagent (Sigma) was added. The sample was vortexed for 10 seconds then placed on dry ice to allow any remaining samples to be processed. All homogenised samples were left at room temperature for

10 minutes. 200μl of chloroform was subsequently added, vortexed for 15 seconds and allowed to stand for 2-15 minutes at room temperature. The samples were then spun for 20 minutes, at 1,3000rpm and 4°C, in a bench top centrifuge. The upper aqueous phase was carefully transferred to a fresh RNase free 2ml centrifuge tube. The RNA was precipitated by adding 500μl of isopropanol, mixing well and leaving at room temperature for 10 minutes. Centrifuging then at 1,3000rpm for 10-15 minutes and 4°C helped pellet the RNA. The supernatant was removed and the white pellet was washed with 1ml of 75% ethanol (absolute ethanol diluted with DEPC treated water 1:3). The samples were briefly vortexed to dislodge the pellet and centrifuged again for 10 minutes at 4°C and 1,3000rpm. The supernatant was removed and the pellet air-dried for 10 minutes. The RNA was re-suspended in 40-60μl of DEPC water.

2.2.3.2. RT-PCR

Reverse transcription – polymerase chain reactions (RT-PCR) were carried out by two-steps methods. Using RT superscript II for the first strand cDNA synthesis by combining 2μg template total RNA, 2μl 10μM oligo dT-18, sample were incubated at 70°C for 10 minutes before immediately cooling on ice. Subsequently the reaction was made up to 20μl by adding the following components: 4μl 5× first strand buffer (250mM Tris pH 8.3/375mM KCl/15mM $MgCl_2$), 2μl 0.1M DTT, 1μl 10mM dNTPs mix and proper amount of DEPC treated water. The mix was incubated at 42°C for 2 min before add into 1μl (200u) RT Superscript II. Subsequently, proceed at temperature 25(10')-42(50')-70(15'to inactivate the enzyme) for indicated time.

For subsequent normal PCR, use 2 μl of above mixture as template, 2.5 μl of DMSO added for 50 μl of reaction volume before PCR (for disrupting the secondary structure of the single DNA strand).

2.2.4. Protein analysis

2.2.4.1. Denaturing SDS-polyacrylamide gel electrophoresis

All denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Mini-blot Protean system (BioRad).

Gel preparation

Different percentage gels were used depending on the size of the protein that was to be resolved. All gels were made fresh on the day of use. The resolving gel was poured between two glass plates then overlaid with 2mm of isopropanol. The gel was allowed to set for a minimum of 25 minutes. Isopropanol was removed and washed using water, and a stacking gel was poured onto the top of the resolving gel. A comb was inserted, ensuring no bubbles were trapped and the whole gel left to set for at least 25 minutes.

Reagents and amount used for different percentage resolving gels

Resolving gel components[a]	Volume for different percentages of gels (in ml)[d]		
	7%	10%	12%
H_2O	5.5	4	3
30% acrylamide mix[b]	3.5	5	6
1M Tris-HCl (pH8.8)	5.7	5.7	5.7
10% SDS	0.15	0.15	0.15
10% ammonium persulfate[c]	0.15	0.15	0.15
TEMED	0.01	0.006	0.006

Stacking gel components[a]	Component volume (in ml)	
	5ml	10ml
H_2O	2.7	6.8
30% acrylamide mix[b]	0.67	1.660
1.5M Tris-HCl (pH8.8)	0.5	1.26
10% SDS	0.04	0.1
10% ammonium persulfate[c]	0.04	0.1
TEMED	0.004	0.01

[a]Add in stated order, mixing between subsequent additions. [b]30% Acrylamide/Bis solution 37.5:1. [c]Make-up fresh before use. [d]Recipes prepare solution sufficient for two gels, 1.5mm thick or four gels , 0.75mm thick (7 × 10cm).

Yeast crude protein extraction

Overnight yeast cultures raised in SD selection media and 3 OD_{600} units of cell pelletes obtained from each culture by centrifugation at 3500 rpm. Immediately the pelletes were frozen in liquid N_2 and samples were boiled for 5 min, these treatments were repeated for at least 3 times. Directly 200µl of 2x loading buffer with freshly added DTT was mixed with samples that can be stored at –20 until use. Samples need to be boiled for 5 min and centrifuged for 5 min at 13,000 rpm before 20 µl of supernatants loaded on gel for Western blotting.

Western blot

Proteins resolved on acrylamide gels were transferred to Hybond – ECL (nitrocellulose) membrane (Amersham pharmacia biotech) after being released from the glass plates and having their stacking gel removed with a scalpel. The electroblot apparatus (Mini-blot Protean III; BioRad) was assembled. The ECL membrane was pre-equilibrated by immersing in transfer buffer. Transfer was carried out either at 40mA overnight or 250mA for 1 hour 30 minutes at 4°C.

The transfer cassette was dismantled and the membrane washed 5 min with water, then stain with Poncean (1:20 v/v, stain:water) for 15 seconds to check for equal loading before rinsing in excess volumes of water. The membrane was then washed with PBS-T for 5 min and left to block at room temperature in blocking buffer for 2 hours on a rotary shaker. The blocking solution was removed and the membrane washed briefly in PBS before the addition of the primary antibody. The optimum dilution of a particular primary antibody was determined beforehand. The membrane was incubated in the presence of the primary antibody for approximately 1 hour 30 minutes at room temperature on a rotary shaker. The membrane was briefly rinsed in PBS-T, then washed with excess PBS-T for 3 $\times$ 5 minutes.

A horseradish peroxidase (HRP) chemiluminescence system was used to detect bound antigen/primary antibody conjugates. A suitable secondary antibody (anti-IgG) was added at an optimised dilution of between 1:5000 and 1:15000, in PBS or blocking milk solution. The membrane was incubated at room temperature for a maximum of 1 hour on a rotary shaker, rinsed briefly in PBS-T, then washed with excess PBS-T for 3 $\times$ 5 minutes. Each membrane was developed using ECL detection reagents according to manufacturer's protocol. Any signal was detected by exposing the membrane to film (Hyperfilm ; Amersham pharmacia biotech) from 1/2 min to 1 hour.

2.2.5 Transformation of *E. coli*

2.2.5.1 Preparation of electro-competent E. coli cells

10 ml of an overnight culture of *E. coli* strain (DH5α) was added to 1 litre of LB broth and shaken at 37°C until the bacterial growth reached an OD= 0.5-0.6. The bacteria were pelleted at 5000 x g for 20 minutes at 4°C and the pellet gently resuspended in ice-cold sterile water. The cells were pelleted as before and again resuspended in ice-cold water. The process was repeated twice. Finally the cells were

gently resuspended in a 1/100 volume of the initial culture in 10% sterile glycerol, pelleted once more and then resuspended in 5 ml 10% glycerol. 50 µl aliquots of cells were frozen in liquid nitrogen and stored at –80 till use.

2.2.5.2 Transformation of electro-competent E. coli cells

20 to 50 ng of salt-free ligated plasmid DNA (or ~1µl of ligated mix from 10 µl ligation system) was mixed with 50 µl of electro-competent cells, and transferred to the 1mm cold BioRad electroporation cuvette. The BioRad gene pulse apparatus was set to 25 µF capacitance, 1.8 kV voltage and the pulse controller to 200 ohms. The cells were pulsed once at the above settings for a few seconds and 500 µl of SOC medium was immediately added to the cuvette and the cells were quickly resuspended and incubated at 37°C for 1 hour. A fraction (~150-300µl) of the transformation mixture was plated out onto selection media plates.

2.2.6 High-efficiency transformation of yeast competent cells

The protocol
(modified from Gietz, R.D. and R.A. Woods, 2002)

1. Start an overnight culture in YPAD (15 ml) supplemented with antibiotics at 30 °C
2. Start a new culture in 100 ml YPAD using 5 x10^8 cells in total and grow for 5-6 hours at 30 °C (no more than 6 hr).
3. Centrifuge 3500 rpm for 3 min.
4. Resuspend the cells in 25 ml of sterile water and centrifuge again.
5. Resupsend the cells in 1 mL of 100 mM LiAc (freshly made from a 1M stock)
6. Centrifuge at 6000 rpm for 30sec.
7. Resuspend the cells in 500 µl of 100 mM LiAc.

8. For 1 transformation Use 50-100 ul of cells and spin down (15 s, 6000 rpm). Remove the supernatant, then add 1 µg of plasmid DNA diluted in water as 5ul. Vortex at low speed 2sec and, under vortexing, add 300 ul of Transformation Mix. Vortex for 5 more seconds.
9. Incubate at 30 °C for 30min. Invert tubes every 10 min.
10. Incubate at 42°C for 45min (time various on strains). Invert tubes every 10 min.
11. Centrifuge 6000 rpm, 10-15 second.
12. Eliminate supernatant. Add carefully 200 uL of sterile water, set for 10-30 min at RT. Gently resuspend the cells by inverting the tubes. Plate a dilution of the transformed mixture on selective plate to estimate the number of transformants. Plate the rest of the transformed mixture on selection plates.

	Yeast Transformation Mix			
	Times	x1	x 5	x 10
PEG 50%		680	3400	6800
1M LiAc		100	500	1000
Carrier DNA		140	700	1400
water		80	400	800

Library DNA transformation into strain YM4271 (*MATα*)

Several independent transformations were carried out according to above protocol, and transformants were selected on ~50 big plates (Φ145mm) with SD/-Trp agar and incubate for 2-3 days at 30 °C until colonies appears with size of the tip of a pin. All colonies were collected with chilled YPAD broth and stored as many 15 ml falcon aliquots with glycerol at 25% final concentration. In total, ~$2x10^6$ independent transformants were obtained.

2.2.7 Yeast two-hybrid screening via interaction mating methods

Briefly, overnight bait cultures were raised in proper SD media containing 4% glucose, and the frozen library aliquots were re-generated in 10-20 times volume of pre-warmed YPAD for 20 min. 20 OD of each bait culture was mixed with ~10 OD of library cells and subjected to mating for 4.5 hr in YPAD/PEG solution. Inducing media was used for the expression of the prey library fusion and incubate for ~6 h, cells were collected and diluted to ≤ 5 OD before plating ~6x10^6 diploid cells onto selection inducing plates. Plates were incubated for 2-5 days for checking of appearing of colonies (Fig. 12 for overview; described in Kolonin et al (2000)).

2.2.8 Single-cell transient assay in barley epidermal cells using particle bombardment

Overview

A reporter plasmid containing *Mlo* and *GUS* genes (*GUS* gene alone in the case of *Mlo* genetic background), and the respective effector plasmids were mixed prior to the coating of particles (molar ratio 2:1, respectively, maximum 5µg DNA). The bombarded leaves were transferred onto 1% agar plates supplemented with 85 µM Benzimidazol and incubated at 18 ^{o}C for 15 h before high-density inoculation with *Bgh* spores. Leaves were stained for GUS and single leaf epidermal cells attacked by *Bgh* germlings were evaluated under the microscope at 48 h after spore inoculation. In dsRNAi single-cell silencing experiments, particles were co-coated with a construct encoding an intron-spliced dsRNAi construct targeting *HvRAR1* or *HvSGT1* according to Azevedo et al., (2002) (molar ratio 1:1:1, 5µg total DNA). In the gene silencing experiments the bombarded leaves were incubated at 18 °C for 48 or 96 h before high-density inoculation to allow turnover of preformed RAR1 or SGT1.

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

Material preparations

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

Sucrose and Benzimidazol agar (1-1.5%) plates

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

Spermidine solution (0.1M); $CaCl_2$ solution (2.5M); ethanol (70% and pure), glycerol (50% in water)

Particle bombardment

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

Fungal inoculation

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

GUS staining

Infiltration with GUS staining solution into bombarded leaves in falcon, leave for at least 10 h at 37 °C

GUS destaining and fixing

Remove GUS staining solution and add in destaining solution

Microscopy

Use coomassie solution to stain fungal surface structure and evaluate haustorium index (%) under light microscopy.

Other materials required and recipes

Consumables

Macrocarrier

Rupture disc

Gold particles

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

Gus staining solution (1L)

1M Na_2HPO_4	57.7 ml
1M NaH_2PO_4	42.3 ml
0.5M Na_2EDTA	20.0 ml
$K_4Fe[CN_6]$	2.112 g
$K_3Fe[CN_6]$	1.646 g
Triton X-100 (v/v)	0.1%
methanol (v/v)	20%
X-gluc	1 g

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

Destaining solution

stock solution

50% glycerol

25% lactic acid

H_2O

work solution

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

Coomassie solution

coomassie (w/v)	0.6%
methanol (or ethanol)	

coomassie: Serva Blue R, from Serva

3. Isolation and characterization of a new *Mla* resistance specificity: *Mla*12

3.1. Introduction

Mla12 is one of the *Bgh* resistance specificities that was mapped to the *Mla* locus on barley chromosome 5S (1HS; Schwarz et al., 1999). A cytological comparison of near-isogenic *Mla12* resistant and *mla12* susceptible barley lines in the genetic background of cultivar Pallas revealed in the resistant line an epidermal cell death response concomitant with the establishment of *Bgh* haustoria (Görg et al., 1993). Similarly, a time course analysis of cytological events during *Mla12*-mediated resistance in cv. Sultan 5 showed host cell death at later stages during fungal pathogenesis (Freialdenhoven et al., 1994). In this case, fungal growth proceeded within 36 h after *Bgh* spore inoculation to the stage of identifiable differentiated haustoria at 60% of interaction sites. Cell death of attacked epidermal cells, measured by whole-cell autofluorescence, reached a maximum at approximately 48 h (60% interaction sites; Freialdenhoven et al., 1994). These data demonstrate that the timing of *Mla12*-triggered cell death relative to *Bgh* growth stage is similar though not identical in different host genetic backgrounds, terminating fungal growth after the complex process of epidermal cell wall penetration. It is possible though speculative that this 'post penetration resistance' reflects a relatively late release of the cognate *AvrMla12* effector during pathogenesis, e.g. coincident with haustorium differentiation.

The availability of susceptible *mla12* mutants greatly facilitated the molecular isolation of *Mla12*. Sultan 5 seeds were chemically mutagenized by EMS or NaN_3 and 25 susceptible mutants were recovered from M_2

populations (Torp and Jørgensen, 1986; Jørgensen, 1988). Genetic analysis (after crosses with the resistant Sultan 5 and a line lacking *Mla12*) indicated that susceptibility in mutants M66 and M86 is likely due to mutations in *Mla12*, whereas susceptibility in another line, M22, possibly resulted from extragenic suppressor mutations of *Mla12* function (Jørgensen, 1988, 1996). The gene required for *Mla12* function was designated *Rar2* (Freialdenhoven et al., 1994). However, a closer investigation of the original data from the genetic analysis of mutant M22 (Jørgensen, 1988; Freialdenhoven et al., 1994) suggested another possibility: a mutation in *Mla12* leading to a partially susceptible phenotype.

To isolate *Mla12*, a genomic cosmid library was constructed using DNA from cv Sultan 5. The library was screened with a DNA probe corresponding to the LRR region of MLA1 (Zhou et al., 2001), a known polymorphic region in *NB-LRR* type genes. By using a DNA probe representing the LRR region of MLA1, it was expected to isolate from cv. Sultan 5 only genes that are highly sequence related to *Mla1* rather than any *RGH*. Sixteen cosmid clones were isolated from the cosmid library. Low-pass DNA sequencing of the cosmid clones revealed that all of them contain NB-LRR-type *RGHs*. Two clones, designated Sp14-1 and Sp14-4, contain identical *RGHs* showing ~90% sequence identity to *Mla1* and *Mla6* in deduced exon and intron sequences. A closer comparison of the *NB-LRR* gene in Sp14-4 with *Mla1* and *Mla6* revealed an identical 5' untranslated small open reading frame of nine amino acids and the same intron-exon structure (Halterman et al., 2001; Zhou et al., 2001). These genes share a simple sequence repeat $(AT)_n$ in intron 3, although the exact numbers of the repeats differ (see Fig. 15; below). Therefore, the *RGH* in Sp14-4 was considered a candidate *Mla12* gene that encodes a predicted CC-NB-LRR-CT protein of 108 kD sharing 89% identical residues with MLA1 and 92% identical residues with MLA6 (Fig. 2).

3.2. Characterization of susceptible *mla12* mutant alleles

To confirm the identity of the putative *Mla12*, genomic DNA was

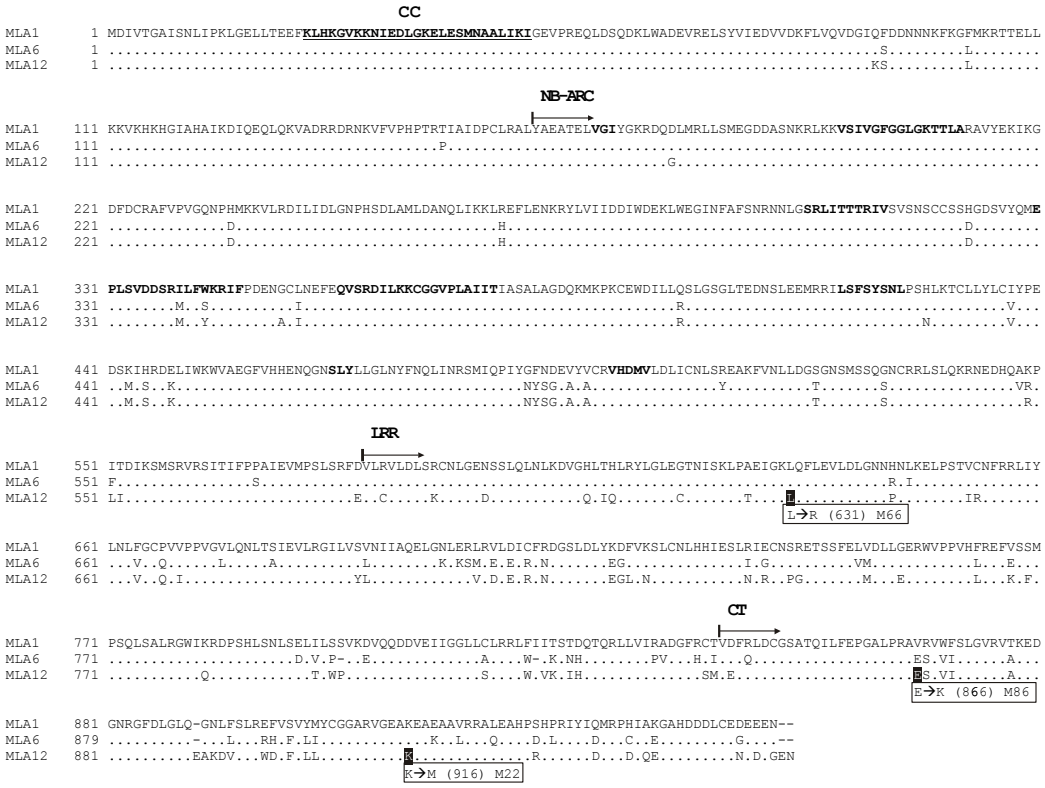

Figure 2. Amino acid sequence alignment of deduced products of the *Mla1*, *Mla6*, and *Mla12* genes.

Residues identical to those in MLA1 are shown as dots, and deletions are shown as hyphens. A predicted CC structure is underlined. The starts of the NB, LRR, and CT regions are indicated with arrows and are operational according to Zhou et al. (2001). Boldface letters in the NB-ARC domain indicate amino acid motifs conserved among known NB-LRR proteins. Boxes indicate amino acid exchanges identified in three susceptible *Mla12* mutants, and affected residues are shaded in black.

isolated from the *Mla12* mutants M86 and M66 and the putative *rar2* mutant M22. The DNA was used as a template for PCR amplification of the respective *Mla12* mutant alleles. *Mla12*-specific primers were designed based on the sequence alignment of known *Mla1*, *Mla6*, *Mla1-2*, *RGH1a*, and the putative *Mla12*. PCR products were purified and then sequenced directly.

DNA sequence analysis of the candidate *Mla12* in the susceptible mutants M66 and M86 revealed in each a single nucleotide substitution compared with the wild-type gene derived from the cosmid clone Sp14-4. The substitutions replace amino acid Leu^{631} with Arg in the second LRR of the deduced candidate MLA12 protein in M66 and amino acid Glu^{866} with Lys in the CT region in M86, respectively (Fig. 2). Therefore, the Sp14-4–derived candidate gene probably is *Mla12*.

DNA sequence analysis of the candidate *Mla12* in M22 plants revealed a single nucleotide substitution that replaces amino acid Lys^{916} with Met in the CT region (Fig. 2). Previous DNA marker-based mapping of susceptibility conferred by the M22 mutant revealed its location on chromosome 5(1H) at the *Mla* locus between restriction fragment length polymorphism markers MWG036 and MWG068 (Schüller et al., 1992). This finding suggested that susceptibility might be caused by a mutation in *Mla12* or in a tightly linked gene. The intragenic single amino acid replacement further suggests that M22, like M66 and M86, likely is a mutant allele of *Mla12* (see below).

3.3. Over-expression of *Mla12* alters the resistance kinetics but retains *Rar1* dependence

3.3.1. Over-expression of *Mla12* alters the resistance kinetics

To test directly the function of the candidate *Mla12* gene, Sp14-4 DNA was delivered into epidermal cells of detached barley leaves by particle bombardment. Transformed cells were tested for their ability to activate race-specific powdery mildew resistance upon inoculation with *Bgh* conidiospores

of isolates expressing or lacking *AvrMla12* (isolate A6 harbouring *AvrMla6* and *AvrMla12* and isolate K1 harbouring *AvrMla1*) (Zhou et al., 2001). Infection phenotypes of transgene-expressing epidermal cells were microscopically inspected at 48 h after inoculation by scoring the presence or absence of intracellular *Bgh* haustoria at single interaction sites. Unlike control bombardments with cosmid DNA harbouring *Mla1* or *Mla6*, which are known to mediate race-specific resistance in the transient gene expression assay (Halterman et al., 2001; Zhou et al., 2001), delivery of Sp14-4 DNA failed to trigger detectable resistance upon inoculation with *Bgh* strains A6 and K1 (data not shown). This effect may be caused by insufficient 5' flanking regulatory sequences (~400 bp upstream of the transcription start) in cosmid Sp14-4, driving expression of the candidate *Mla12*, or delayed activation of *Mla12* compared with *Mla1* and *Mla6* resistance.

To examine this possibility further, the coding region of the *Mla12* candidate was subcloned under the control of the strong maize ubiquitin promoter and the nopaline synthase (*Nos*) terminator. DNA of this over-expression construct and two similar control over-expression plasmids harbouring *Mla1* or *Mla6* were delivered into leaf epidermal cells of barley cv Ingrid lacking *Mla12* and *Mlo* (Fig. 3A). Delivery of each plasmid DNA together with an *Mlo*-expressing construct resulted in a haustorium index of 2 to 5% upon challenge with the *Bgh* isolate containing the cognate *Avr* genes, whereas the control compatible interactions showed an index of ~80%. Note that the very high level of haustorium incidence found in the compatible interactions likely is the result of co-bombardment of the race-nonspecific defense modulator *Mlo*, which renders transformed epidermal cells super-susceptible to the fungus (Kim et al., 2002). These data provided evidence that the candidate *Mla12* gene subcloned from cosmid Sp14-4 triggered *AvrMla12*-dependent *Bgh* growth termination. Interestingly, bombardments with empty vector DNA into epidermal cells of cv. Sultan 5, which contains *Mla12*, resulted in a high haustorium index of 45% when inoculated with the incompatible isolate *Bgh* A6 (Fig. 3B). This finding suggests that *Mla12*

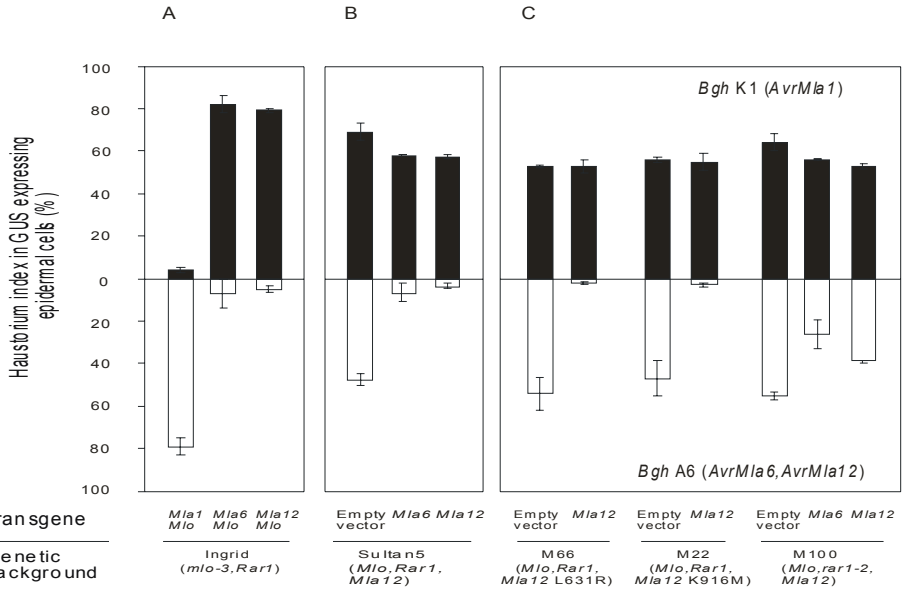

Figure 3. Complementation of susceptible *Mla12* mutants by overexpression of *Mla12* resistance.

Relative single cell resistance/susceptibility upon delivery of various *Mla* transgenes at 48 h after spore inoculation is indicated by haustorium indices of attacked β-glucuronidase (GUS)–expressing cells (%). Data shown were obtained by bombardment of plasmid DNAs into epidermal cells of detached barley leaves (described by Shirasu et al., 1999b; Zhou et al., 2001). A β-glucuronidase reporter gene was used to identify transformed cells.

(A) The indicated transgenes were tested in detached leaves of barley cv Ingrid harboring *mlo-3 Rar1*. In this line, broad-spectrum *mlo-3* resistance was complemented by cobombardment with a plasmid expressing wild-type *Mlo*; this renders cells supersusceptible to all tested *Bgh* isolates (Zhou et al., 2001; Kim et al., 2002). Results obtained with the *Bgh* isolate K1 (*AvrMla1*) are shown by closed columns, and results obtained with isolate A6 (*AvrMla6* and *AvrMla12*) are shown by open columns in downward orientation. The data shown are means of at least three independent experiments (SD indicated). Each experiment involved light microscopic examination of at least 100 interaction sites between a single *Bgh* sporeling and an attacked epidermal cell.

(B) The indicated transgenes and an empty vector control were delivered into epidermal cells of cv Sultan 5 containing *Mla12 Mlo Rar1*. Experimental conditions and symbols are identical to those in **(A)**.

(C) Transgene *Mla12* or an empty vector control was delivered into epidermal cells of two susceptible *Mla12* mutant lines (M66 and M22). Transgene *Mla6* or *Mla12* or an empty vector control also was delivered into the *rar1-2* mutant line M100. Experimental conditions and symbols are identical to those in **(A)**.

resistance is not effective before haustorium development, consistent with the previous quantitative inspection of single interaction sites in resistant *Mla12* wild-type and susceptible mutant leaves (Freialdenhoven et al., 1994). However, when the putative *Mla12* was over-expressed in cv. Sultan 5 usingthe single cell expression assay, the haustorium index was reduced to ~2%, similar to the level conferred by *Mla6* (Fig. 3B). Apparently, over-expression of the candidate *Mla12* shifted the resistance response from post-haustorium growth arrest to an abortion of fungal development before the penetration of the epidermal cell wall and the formation of haustoria.

3.3.2 Over-expression of *Mla12* complements mutant phenotypes but retains *Rar1* dependence

To corroborate the function of *Mla12*, the over-expression construct of *Mla12* was bombarded into epidermal cells of mutant lines M66, M22, and M100 (the latter contains the severely defective *rar1-2* allele) (Shirasu et al., 1999a) (Fig. 3C). In these experiments, full *AvrMla12*-dependent resistance was restored in both M66 and M22 plants, demonstrating that the mutant phenotypes were complemented by the candidate *Mla12*. By contrast, neither over-expression of *Mla6* nor of the candidate *Mla12* restored full resistance in the *rar1-2* mutant line M100. The *Mla12* over-expression phenotype was affected more strongly than the *Mla6* response in the *rar1* mutant background.

The data obtained from over-expression of *Mla12* in different genetic backgrounds strongly support the idea that the *RGH* in cosmid Sp14-4 is a functional *Mla12*.

3.4. *Sgt1* is required for *Mla12* resistance

Double stranded RNA interference (dsRNAi) gene silencing of *HvSgt1* in a single-cell expression system compromised *Mla6*- but not *Mla1*-mediated resistance function (Azevedo et al., 2002). This technique was used to test a potential SGT1 requirement for *Mla12*-mediated resistance to *Bgh*. Detached barley leaves (*Rar1* genotype) were co-bombarded with a dsRNAi vector for

silencing *Sgt1* and a *Mla12* over-expression construct. In comparison to a control with an empty dsRNAi vector I recorded about 10% increase in *Bgh* haustorium index (2% and 13% with and without including dsRNAi effector construct at 96 h after inoculation), indicating that *HvSgt1* is required for *Mla12*-triggered resistance.

3.5. Context-dependent function of conserved MLA residues Leu^{631} and Lys^{916}

The amino acid substitutions in the susceptible *mla12* mutants M66 ($L^{631}R$) and M22 ($K^{916}M$) affect residues that are conserved in MLA1 and MLA6, whereas the substitution in mutant M86 ($E^{866}K$) changes a nonconserved residue (Figure 1). To investigate the importance of Leu^{631} and Lys^{916} in *Mla1*- and *Mla6*-triggered resistance, the same amino acid substitutions were introduced into *Mla1* and *Mla6* under the control of the ubiquitin promoter and were reintroduced into *Mla12* for comparison and confirmation. Wild type and mutant variant plants were tested in the transient gene expression system. This analysis showed that *Mla12* mutant variant $L^{631}R$ impaired *AvrMla12*-dependent resistance fully (84%) and $K^{916}M$ impaired it partially (31%), indicating that the MLA12 ($K^{916}M$) variant protein retains residual activity (Fig. 4). This observation is consistent with the fully compromised and partially impaired *Mla12* resistance reported for M66 and M22 mutant plants (infection types 4 and 2/3, respectively) (Torp and Jørgensen, 1986) and validates the usefulness of the single-cell assay to evaluate *Mla12* activity using the strong ubiquitin promoter. Surprisingly, despite an overall sequence relatedness of 90% between the tested MLA proteins, none of the amino acid replacements in MLA6 or MLA1 resulted in a detectable change of resistance activity compared with that in the respective wild-type genes (Fig. 4). Thus, it is possible that other regions are critical for R protein function in MLA1 and MLA6 (see below). Alternatively, other residues that are absent or polymorphic in MLA12 might compensate for the functional contributions of Leu^{631} and Lys^{916} in the MLA1/MLA6 substitution mutants.

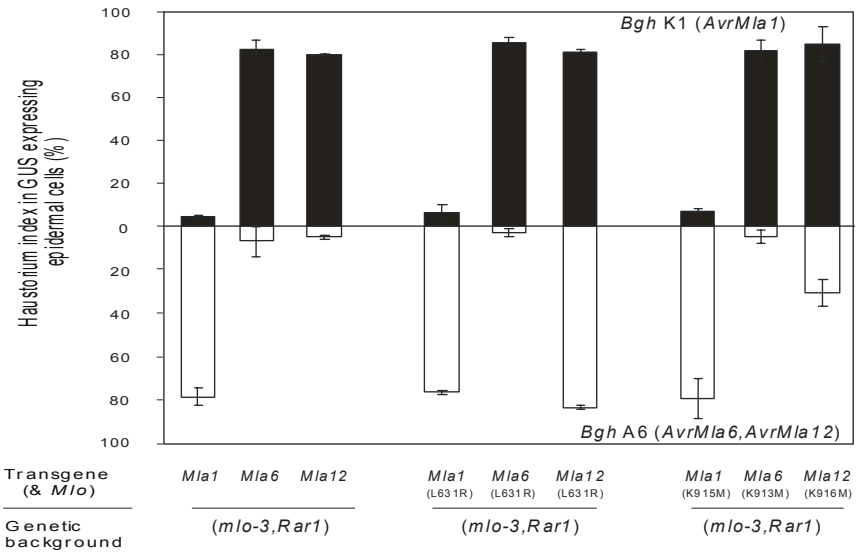

Figure 4. Context-dependent functions of conserved MLA residues Leu-631 and Lys-916.

Mean values of single cell resistance/susceptibility (%) are shown at left after delivery of *Mla1*, *Mla6*, or *Mla12* into the genetic background of cv Ingrid (*mlo-3 Rar1*). Results obtained with L631R variants of *Mla1*, *Mla6*, and *Mla12* are shown in the middle. Results obtained with *Mla1*, *Mla6*, and *Mla12* variants each containing a K to M substitution at the indicated positions are shown at right. Experimental conditions and designations are identical to those in Figure 2. GUS, β-glucuronidase.

3.6 Discussion: Altering resistance response kinetics by *Mla* dosage

An intriguing feature of *Mla*-mediated *Bgh* immunity is the diversity of macroscopically and microscopically visible infection phenotypes determined by different *Mla R* specificities. A quantitative analysis of single interaction sites in nearly isogenic lines containing different *Mla* genes revealed for *Mla1* and *Mla6* early termination of *Bgh* growth coincident with haustorium differentiation (Boyd et al., 1995). Rarely were interaction sites found permitting the development of elongating secondary hyphae. By contrast, *Mla3* and *Mla7* mediated cessation of fungal growth at a later stage of the infection process, permitting frequently the growth of elongating secondary hyphae on the leaf surface in addition to haustorium differentiation. These *Mla* gene-specific differences correlated with the timing of a cell death response that was either rapid, involving attacked epidermal cells, or slower, including epidermal and subtending mesophyll cells (Boyd et al., 1995). Similarly, a late cell death–associated resistance is characteristic for lines carrying *Mla12*, permitting indistinguishable fungal growth for up to 36 h after *Bgh* spore inoculation and a high haustorium index of ~60% on both *Mla12*-resistant and *Mla12*-susceptible mutant plants (Freialdenhoven et al., 1994). It is possible that differences in the timing of *Mla* resistance responses are the indirect consequence of different infection stage-specific delivery systems for particular *Bgh* AVRMLA effector proteins (e.g., delivery of AVRMLA12 only after or coincident with haustorium differentiation). Precedence for this idea is found in the expression of *Cladosporium fulvum AVR9*, which is induced strongly upon a switch from surface to intercellular growth of the fungus in leaves, which may be cued by fungal nitrogen starvation (Van Kan et al., 1991; Perez-Garcia et al., 2001).

In this study it has been shown that slow *Mla12*-triggered resistance was altered dramatically to a rapid response by *Mla12* over-expression, leading to almost complete abortion of *Bgh* attack before haustorium differentiation (Fig. 3). The retained *Rar1* dependence of the *Mla12* over-expression phenotype corroborates this as an authentic but kinetically altered

response. Because the rapid response retained AVRMLA12 dependence, the *Bgh* effector protein must be, like AVRMLA1 and AVRMLA6 (Halterman et al., 2001; Zhou et al., 2001) (Fig. 3), delivered before or during the switch from surface to invasive pathogen growth. The rapid *Mla12* over-expression response suggests that normal cellular amounts of MLA12 or protein complexes containing MLA12 are rate limiting for the onset or speed of the resistance. This finding is consistent with previous results demonstrating markedly reduced resistance in plants that are heterozygous for *Mla12* (Torp and Jørgensen, 1986). Further evidence that MLA levels are regulated *in planta* has recently been reported by Halterman et al. (2003). *Mla6* and *Mla13* transcription levels were induced rapidly between 16 to 24 h post inoculation of a incompatible pathogen. The time points correspond to that when the fungus made an intimate contact with the host plant, suggesting the transcripts accumulations are induced upon the pathogen recognitions (Halterman et al., 2003).

4. Structure and function analysis of MLA protein by domain swapping: the LRR-CT unit in MLA1 and MLA6 determines recognition specificity

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4.1. Introduction

Sequence analysis of isolated *Mla* alleles indicate a common structure of the CC-NB-LRR-CT type R proteins, sharing over 90% overall sequence similarity. The most divergent region is the LRR-CT part (~87% sequence similarity), consistent with the notion that the LRR domain might mediate protein-protein interaction, and hence the specificity determinant. Despite the overall similarity in sequence and in the gene structure, isolated *Mla* resistance specificities differ in the requirement for their function the *Rar1* and *Sgt1* gene (Jørgensen et al*,* 1996; Freialdenhoven et al*,* 1994; Schulze-Lefert and Vogel, 2000; summarised in Table 2). It was assumed that the existence of a convergence point in signalling pathways triggered by multiple *Mla* genes and suggests a function for *Rar1* and *Sgt1* downstream from pathogen recognition, whereas the functional independence on *Rar1* of *Mla1* and other *Mla* specificities points to the existence of more than one race-specific resistance signalling pathway (Schulze-Lefert and Vogel, 2000). How can both structural and sequence related NB-LRR-CT proteins at *Mla* locus mediate distinctive specificities and feed into separate signalling pathways?

4.2. The CT domain in MLA proteins is also subject to diversifying selection

Attempts to identify regions determining recognition specificity in R-genes point to the LRR regions in some successful domain swapping experiments, as well as region outside the LRR domain, for example, the TIR

Table 2. *Rar1/Sgt1* requirement of molecularly isolated *Mla* alleles

Allele	*Rar1* requirement	*Sgt1* requirement
Mla1	no	no
Mla6	yes	yes
Mla12	yes	yes
Mla13	yes	yes

Table 3. Comparison of average polymorphic residues in MLA and L NB-LRR proteins

Region	MLA[a]	L[b]
TIR	---	3.9
CC	1.5	---
NB-ARC	13.0	12.0
LRR	31.0	42.0
CT	19.0	---

[a] Calculated on the basis of MLA1, MLA6, MLA12 and MLA13

[b] Calculated on the basis of 11 L proteins (L1, L2, L5, L6, L7, L8, L9, L10, L11, L, LH)

domain of the L alleles for flax rust resistances can also determine specificity (Ellis et al, 1999; Luck et al, 2000). A comparison of average polymorphic residues for MLA and L proteins in different domains revealed that comparable polymorphism in corresponding domains (Table 3), implicating again that the respective LRR region is most relevant in specificity recognition. Unexpectedly, a relatively high polymorphism is also observed in the CT region of MLA compared to its CC and NB-ARC regions (Table 3). Moreover, in the middle of the CT domain a hypervariable region was identified, which shows an increased ratio of nonsynonymous (k_a = 15.4) to synonymous (k_s = 9.6) nucleotide substitutions [based on *Mla1*, *Mla6*, *Mla12*, and *Mla13* sequences (Halterman et al., 2003); significant at P < 0.1%], indicating the operation of diversifying selection and hence an important role in function or/specificity.

4.3. Domain swaps between MLA1 and MLA6 reveal determinants for recognition specificity reside in LRR-CT unit

A series of reciprocal domain swaps between *Mla1* and *Mla6* were constructed to identify regions that are critical for MLA function and respective specificities (Fig. 5). The maize ubiquitin promoter drove the expression of each chimeric gene, and their function was tested after bombardment into leaf epidermal cells by spore inoculation with *Bgh* isolates K1 (*AvrMla1*) and A6 (*AvrMla6*) at 15 h after delivery. Recognition specificity and activity of the chimeras were compared with those of the respective *Mla1* and *Mla6* wild-type genes whose expression was driven by either native regulatory 5' sequences or the strong ubiquitin promoter (Fig. 5A). No significantly different activity was seen using constructs driven by the native or the strong ubiquitin promoter. Full *AvrMla6*-dependent recognition specificity was retained in chimeras containing the complete MLA1-derived CC-NB domains and in chimeras containing progressively more MLA1-derived N-terminal LRR repeats (constructs 16666, 11666, and 11166; Fig. 5B). Activities mediated by chimeras containing only MLA6-derived LRRs 3 to 11 (11661) or only the

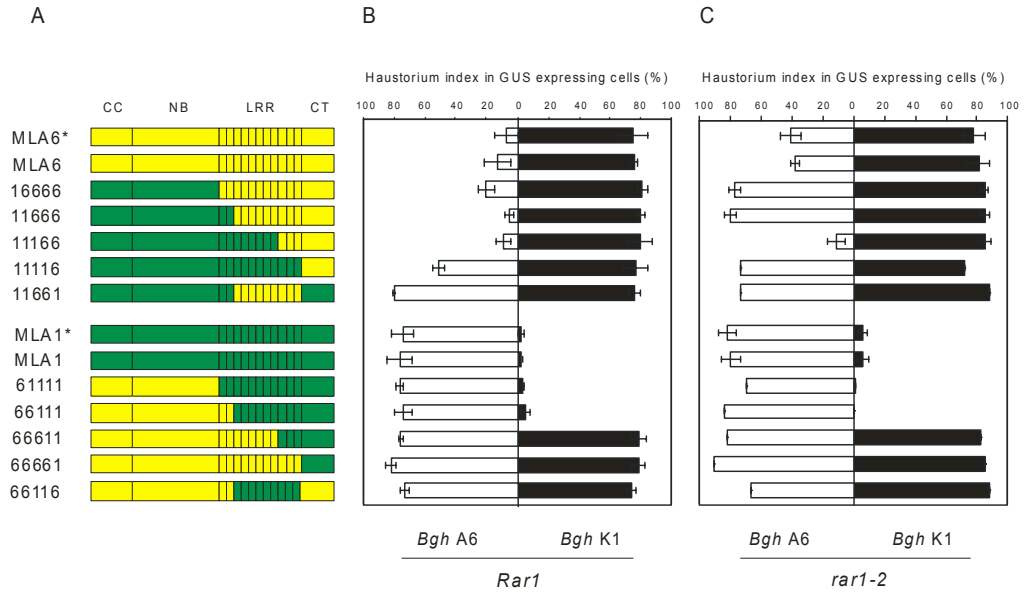

Figure 5. Domain swaps between MLA1 and MLA6 reveal determinants for recognition specificity and RAR1 dependence.

(A) Schemes of MLA6 (yellow), MLA1 (green), and 10 chimeras are shown. The relative positions of the CC, NB, LRR, and CT parts are indicated at top, and acronyms for each chimera are shown at left. The stars indicate gene expression driven by native 5' flanking sequences; the strong ubiquitin promoter drove the expression of all other genes.

(B) All genes shown in **(A)** were expressed in the *Rar1* wild-type background, and mean values of single cell resistance/susceptibility were scored microscopically upon challenge inoculation with *Bgh* isolates A6 or K1. Experimental conditions and designations are identical to those in Figure 2. GUS, β-glucuronidase.

(C) All genes shown in **(A)** were expressed in the *rar1-2* mutant background, and mean values of single cell resistance/susceptibility were scored microscopically upon challenge inoculation with *Bgh* isolates A6 or K1. Experimental conditions and designations are identical to those in Figure 2.

MLA6-derived C terminus (11116) were inactive or severely impaired, respectively. These data suggest that MLA6 LRRs 9 to 11 act together with the cognate C-terminal domain to confer *AvrMla6* recognition specificity.

Reciprocal domain swaps showed that *AvrMla1*-dependent activity was retained upon replacement of the entire MLA1 CC-NB domain only and upon additional replacement of LRRs 1 and 2 (constructs 61111 and 66111). Interestingly, longer substitutions up to LRR 8 rendered the 66611 chimera fully inactive, although the reciprocal construct 11166 fully retained *AvrMla6*-dependent activity. Substitutions containing LRRs 3 to 11 (construct 11661) also compromised *AvrMla1* recognition specificity. Because chimeras containing only MLA1-derived LRRs 3 to 11 (66116) or only the MLA1-derived C terminus (66661) were inactive, it is therefore concluded that MLA1-derived LRRs 3 to 11 together with the cognate C-terminal domain are required for MLA1 recognition specificity.

4.4. Uncoupling the RAR1 requirement from MLA6 recognition specificity

The sequence differences between *Mla1* and *Mla6* are unusually small, however, these differences affect both recognition specificity and the use of *Rar1*. As more divergence is observed in the LRR-CT region of both MLA, it is reasonable to assume that region(s) that affect the *Rar1* dependence are probably also located in the LRR-CT region. To gain more insight in that direction, the activities of wild-type MLA1 and MLA6, as well as the MLA chimeras were assessed in the *rar1-2* genetic background (Fig. 5C). The *rar1-2* mutation leads to a transcript-splicing defect, and a RAR1 antiserum fails to detect RAR1 signals on protein gel blots (Azevedo et al., 2002). Delivery of wild-type MLA1 or MLA6 plasmid DNA in *rar1-2* leaf epidermal cells led to fully retained or partially compromised resistance (4 and 39% haustorium index, respectively) (Fig. 5C). No significant differences were found between wild-type constructs driven by the native and strong ubiquitin promoters. Thus,

Mla6 function is compromised partially by the *rar1-2* mutation compared with bombardments of the same constructs in the *Rar1* background (Fig. 5B,C).

Remarkably, delivery of the three chimeras conferring *AvrMla6*-dependent resistance in *Rar1* plants (16666, 11666, and 11166) displayed either full RAR1 dependence (constructs 16666 and 11666, each showing 80% haustorium index) or uncoupled RAR1 dependence from recognition specificity (construct 11166, showing 10% haustorium index) in the *rar1-2* background. Neither of the two chimeras that retained *AvrMla1*-dependent resistance activity (61111 and 66111) was impaired functionally upon delivery in *rar1-2* mutant plants. Unless MLA6 accumulation is self-limited, the data suggest that RAR1 dependence cannot be overcome by *Mla6* over-expression and appears to be modulated by both the CC-NB and LRR regions.

It was observed that an *Arabidopsis rar1* mutant line failed to accumulate a CC-NB-LRR protein, RPM1 (Tornero et al. 2002). Therefore it is reasonable to also test whether MLA6 becomes unstable in the *rar1-2* mutant background. The activity of MLA6 can be inferred as its stability indirectly in the single cell assay. At 96 h after delivery, MLA6 remained as active as at 15 h after delivery (39% haustorium index), suggesting that MLA6 level required for its functionality is still present at 96 h after delivery in *rar1-2* plants (see below for examples of unstable MLA variants 16666 and 11666).

4.5. SGT1 is associated with RAR1 in MLA mediated resistances

Previously it was shown that barley *Sgt1* (*HvSgt1*) is required for *Mla6*- but not *Mla1*-mediated resistance using double-stranded RNA interference (dsRNAi) gene silencing of *HvSgt1* in a single-cell expression system (Azevedo et al., 2002). It is then prompted to examine in the *Rar1* wild-type background the SGT1 requirement of MLA chimeras that retain MLA6 recognition specificity (constructs 16666, 11666, and 11166 in Fig. 5A) using the same silencing techniques. In these experiments, *Bgh* spore inoculations were performed at 48 or 96 h after particle delivery, and the leaf tissue was fixed for microscopic analysis 48 h after spore inoculation (Fig. 6).

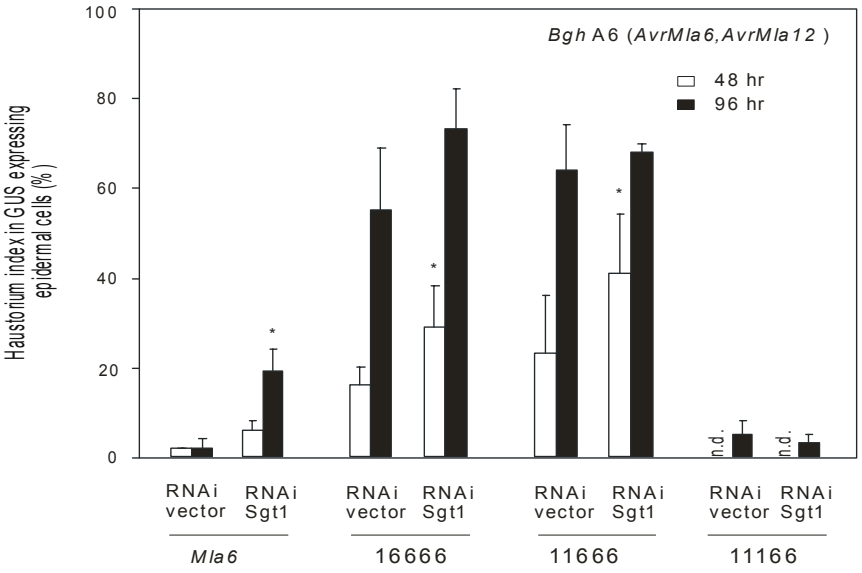

Figure 6. Single cell silencing of Sgt1 by dsRNAi.

Wild-type *Mla6* or chimeras retaining *AvrMla6*-dependent recognition specificity were coexpressed with a *HvSgt1* dsRNAi-silencing plasmid (Azevedo et al., 2002) in the *Rar1* wild-type background using a modified single cell transient gene expression assay (Azevedo et al., 2002). After delivery of plasmid DNAs into epidermal cells, detached barley leaves were incubated for 48 h (open bars) or 96 h (closed bars). Subsequently, leaves were inoculated with spores of *Bgh* isolate A6 (*AvrMla6*) and incubated for another 48 h. Microscopic scoring of single interaction sites was identical to that described for Figure 2. Asterisks indicate haustorium indices that are significantly different ($P < 0.05$) from bombardments using empty dsRNAi vector controls. GUS, β-glucuronidase; n.d., not determined.

Co-bombardment of SGT1 dsRNAi DNA with a plasmid driving wild-type *Mla6* from the ubiquitin promoter resulted in a small but significantly increased haustorium index (19% at 96 h after delivery) compared with delivery of an empty dsRNAi vector control (2%). This result is consistent with previous data (Azevedo et al., 2002). Unexpectedly, the functioning of chimeras 16666 and 11666 was partially impaired at 48 h after delivery in co-bombardment experiments with the empty vector dsRNAi control. This phenomenon was time dependent in that the chimeras were almost completely inactive at 96 h after delivery. This finding may indicate that the two chimeric MLA proteins are less stable or that fewer or less active recognition complexes are formed compared with complex formation in the MLA6 wild-type protein. Nevertheless, at 48 h after delivery, co-bombardment of plasmids 16666 and 11666 with SGT1 dsRNAi DNA significantly enhanced the haustorium index compared with that in empty vector controls ($P < 0.05$), indicating at least a partial requirement of the chimeras for *Sgt1*. By contrast, the 11166 chimeric protein retained full activity upon co-bombardment with the empty dsRNAi plasmid control, and its function remained unaffected by *Sgt1* silencing even at 96 h after delivery (Fig. 6). Unlike wild-type *Mla6*, *AvrMla6*-dependent resistance conferred by the 11166 variant appears to be uncoupled from both *Rar1* and *Sgt1* dependence (Fig. 5C and 6).

5. RAR1 is not sufficient to increase MLA steady-state protein levels in *Saccharomyces cerevisiae*

5.1. Introduction

It was previously reported that in uninfected *Arabidopsis* plants steady-state levels of the R protein RPM1 are greatly reduced in a *rar1* null mutant background (Tornero et al., 2003). Recently, a similar behaviour was observed for MLA proteins in transgenic barley plants in a *rar1* mutant background (Bieri S., MPIZ unpublished data). MLA6 protein level is significantly decreased in healthy *rar1* mutant plants. Surprisingly, MLA1 protein abundance is also reduced in *rar1* mutants although *Mla1* resistance was shown to function independently of *Rar1* (Jørgensen, 1996; Zhou et al., 2001). In *Rar1* wild-type plants, MLA1 accumulates to four-fold higher levels than MLA6, whereas in *rar1* mutants MLA1 abundance is decreased to similar levels as MLA6 in a *Rar1* wild-type background. Moreover, protein accumulation of chimera 11166, previously shown to recognize *AvrMla6* and to function independently of *Rar1* (Fig.5 and Chapter 4.4), accumulated to levels that are indistinguishable from MLA1 abundance in a *Rar1* wild type background. Conversely, steady state levels of chimera 11666, also recognizing *AvrMla6*, was much lower than wild type MLA6 abundance in *Rar1* wild type plants. The resistance activity of this chimera was fully inactivated in a *rar1* mutant background (Fig.5 and Chapter 4.4). Given the fact that RPM1 and MLA are NB-LRR R proteins from dicots or monocots respectively, it is possible that one function of RAR1 involves stabilization and/or folding of NB-LRR R proteins. It remains unclear whether this is a direct or indirect RAR1 activity. MLA protein accumulation appears to be intrinsically sensitive to elevated temperature *in planta* because protein levels of both wild-type MLA1 and MLA6 are markedly decreased after the

plants are shifted from 17 °C to 37 °C (Mauch S., MPIZ unpublished data). This phenomenon is not a general feature of plant proteins as Ponceau staining revealed an unchanged protein pattern before and after the temperature shift. In addition, proteins like HSP90, RAR1, and SGT1, that might regulate MLA stability, do not exhibit temperature sensitivity at the above tested temperature conditions. It remains unclear whether this temperature sensitive accumulation of MLA proteins is related to the potential role of RAR1 in NB-LRR stabilization and/or folding.

To further investigate the role of RAR1 in MLA protein stability, yeast was used for heterologous expression of MLA proteins in the present study. MLA1 or MLA6 C-terminal HA-tagged variants, as well as two HA-tagged chimeric protein variants (11166 and 11666) were expressed in the presence or absence of HvRAR1 in yeast cells. The potential role of RAR1 in MLA stabilization/folding was tested at standard yeast growth temperature of 30 °C and at an elevated temperature of 37 °C. Protein steady-state levels of respective MLA variants were evaluated and compared by Western blot analysis of yeast crude protein extracts.

5.2. RAR1 does not alter MLA steady-state protein levels in yeast at standard growth temperature

To express MLAs in yeast, the full-length cDNA of *Mla1* and *Mla6* were isolated by RT-PCR and subcloned into a shuttle expression vector under the control of the inducible *GAL* promoter. Sequences corresponding to the full-length cDNA of MLA chimeras 11166 and 11666 were also generated and subcloned into the same vector. A single HA epitope tag was added to the end of all *Mla* cDNA sequences for subsequent immunodetection. Four *Mla* containing plasmids were transformed into the same yeast strain using the LiAc transformation method (Gietz and Woods, 2002) and yeast transformants were selected by Tryptophan prototrophy.

For RAR1 expression in yeast, the full-length *Rar1* cDNA sequence was fused to a single myc epitope tag at the 3'-end of the sequence and

subcloned into expression vector *pLexA*. This vector served originally as bait vector in the LexA system and was modified in the present study such that *Rar1* expression is driven by the strong constitutive *Adh* promoter. In addition, sequences encoding the LexA DNA binding domain were removed. Presence of resulting expression vector *pQSHvRar1-myc* in yeast can be selected by histidine prototrophy. To achieve co-expression of RAR1 with the tested MLA derivatives, both *Rar1* and respective *Mla* containing plasmids were co-transformed into the same yeast strain and colonies were selected for the presence of both plasmids.

Yeast cultures were raised from strains containing one plasmid to express individual MLA derivatives alone or in the presence of HvRAR1. The yeast cultures were grown at 30 ^{o}C overnight and subjected to induction for 4 h. Cell pellets were obtained from yeast cultures grown to similar OD_{600} units. Crude extracts were made from these pellets by freezing in liquid N_2 and then boiling for 5 min for at least three rounds. Equal volumes of crude extracts dissolved in loading buffer were separated by SDS-PAGE. After immuno-blotting with the anti-HA antibody, MLA proteins were detected at the expected molecular weight in all samples (lane 2 to 9, top panel, Fig. 7). MLA1-HA expressed in transgenic barley plants showed an electrophoresis mobility that was indistinguishable from MLA1-HA expressed in yeast (lane1, Fig. 7). This indicates absence of plant- or yeast-specific modifications of the MLA protein. RAR1 appears to accumulate to similar levels in all yeast cell cultures expressing the tested MLA derivatives (bottom panel, lane 6 to 9, Fig. 7). A similar RAR1 level is observed in yeast cells expressing RAR1 alone or together with MLA proteins (lane 10, Fig. 7). MLA abundance was indistinguishable for each tested MLA derivative in the presence or absence of HvRAR1(top panel, lane 2 to 9, Fig. 7). In summary, full-length MLA proteins can be expressed in the heterologous yeast system but co-expression of RAR1 is apparently insufficient to elevate MLA steady-state levels. These finding shows that RAR1 does not directly affect MLA abundance in yeast or that MLA folding/stabilization might require additional plant-derived proteins.

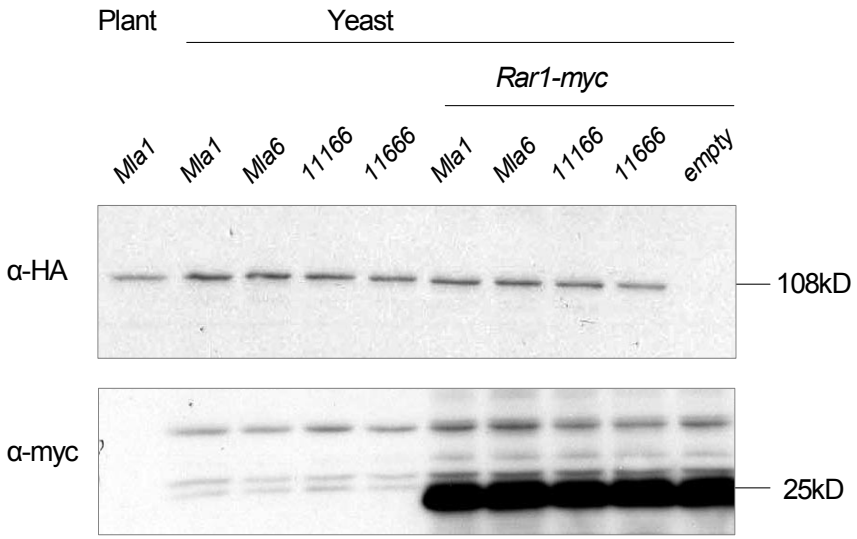

Figure 7. RAR1 is not sufficient to alter MLA steady-state protein levels in yeast.

Individual MLA-HA was expressed in yeast in the absence or presence of RAR1-myc. Yeast crude extracts were prepared after 6 hr of induction with galactose. Equal amounts of extract were separated on SDS-PAGE and were immunoblotted with anti-HA (top pane) or anti-myc (bottom pane) antibody. Crude extracts were from Mla1-HA transgenic barley leaves (lane 1); or from yeast expressing respective MLA-HA alone (lane 2-5), or co-expressing with RAR1-myc (lane 6-9); or from yeast expressing RAR1-myc alone (lane 10).

5.3. RAR1 does not impair MLA protein abundance in yeast at elevated temperature

To better understand the role of RAR1 in MLA accumulation, yeast strains transformed with plasmid expressing various MLA HA-tagged variants were subject to temperature shift experiments. Yeast cells were tested in the absence or presence of co-expressed HvRAR1. Cell cultures were cultivated at 30 ^{o}C overnight before induction of MLA-HA expression by galactose. Individual yeast cell cultures were then divided into two portions; one half was grown at 30 ^{o}C for 2 h and crude extracts prepared from these samples were used as controls. The other half was shifted to 37 ^{o}C for 2 h. All MLA derivatives accumulated to similar levels at 30 ^{o}C in a RAR1 independent manner (compare lane 2 to 5 with lane 11 to 14, Fig. 8). Likewise, at an elevated temperature of 37 ^{o}C, RAR1 did not significantly alter the abundance of any of the tested MLA derivatives (compare lane 11 to 14 with lane 15 to 18, Fig. 8). Thus, the observed temperature sensitive MLA accumulation *in planta* is not recapitulated in yeast cells despite co-expression of HvRAR1. This suggests a contribution of additional plant-specific components mediating *in planta* temperature sensitivity.

5.4. Discussion

Although biochemical analysis in both *Arabidopsis* and barley suggests that RAR1 may have a role in stabilizing NB-LRR R proteins *in planta* (Tornero et al., 2002; Bieri et al., unpublished), the exact molecular mechanism is still unclear. More recently, the cytosolic HSP90 family has been genetically identified as a component required for the function of some NB-LRR R proteins, e.g., *Arabidopsis* RPM1 and RPS2, potato RX, tobacco N (Hubert et al., 2003; Takahashi et al., 2003; Lu et al., 2003; Liu et al., 2004). Furthermore, biochemical evidences indicate HSP90 physically associates with RPM1 or N proteins *in planta* (Hubert et al., 2003; Liu et al., 2004). It was also demonstrated that HSP90 is critical for RPM1 and RX stability in plants. Mutant alleles of one of four genes encoding cytosolic HSP90 severely compromise RPM1 protein accumulation in *Arabidopsis* while VIGS silencing

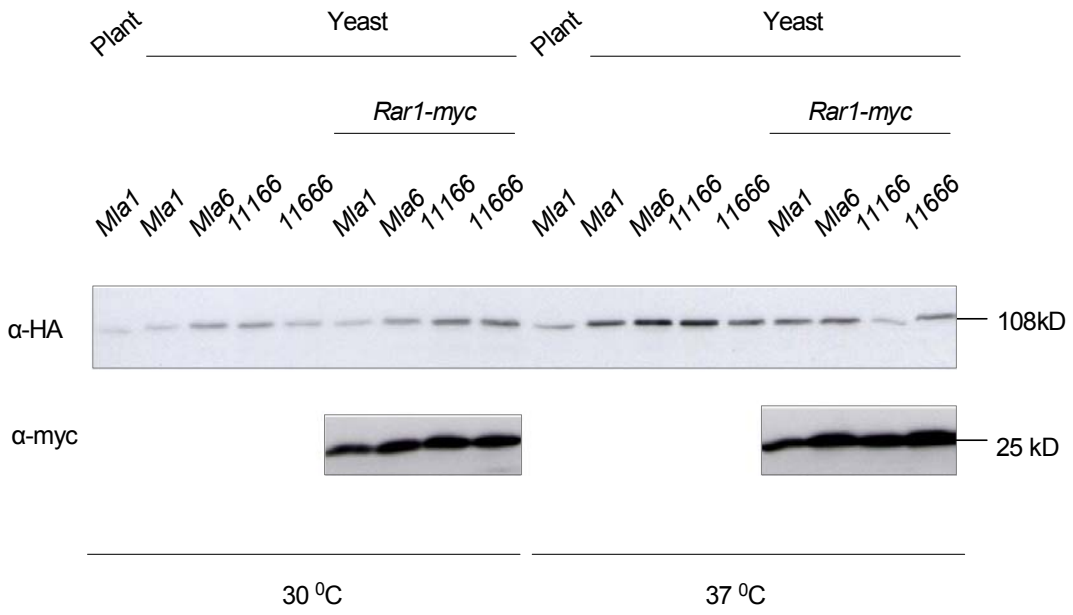

Figure 8. RAR1 does not alter MLA steady-state protein levels in yeast at standard and elevated growth temperature.

Yeast expressing individual MLA-HA alone or co-expressing with RAR1-myc were grown at standard (30 ^{o}C) or elevated (37 ^{o}C) growth temperature. Crude extracts were made from these yeast after galactose induction of MLA expression. Equal amounts of crude extracts were separated on SDS-PAGE and analysed by immunoblotting with anti-HA (top panel) or anti-myc (bottom panel) antibodies. Crude extracts were from *Mla1-HA* transgenic barley leaves (lane 1 and lane 10); or from yeast expressing respective MLA-HA alone (lane 2-5 and lane 11-14), or co-expressing with RAR1-myc (lane 6-9 and lane 15-18).

of *NbHSP90* impairs RX protein accumulation in *Nicotiana benthamiana* (Hubert et al., 2003; Lu et al., 2003). Interestingly, HSP90 has been co-immunoprecipitated with RAR1 in *Arabidopsis*, or shown to directly interact with RAR1 in yeast two-hybrid assays (Hubert et al., 2003; Takahashi et al., 2003). These findings may indicate that RAR1 assembles together with HSP90 and R proteins in a hetero-complex. Animal homologs of RAR1 and SGT1 share structural features (the CS domain) with other co-chaperones previously shown to bind HSP90 (Dubacq et al., 2002; Garcia-Ranea et al., 2002). Therefore, it has been proposed that R proteins might be HSP90 client proteins and RAR1 could function as HSP90 co-chaperone possibly regulating the stability and activity of NB-LRR R proteins (Hubert et al., 2003; Takahashi et al, 2003). However, how these heterocomplexes could regulate R protein activity/stability is still unknown.

In the present study, co-expression of HvRAR1 together with various MLA derivatives in yeast was not sufficient to alter MLA abundance. These findings differ from greatly reduced MLA levels seen *in planta* in *rar1* mutant plants. One possibility is that additional (yet unknown) plant components must be co-expressed in yeast to mimic the effects seen *in planta*. Potential candidates might be HvHSP90 and/or HvSGT1. In this model, RAR1 does not directly stabilize MLA. It is conceivable that RAR1 stabilizes MLA only if the latter is physically associated together with other (unknown) host factors in a presumptive 'recognition complex'.

MLA protein levels are markedly decreased at elevated temperature *in planta* (Mauch S., MPIZ unpublished). This might indicate that MLA proteins are intrinsically temperature sensitive. Alternatively, presumptive MLA containing hetero-complexes may become unstable at elevated temperature, thereby indirectly triggering degradation of free MLA *in planta*. In yeast cells, MLA abundance remained unchanged upon a temperature shift from 30 ^{o}C to 37 ^{o}C, suggesting that MLA proteins are not *per se* unstable in this eukaryote. However, since the standard growth condition of barley seedlings was at

approximately 20^{o}C, MLA accumulation in yeast should be re-evaluated at a comparable temperature.

6. Identification of MLA interactors using yeast two-hybrid selection

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6.1. The LexA yeast two-hybrid system and interaction mating method

6.6.1. The LexA yeast two-hybrid system

The LexA two-hybrid system is a LexA-based interaction trap for detecting specific protein-protein interactions in yeast (Gyuris et al., 1993; Mendelsohn & Brent, 1994). In this system the DNA binding domain (BD) in the bait vector is the entire bacterial LexA protein (Ebina et al., 1983). The activation domain (AD) in the prey vector is provided by an 88-residue acidic *E. coli* peptide (B42) that activates transcription in yeast (Ma & Ptashne, 1987). An interaction between a protein of interest (fused to the BD) and a library-encoded protein (fused to the AD) creates a novel transcriptional activator with binding affinity for *LexA* operators. This factor then activates reporter genes having upstream *LexA* operators, which makes the protein-protein interaction phenotypically detectable. A dual reporter system is used for detecting protein-protein interactions, consisting of: (*i*) the *LEU2* nutritional reporter gene, preceded by six copies of the *LexA* operator to which the DBD binds, is stably integrated into the yeast genome and its activation allows for Leucine autotrophic growth selection; (*ii*) the *LacZ* gene on an autonomously replicating plasmid is preceded by eight copies of *LexA* operator and allows for a β-galactosidase assay with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as substrate.

The LexA system provides several advantages compared to other genetic selection systems for protein-protein interactions. Firstly, the promoters of the two reporters differ in the sequences flanking the *LexA* operators, and this sequence dissimilarity helps to eliminate some false positive clones and to confirm the positive two-hybrid interactions. Secondly, the inducible yeast *GAL1* promoter drives the expression of AD-fusion

proteins. Inducible expression provides more opportunity for the prey fusion proteins with toxicity to the yeast host to survive and thus will less likely be eliminated from the pool of potentially interacting proteins. Thirdly, both the *LacZ* and *LEU2* reporters are under control of multiple *LexA* operators. This allows several BD-bait fusion proteins to bind to each promoter, thereby effectively amplifying the intensity of weak signals (Golemis et al., 1996).

6.1.2. The interaction mating method

The interaction mating method takes advantage of the fact that yeast haploids of two opposite mating type can mate to form diploid cells and hence presents a convenient method of introducing two different plasmids into the same yeast cells (Finley & Brent, 1994; Harper et al., 1993). This method provides at least three benefits:

1). It significantly reduces the labor and time involved in performing a two-hybrid library screening when several baits will be used to screen a single library. In this case, different baits transformed into yeast of one mating type can be used in parallel to screen a library transformed into an opposite mating type yeast in a single high-efficiency transformation, hence eliminates the need for many library-scale yeast transformations (which can vary 10- to 100-fold in their efficiency). It is especially useful when a constitutively expressed bait interferes with yeast viability. In such cases it is difficult to achieve high-efficiency transformation.

2). In the interaction mating method the actual selection for interactors will be conducted in diploid yeast cells, which are more vigorous than haploid yeast and generally can better tolerate expression of toxic proteins. Thus, it may improve the chances of finding rare protein-protein interactions.

3). In diploids the reporters are less sensitive to transcription activation than they are in haploids. It may provide an additional way to reduce the background from baits that weakly auto-activate transcription of reporters.

6.2. Construction of multiple LexA-MLA fusion baits using domains or full length sequences of MLA1 and MLA6

MLA proteins belong to the CC-NB-LRR-CT subclass of R proteins. Operationally defined domains or regions in R proteins, e.g. CC, NB-ARC and LRR-CT, are structurally distinct and are believed to have a distinctive function (Ellis et al, 2000; Dangl and Jones, 2001; the present study). Nevertheless, indirect evidence suggests conformational alterations in the potato RX protein after perception of the corresponding effector protein (Moffett et al., 2002; Hwang and Williamson, 2003). This suggests that the full-length protein in non-challenged plants might exist in a closed conformation that might prevent interactions with a subset of potential host interactors. To bypass this potential complication, LexA fusion baits were constructed by using partial cDNA sequences encoding distinct or overlapping domains (i.e. CC, NB, CC-NB and LRR-CT) of either MLA1 or MLA6. In addition, fusion baits using both full-length *Mla1* and *Mla6* cDNA were also constructed (Fig. 9).

Other considerations were also taken into account when making baits. First, preliminary observations indicated that fusion bait with the entire MLA1 or MLA6 CC domain activates reporters in the absence of a fusion prey protein. Therefore, truncations at either C- or N-terminal, or at both ends of the CC domains were introduced to make multiple CC-containing bait variants (Fig. 9, only one truncated variant is shown). Second, mutagenesis of genes encoding the NB-LRR R proteins RX or L identified in either protein single amino acid substitutions in a conserved motif of the NB-ARC domain that confers either AVR-effector independent HR, dwarfism, or lethality (Bendahmane et al., 2002, Jeff Ellis unpublished data). The corresponding conserved residues in MLA proteins are located at the C-terminal end of the NB-ARC domain and define a so-called ‘VHDM’ motif . Mutants carrying these amino acid substitutions confer (Bendahmane et al., 2002, Jeff Ellis unpublished data). It is proposed that these gain-of-function phenotypes might disrupt intramolecular interactions in the NB-ARC region (Bendahmane et al.,

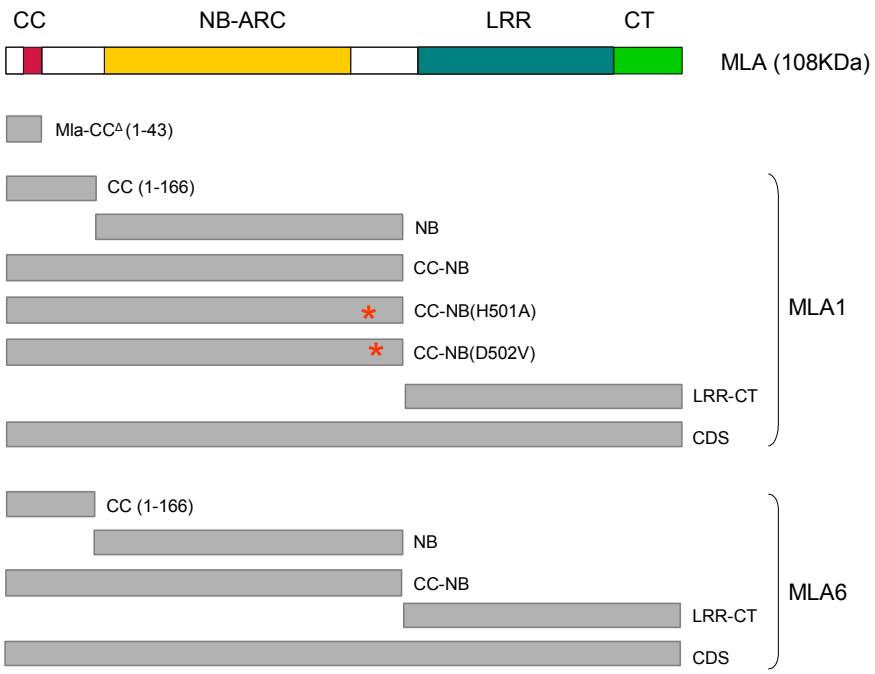

Figure 9. Graphic representation of MLA full-length protein and MLA baits used for yeast two-hybrid screenings.

Modular structure of MLA are shown on the top. The name of the baits are shown on the right. The Mla-CC$^{\Delta}$(1-46) bait containing a truncated CC domain and is conserved between MLA1 and MLA6 sequence. The relative position of the amino acid substitution in the VHDM motif is shown in star in two mutated versions of MLA1-CC-NB bait.

2002). To increase the chances of identifying MLA interactors, single amino acid substitutions were introduced into the VHDM motif in MLA1, generating fusion baits MLA1-CC-NB (H501A) or MLA1-CC-NB (D502V). In total, 18 different LexA-MLA fusion baits were constructed (Fig. 9, some autoactivating baits are not shown).

6.3. Transforming yeast strain EGY48 (*MATα*) with bait plasmid and characterization of bait strains

To perform a yeast two-hybrid screen by the interaction mating method, the bait plasmids and the prey library DNA were separately pre-transformed into two yeast strains of different mating types. The resulting bait strains were then tested for reporter autoactivation and only those that did not activate the reporters were used for subsequent screenings. Individual MLA bait constructs were transformed into the yeast strain EGY48 (mating type: *MAT**α***) that carries an autonomous plasmid (p8op-*LacZ*) in which the *LacZ* reporter gene is integrated. All resulting bait strains were then tested for reporter gene activation by each baits itself. Unexpectedly, almost all baits containing the CC domain autoactivated both reporters (*LacZ* and the *LEU*) for unknown reasons, except MLA-CC$^{\Delta}$(1-46) that contains only the first 46 amino acids of the MLA N-terminus. Other baits did not activate the two tested reporters. Notably, baits containing either wild type MLA1-CC-NB or MLA6-CC-NB were not autoactive, and the same is true for two variants containing mutant VHDM versions, MLA1-CC-NB(H501A) and MLA1-CC-NB(D502V), although they contain the intact CC domain.

Some bait strains were also tested for the expression of the fusion proteins. Crude protein extracts from individual bait strain cultures were prepared and separated by SDS-PAGE. The fusion proteins were detected by immuno-blotting using a commercial LexA-antiserum. All bait strains tested express individual bait proteins to a level that was easily detectable (Fig. 10). However, two full-length fusions (MLA1-CDS and MLA6-CDS) proteins accumulated to lower levels compared to other bait proteins (Lane 5 and 11,

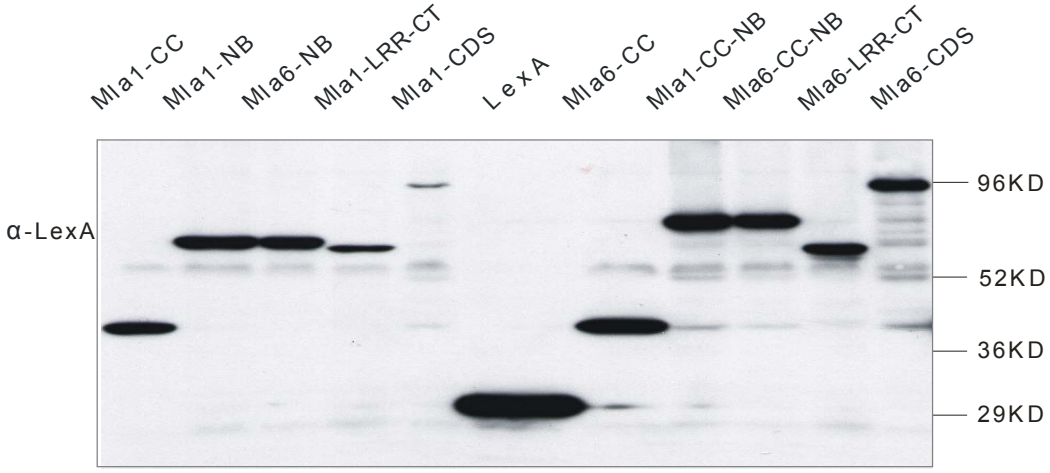

Figure 10. LexA-MLA fusion bait proteins are expressed in yeast.

Yeast culture of respective bait yeast strains were raised overnight. Crude extracts were made from these cultures and separated on SDS-PAGE. Anti-LexA antibody was used for subsequent immunoblotting detection of bait protein expression. Crude extracts were from yeast expressing either full-length CC domains (lane 1 and 7), NB-ARC domains (lane 2 and 3), LRR-CT regions (lane 4 and 10), CC-NB-ARC regions (lane 8 and 9), both full-length proteins (lane 5 and 11), or the LexA DNA binding domain alone (lane 6).

Fig. 10). In addition, the size of these two fusion proteins is smaller than the expected full-length protein (~130 KDa), indicating a possible truncation from the C-terminal ends for unknown reasons (Lane 5 and 11, Fig. 10).

6.4. A barley prey library suitable for yeast two-hybrid selection by mating type

A barley prey library was created using cDNA synthesized from poly(A)+RNA isolated from mixed leaf tissue samples of barley cultivar Sultan5. Samples were collected from healthy leaf tissue and from *Bgh* challenged leaf material at 6, 12, and 24 h post inoculation with spores of an incompatible A6 isolate (Piffanelli P., and Schulze-Lefert, unpublished data). High purity library plasmid DNA was obtained from crude DNA preparations from *E. coli* cell cultures using the CsCl gradient ultra-centrifugation method. Subsequently, the library DNA was used to transform the YM4271 strain (mating type: *MAT***a**). In total, ~ 2 x 10^6 independent yeast transformants were obtained. This prey library was amplified when the transformants were selected and collected from selection media. Glycerol stocks of this library are kept as individual aliquots at -80 ^oC and can be used for multiple mating type protein-protein interaction screens. The plating efficiency of the frozen cells was determined thereafter at about 5x10^7 CFU/100µl for library glycerol stocks.

6.5. Library screenings using interaction mating methods

For interaction mating, the haploid yeast strain containing the MLA bait was mated with the haploid yeast cells expressing individual prey to allow formation of diploid yeast cells. Briefly, respective EGY48 (p8op-LacZ) (*MAT***α***)* bait yeast cells were used to raise overnight cultures and appropriate OD units of these cultures were combined with YM4271(*MAT***a***)* cells (ratio of 2.5:1).. The mixtures were subjected to mating procedures and the resulting diploid cells were then induced for the expression of the AD-library proteins. On average, mating efficiency was determined to be ~10% (the ratio of zygotes versus viable *MAT***a** cells). On average, each of the baits screened

~$10x10^7$ viable library cells and ~$10x10^6$ diploid cells (i.e. double transformants) were obtained from each screening. Approximately $6x10^6$ diploid cells were plated on inducing synthetic dropout plates lacking Leucine (Gal/Raf/SD/-Leu) to select for positive interaction events between individual bait and library prey fusion proteins that activate the *LEU* reporter. The appearance of colonies was checked during 2-5 days after plating. Individual colonies emerging on the selection media were picked to proceed for subsequent analysis (Fig. 11 for an overview).

6.6. Characterization of cDNA clones isolated from the prey library

6.6.1. Eliminating false positive clones

False positive clones are common in yeast two-hybrid screenings. Two major types of false positive clones might arise in the LexA system. First, some AD-prey fusions may interact with the *LexA* operator flanking promoter sequence, which may activate the reporters. Second, activation of reporter might occur independent of the expression of a library cDNA prey protein. The former class of false positive clones can be eliminated by using the second reporter (*LacZ*) differing in promoter sequences from the one in the *LEU* reporter. The latter type of false positive clones can be eliminated by assessing reporter gene activation under conditions in which the prey proteins are not induced. All colonies picked were replicated on non-inducing media plates to turn off the expression of library proteins under the control of *GAL1* promoter in the prey vector. Colonies were replicated to different indicator plates to reveal these two major types of false positive clones in the LexA system according to the above criteria.

By comparison of the colony numbers before and after the secondary selection described above, it became obvious that most MLA baits identified many false positive interactors from the library (compare lane 2 and 3, table 4). Only few MLA baits identified most of the potential interactors (lane 3, table 4). For example, screenings using MLA-CC$^{\Delta}$(1-46) or MLA1-LRR-CT as bait identified 154 and 72 clones after false-positive elimination steps,

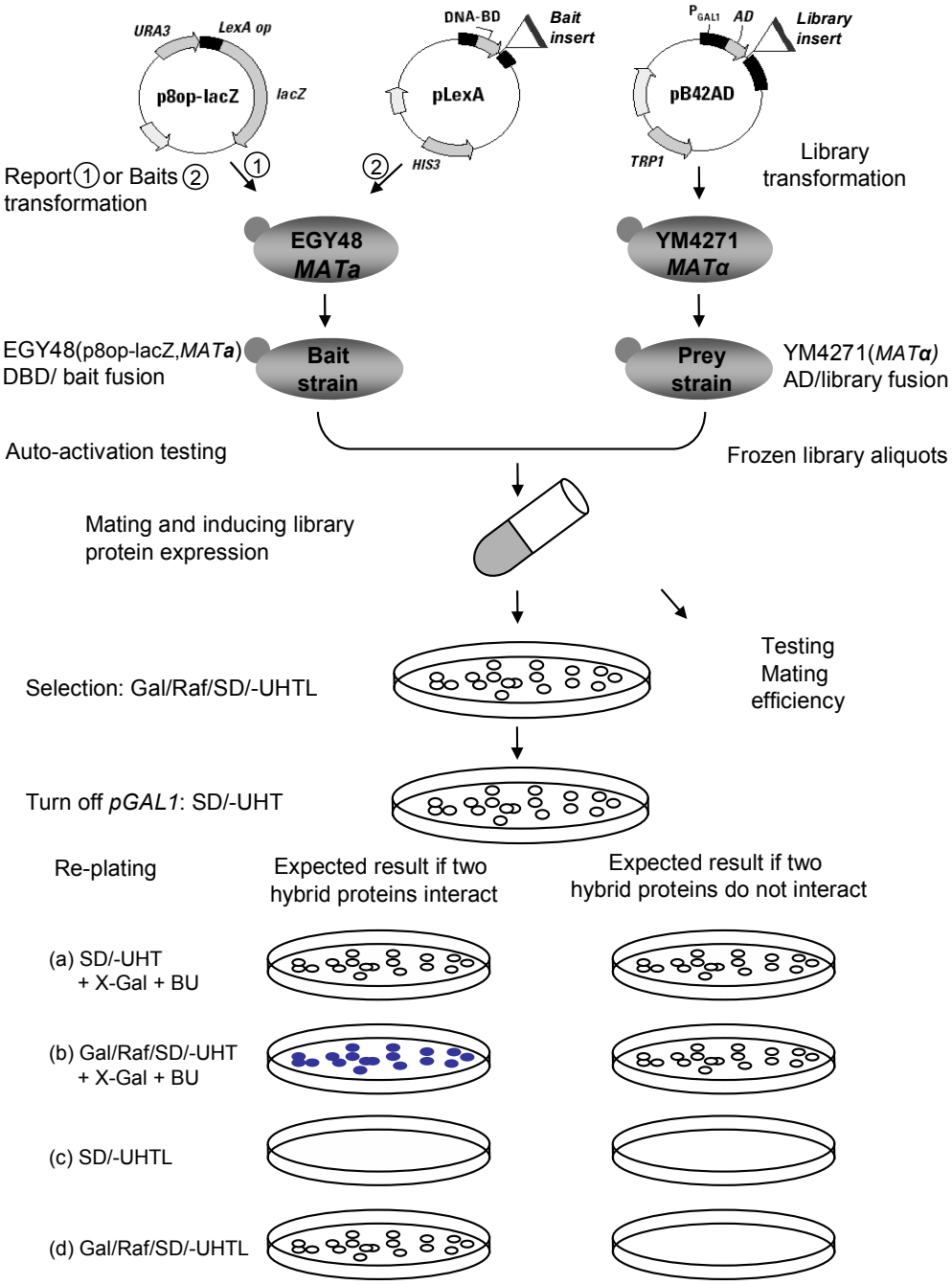

Figure 11. Diagram of major steps of a yeast two-hybrid screening using the interaction mating method.
Expression of the library fusion protein is induced in galactose and raffinose containing medium. Further characterizations of putative interactors are not shown here.

respectively. To process the large number of remaining prey clones, it was necessary to group these further using a DNA fingerprinting method.

6.6.2. Discriminating non-redundant clones from redundant ones

It was expected that several identical prey clones would have passed the genetic selection if the corresponding mRNA accumulates constitutively to high levels or increases in abundance upon *Bgh* pathogen challenge. To eliminate such redundant clones, cDNA inserts were PCR amplified from each prey plasmid DNA using primers flanking the inserts. Amplified products were subsequently digested with restriction enzymes that recognize frequently occurring restriction sites in cDNAs (e.g. *HaeIII*). Those prey clones displaying identical digestion patterns after gel electrophoresis likely represent either identical or highly sequence-related cDNAs. By this means it was possible to identify 89 distinct prey cDNAs with an average insert size of 1.2 kb. Thus, the molecular fingerprinting method eliminated about 90% of the genetically selected 824 prey clones. DNA sequencing was then used for further classification of the identified 89 prey cDNA clones (table 4).

6.6.3. MLA proteins/domains associate with structurally distinct host proteins

A potential sequence relatedness of the identified MLA interactors with known proteins was analyzed using either direct nucleotide-nucleotide BLAST (blastn) or translated polypeptide protein-protein BLAST (blastp) algorithms (Table 5). Some deduced MLA interactors were identified several times in the yeast two-hybrid screens described above although the molecular fingerprinting of the corresponding cDNA clones indicated in many cases clear differences between individual clones. For example, oxygen evolving

Table 4. Selection of prey clones interacting with MLA baits during yeast two-hybrid screenings by interaction mating methods

BD bait	Colonies picked from selection media	Colonies after elimination of false positive clones	Prey clones used for sequencing after DNA fingerprinting
Mla-CC$^{\Delta}$(1-46)	188	154	38
Mla1-CC-NB	20	0	0
Mla6-CC-NB	24	1	1
Mla1-CC-NB(H501A)	50	4	4
Mla1-CC-NB(D502V)	94	8	4
Mla1-NB	94	3	3
Mla6-NB	24	1	1
Mla1-LRR-CT	116	72	32
Mla6-LRR-CT	94	1	1
Mla1-CDS	72	0	0
Mla6-CDS	48	5	5
In total	824	249	89

Table 5. Deduced functions of MLA interactors isolated by yeast two-hybrid screenings.

MLA interactors	Times identified [a]	Bait							
		Mla-CC^A(1-46)	Mla1-LRR-CT	Mla1-CC-NB(H501A)	Mla1-CC-NB(D502V)	Mla6-LRR-CT	Mla1-NB	Mla6-NB	Mla6-CDS
Oxygen evolving protein	20	+							
Carbonic anhydrase	10	+							
Putative WRKY TF	2	+							
bZIP TF	1	+							
Zn transporter	1	+							
Formin protein	1	+							
SGT1	29		+						
Unknown protein	1		+						
3-isopropylmalate dehydrogenase	16			+	+	+	+	+	+

[a] The same interactor may be isolated independently several times as indicated because cDNA clones of the same interactor of variable length are present in the prey library.

+ denotes interaction.

protein and carbonic anhydrase were identified by the MLA-CC$^{\Delta}$(1-46) bait 20 and 10 times, respectively (Fig. 12; Fig. 13). Length variations of cDNAs encoding oxygen evolving protein and carbonic anhydrase can explain why many prey clones were originally assigned to different groups after the DNA fingerprinting. Because DNA sequences were obtained only from the coding DNA strand, comprising on average about 600 bp of prey cDNA, the sequences might still contain few sequencing errors. Despite this caveat, the alignment of carbonic anhydrase cDNA sequences obtained from 10 independently isolated prey clones provides no evidence that these are derived from different sequence-diverged gene family members (see Discussion below). Likewise, the 20 prey cDNA sequences encoding variable-length oxygen evolving proteins are also likely derived from the same gene.

Two prey clones each encoding a putative WRKY transcription factor were also isolated by the MLA-CC$^{\Delta}$(1-46) bait (Table 5). Finally, single prey clones encoding either proteins with homology to bZIP type transcription factors, or Zn^{2+} transporters, or a Formin related protein associated with the MLA-CC$^{\Delta}$(1-46) bait. Interestingly, HvSGT1 was isolated from the library 29 times by the MLA1-LRR-CT bait but not by the corresponding MLA6-LRR-CT bait. This is remarkable since these two MLA LRR-CT regions share ~87% amino acid sequence identity (Table 5).

Six different MLA1 and MLA6 baits identified prey encoding 3-isopropylmalate dehydrogenase (*Leu2* in yeast nomenclature). This enzyme convert β-isopropylmalate to α-ketoisocaproate in the Leucine biosynthesis pathway operated in eubacteria, archaebacteria, fungi and plants (Kohllaw 2003). This pathway is believed to be of ancient original and the catalytic functions of the enzymes in this pathway are largely unchanged throughout evolution (Kohllaw 2003). In the present yeast two-hybrid screening, one of the selection markers used is Leucine prototrophy. It is possible that the prey clones encoded 3-isopropylmalate dehydrogenase complemented the *leu2* auxotrophic mutant phenotype and suvived the selection. Thus, these prey clones are most likely false positives. Indirect evidence comes from DNA sequence similarity analysis, which showed that these prey cDNA sequences

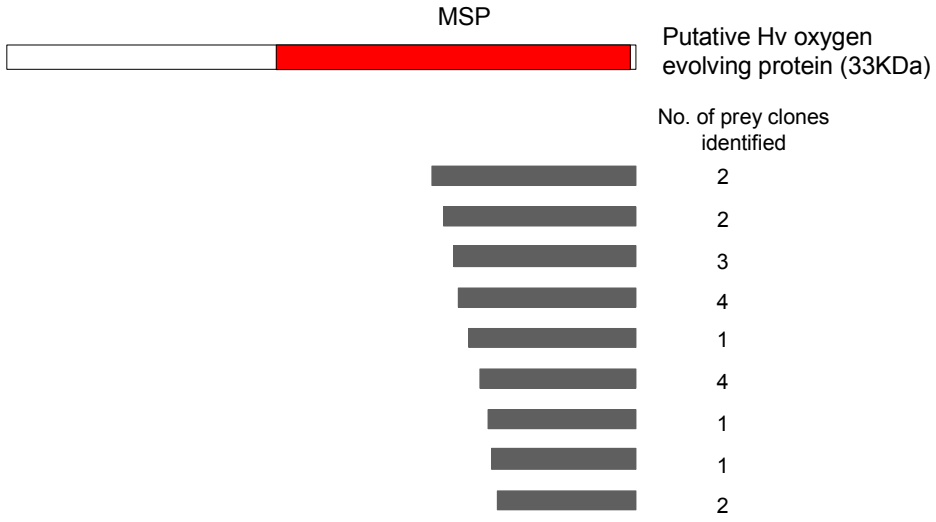

Figure 12. Graphic representation of putative barley oxygen evolving protein and the prey clones identified in the screening by the MLA-CC$^{\Delta}$(1-46) bait.

The relative start and end position of the prey clones are deduced by sequence comparison to the putative barley oxygen evolving protein. The same prey clone was identified independently different times as indicated. MSP, Manganese-stabilising protein conserved domain.

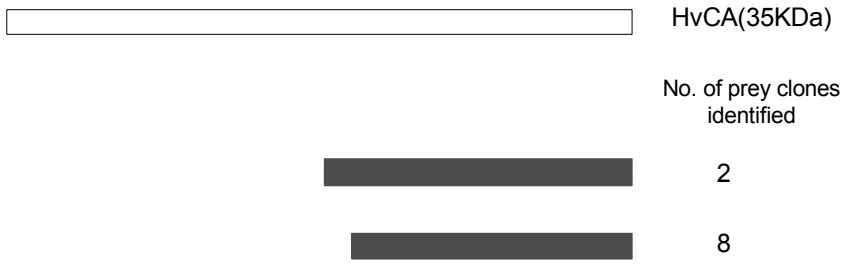

Figure 13. Graphic representation of barley carbonic anhydrase and deduced prey proteins identified in the screening by the MLA-CC$^{\Delta}$(1-46) bait.

The relative start and end position of the prey clones are deduced by sequence comparison to the barley carbonic anhydrase. The same prey clone was identified independently different times as indicated.

encoding 3-isopropylmalate dehydrogenase share higher similarity to fungus-derived 3-isopropylmalate dehydrogenase cDNAs compared to the homologs present in the barley EST database. It is noteworthy that the leaf samples used for prey library construction were taken at various time points after *Bgh* spore inoculations. It was expected that mRNA made from these samples contains also *Bgh*-derived cDNAs. Therefore, it is likely these cDNA inserts are derived from the *Bgh* pathogen.

6.7. Summary and perspective

A yeast two-hybrid screening method that exploits mating of yeast strains with opposite mating types was used to identify MLA interactors. A major advantage of this approach is the possibility of using frozen aliquots of an established prey library stock in yeast strain of one mating type; hence transformation of the prey library in every single screening becomes unnecessary. In the present study, efforts have been made to combine the interaction mating method with the LexA-based yeast two-hybrid system. Extensive optimizations have been applied to mating, selection and subsequent clone characterization steps. A barley cDNA prey library was transformed into yeast strain YM4271 (mating type:*MATa*). After amplification, aliquots of this library were kept as yeast glycerol stocks. It was demonstrated here that it is possible to screen this library many times independently, each time with several baits in parallel. This substantially reduces the time and the costs normally associated with conventional selection methods, enabling parallel screens with diverse baits (Soellick and Uhrig, 2001).

A total of 18 different baits, encoding single domains, two domains or full-length MLA proteins, were originally constructed for the interaction mating screenings. Upon elimination of auto-active baits, 11 could be used to perform screenings. Two baits, MLA-CC$^{\Delta}$(1-46) and MLA1-LRR-CT, identified potentially interesting interactors. One possibility is that these MLA domains preferentially mediate protein-protein interactions. It is worth noting that most of the N-terminal MLA baits containing a full-length CC domain auto-activated the reporters whereas both tested wild type CC-NB-ARC baits did not. This

may suggest that in the CC-NB-ARC baits the CC domain interacts intramolecularly with the NB-ARC region, thereby forming a closed conformation that prevents interaction with prey proteins. This is consistent with experimental data indicating that RX protein activation involves disruptions of intramolecular domain interactions, including the CC and NB-ARC (Moffett et al., 2002). Furthermore, the MLA1-CC-NB(H501A) and MLA1-CC-NB(D502V) did not identify any interacting proteins. This might indicate that the amino acids substitutions introduced in the VHDM motif of MLA are not sufficient to disrupt the potential intramolecular interaction between the CC and NB-ARC domains.

HvSGT1 was identified several times by the MLA1-LRR-CT bait (Table 5; Fig. 14). Since *SGT1* has been shown to be required for many *R* gene mediated resistance responses, the detected physical association in yeast may have significance *in planta*. Interestingly, the finding reported here is reminiscent of the physical association between yeast SGT1 and the yeast LRR-containing adenylyl cyclase (Dubacq et al, 2002). Recently it has been demonstrated that *Arabidopsis* or barley SGT1 co-immunoprecipitates with HSP90 *in planta* (Hubert et al., 2003; Takahashi et al., 2003). Furthermore, SGT1 shares structural features (the TPR and CS domain) with Hop/Sti1 and p23 co-chaperones (Dubacq et al., 2002; Garcia-Ranea et al., 2002). It has therefore been proposed that SGT1 might act as co-chaperone in plants to bind to HSP90 and to regulate R protein stability/activity (Hubert et al., 2003; Lu et al., 2003; Takahashi et al., 2003; Liu et al., 2004). In this context it would be interesting to find out whether yeast HSP90 is also involved in the MLA1-LRR-CT and SGT1 interaction. By extrapolation, it is conceivable that SGT1 associates with MLA proteins in a complex containing HSP90. (see also General Discussion).

Barley carbonic anhydrase (CA) was isolated several times with the MLA-CC$^{\Delta}$(1-46) bait (Table 5). So far two CA isoforms are known to exist in the barley genome and differ only in the 3' untranslated region (Bracey and Bartlett., 1995). The transcripts are derived from nuclear genes but the

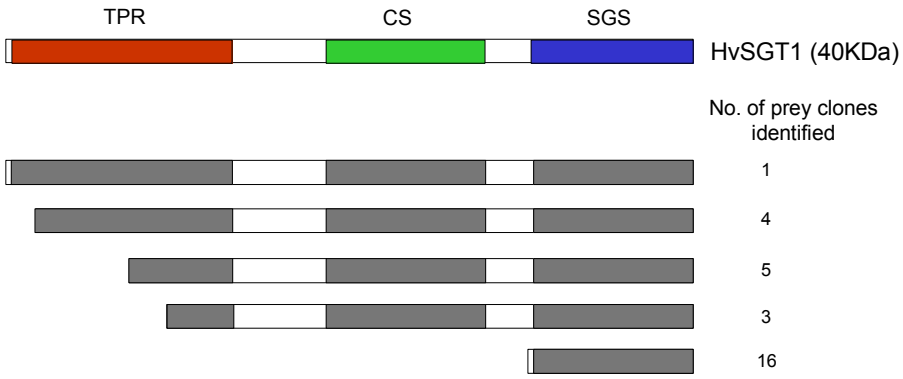

Figure 14. Graphic representation of HvSGT1 protein and the prey clones identified in the screening by the MLA1-LRR-CT bait.

The modular structures of the HvSGT1 protein are shown on the top and the structures of the prey clones were deduced by comparison to the HvSGT1 sequence. The same prey clone was identified independently different times as indicated.

encoded proteins are transported into chloroplasts. It is unknown yet whether other barley CA isoforms are present in the barley genome. Tobacco CA was previously shown to bind to salicylic acid (SA) and is thought to contribute to antioxidant activity by complementing the ability of a CA-like deletion yeast strain to grow aerobically (Slaymaker et al., 2002). Interestingly, silencing of CA gene expression in tobacco leaves suppresses the *Pto*:*avrPto*-mediated HR response, indicating that CA is required for a cell death response upon activation of race-specific resistance. In *Mla* mediated resistance responses, HR cell death is normally confined to epidermal and underlying mesophyll cells at sites of attempted fungal invasion (Hückelhoven et al., 1999). It would be interesting to find out whether barley CA is involved in the HR response that is mediated by MLA R proteins. One might speculate that the biotrophic *Bgh* fungus evolved means to manipulate (inactivate) host factors that are required for R protein triggered resistance responses. In this scenario, MLA proteins might survey (guard) CA proteins that are in transit to the chloroplast. In this context, it should be interesting to find out whether *Bgh* AVR effectors associate with barley CA.

A barley Formin homolog containing the Formin Homology 2 Domain (FH2) was also identified by the MLA-CC$^{\Delta}$(1-46) bait. Formin proteins in animals and fungi control rearrangements of the actin cytoskeleton and are involved in cell polarity processes (Deeks, M., et al., 2002)). Members of this family have been found to interact with Rho-GTPases, profilin and other actin-associated proteins and also play roles in signal transduction processes. Both pharmacological and genetic evidence suggest that actin cytoskeleton rearrangements and processes controlling cell polarity are involved in resistance responses to *Bgh* at the cell periphery. These broad-spectrum resistance responses to powdery mildews and other fungal pathogens prevent a switch from surface to invasive fungal growth (Collins et al., 2003).

AvrMla10 and *Avrk1* have been recently cloned from the *Bgh* genome (Ridout et al., JIC, unpublished). Sequence comparison of the deduced proteins encoded by these two *Avr* genes revealed a conserved core region

sharing 56% identical residues whereas both N- and C-termini are highly sequence divergent. This provides a future opportunity to identify host factors that might be targeted by the fungal effectors using the yeast two-hybrid screening system established in this work. Multiple LexA-AVR fusion baits are under construction and these baits will be used to screen the barley prey library (see also General Discussion).

7. General discussion

7.1. Allelic variants encode MLA R proteins

Eight *NB-LRR* genes are present in a 260-kb interval comprising the *Mla* locus in barley cv Morex and were classified in three dissimilar families (*RGH1*, *RGH2*, and *RGH3*) with <43% amino acid sequence similarity between families (Wei et al., 2002). Computational analysis of the Morex 260-kb sequence contig suggested that a progenitor *Mla* locus harbored at >8 million years before the present one member of each *RGH* family (*RGH1bcd*, *RGH2a*, and *RGH3a*) (Wei et al., 2002). Each of the *Mla* powdery mildew *R* genes identified to date shows highest overall sequence similarity to Morex *RGH1bcd* in coding regions and shares the same exon/intron structure (Fig. 15) (Wei et al., 2002). Unlike *RGH1bcd*, however, each of *Mla1*/*6*/*12*/*13* contains a 5' untranslated open reading frame and, within intron 3, an $(AT)_n$ simple sequence repeat consisting of different repeat numbers (Fig. 15). Also, Morex *RGH1bcd* contains a *BARE1* solo long terminal repeat in intron 3 that is absent in *Mla1/6/12/13*, and the presence of a 29-bp deletion in the LRR region, resulting in a premature stop codon, suggests that it is nonfunctional (Fig. 15). Because Morex lacks a known *Mla* powdery mildew resistance specificity, it has been inferred that *RGH1bcd* is a naturally inactive allele that may have served as progenitor for the other Morex *RGH1* family members (*RGH1a*, *RGH1e*, and *RGH1f*) (Wei et al., 2002).

DNA gel blot analysis and preliminary sequence information obtained from near-isogenic barley lines containing other *Mla* powdery mildew resistance specificities indicate for each line the presence of at least one candidate gene with high sequence relatedness to MLA1/6/12 (Bieri S., Pajonk S., et al., MPIZ, unpublished). Thus, it is possible that most if not all genetically characterized powdery mildew *R* genes at *Mla* are alleles of the

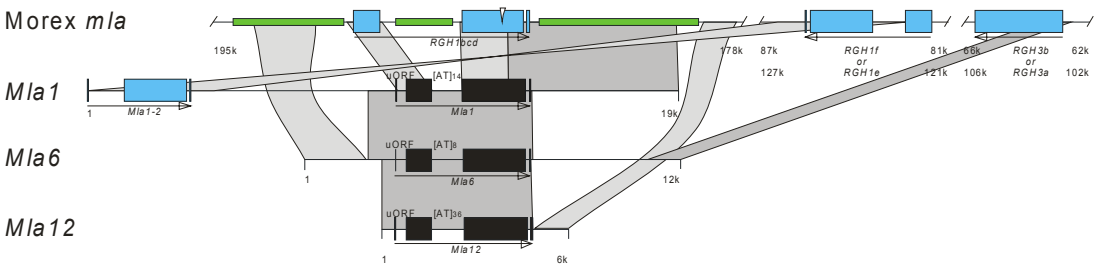

Figure 15. Schemes of the Morex *Mla* locus and genomic regions containing identified *Mla* resistance genes.

DNA sequences encompassing the Morex *Mla* locus (261 kb, in reverse orientation) (Wei et al., 2002) are represented schematically and drawn to scale in the top line (relevant sequences only). Available genomic sequences of *Mla1*, *Mla6*, and *Mla12* and flanking regions are shown below. Coding sequences of functional *Mla R* genes and *RGHs* are boxed and highlighted in black and blue, respectively. A conserved upstream open reading frame (uORF) and a simple $[AT]_n$ microsatellite are shared among functional *Mla R* genes. Green boxes denote retrotransposon sequences: a *BARE1* solo LTR in intron 3 of *RGH1bcd*, *HORPIA2* immediately 3' of *RGH1bcd*, and *ALEXANDRA* 5' of *RGH1bcd*. Dark gray areas denote sequences showing >90% identity, and light gray areas denote sequences showing >75% identity. A possible inversion event could explain the altered relative orientations of homologous genes *Mla1-2* and *RGH1f* as indicated. Note that *RGH1e*/*f* and *RGH3a*/*b* are extremely similar and located within a 40-kb duplicated region (Wei et al., 2002). For this reason, the indicated homologies exist between *RGH1e* and *RGH1f* and between *RGH3a* and *RGH3b*. Arrows indicate the relative orientations of genes (5' to 3'). Borders of Morex sequences are indicated in kb according to accession AF427791.

molecularly validated *Mla* resistance specificities to *Bgh*. The presence of the $(AT)_n$ microsatellite in all *Mla R* genes identified to date and its absence in currently available *Mla RGHs* are consistent with this hypothesis, because recent findings indicate that most microsatellites reside in regions predating recent genome expansion in plants (Morgante et al., 2002).

The very high level of DNA sequence conservation in exon and intron sequences of identified *Mla R* genes (average overall identity of 94 and 93%, respectively) may be indicative of selective constraints acting on both coding and noncoding regions. By contrast, inspection of flanking regions revealed evidence for extensive intralocus recombination events that reshuffled both genes and intergenic regions (Fig. 15). For example, sequences located immediately 3' of *Mla12* were found 5.5 Kb downstream of *RGH1bcd*, indicating an extensive intralocus insertion/deletion event. Morex *RGH1f/e* exhibited highest sequence relatedness to the *Mla1* paralog *Mla1-2*; their altered relative orientation to *RGH1bcd* and *Mla1*, respectively, suggests the occurrence of an intralocus inversion event (Fig. 15).

7.2. Determinants of MLA recognition specificity

Functional analysis of reciprocal domain-swap constructs between *Mla1* and *Mla6* revealed an essential role of the LRR-CT unit in specificity determination (Fig. 5B). It was demonstrated in this study that distinct regions in the LRRs of MLA1 and MLA6 (LRRs 3 to 11 and 9 to 11, respectively) are necessary for cognate AVRMLA perception. This finding is in agreement with LRRs representing the most variable part of MLA and other characterized NB-LRR-type R proteins (Botella et al., 1998; McDowell et al., 1998; Meyers et al., 1998; Ellis et al., 1999; Halterman et al., 2001). It is also consistent with the finding that potentially solvent-exposed residues in MLA LRRs and those of other NB-LRR R proteins are subject to diversifying selection (Botella et al., 1998; McDowell et al., 1998; Meyers et al., 1998; Halterman et al., 2001). One interpretation of these data is that the diversified regions are involved in ligand-specific recognition.

LRRs have been demonstrated to function as specificity determinants of membrane-anchored R proteins (Van der Hoorn et al., 2001; Wulff et al., 2001). Successful domain-swap experiments have been reported only for intracellular TIR- NB-LRR-encoding resistance alleles at the *L* locus in flax to the flax rust fungus (Ellis et al., 1999; Luck et al., 2000). Both MLA and L proteins exhibit comparable average polymorphisms in corresponding domains (Table 3). Unlike this study involving CC-NB-LRR proteins, the analysis of L chimera functions suggested that both TIR-NB and LRR regions can determine specificity differences (Ellis et al., 1999; Luck et al., 2000). Although it is possible that the CC-NB domain is irrelevant for specificity determination, more diverged CC-NB domains from other MLA proteins must be tested before any generalization can be made from the observations based on MLA1 and MLA6 chimeras.

Reciprocal swaps of the CT domains between MLA1 and MLA6 resulted in nonfunctional chimeras (11116 and 66661; Fig. 5B). One interpretation is that cognate LRR-CT units are required for MLA specificity determination, which was also supported by the finding that two of three single-amino acid replacements in mutant MLA12 variants affect CT amino acids and the third affects an LRR residue (Fig. 2). Additional evidence for a role of the MLA CT in specificity determination comes from the identification of a hypervariable region in the middle of this domain (residues 893 to 945 in MLA1). This hypervariable region shows an increased ratio of nonsynonymous (k_a = 15.4) to synonymous (k_s = 9.6) nucleotide substitutions (based on *Mla1*, *Mla6*, *Mla12*, and *Mla13* sequences (significant at P < 0.1%), which is indicative of the operation of diversifying selection. This is reminiscent of the C-terminal non-LRR domain of *P* locus genes that encode flax TIR-NB-LRR proteins, which also was found to contain a region that is subject to diversifying selection and might contribute to specificity determination (Dodds et al., 2001).

7.3. Potential roles of RAR1 and SGT1 in MLA-mediated resistances

By genetic studies it has been well documented that RAR1 is required for members from both TIR- and CC- NB-LRR subtype R proteins against oomycete, bacterial, fungal and viral pathogens (Freialdenhoven et al, 1994; Jørgensen, 1996; Liu et al., 2002a; Muskett et al., 2002; Tornero et al., 2002). Likewise, gene silencing or loss-of-function alleles of *sgt1* demonstrated that SGT1 also plays an important role in race-specific disease resistance to different pathogen classes mediated by the two major structural subtypes of NB-LRR R proteins (Azevedo et al., 2002; Austin et al., 2002; Liu et al., 2002b; Peart et al., 2002; Tör et al., 2002). Significantly, VIGS mediated silencing of *Sgt1* in *Nicotiana benthamiana* demonstrated that SGT1 is also required for certain non-host resistance responses (Peart et al., 2002). Furthermore, SGT1 physically associates with RAR1 in plant and in yeast, and both proteins co-operate in some R protein mediated resistance against different pathogens (Azevedo et al, 2002; Austin et al., 2002; Liu et al., 2002b).

Genetic analysis also provided strong evidence that RAR1 is essential for the function of a subset of *Mla*-encoded *R* specificities (Jørgensen, 1988; Freialdenhoven et al., 1994). Gene silencing in single barley epidermal cells of *Rar1* and/or *Sgt1* significantly compromised *Mla6* and *Mla12*-mediated resistance. In contrast, *Mla1* resistance was only slightly affected in these experiments (Azevedo et al., 2002; this study). Using transient gene expression in *rar1* mutant plants, *Rar1* was shown to be required for *Mla13*-mediated resistance (Halterman et al., 2003).

A rapid and coordinate increase in the accumulation of *Mla13* transcripts, and *Rar1* and *Sgt1* transcripts was observed within roughly the same time frame when *Bgh* haustoria make contact with the host cell plasma membrane (around 16-24 h after inoculation; Halterman et al., 2003). It remains unclear whether the pathogen responsiveness of *Mla*, *Rar1*, and *Sgt1* gene expression is required for an effective resistance response. In the present study, functional analysis of chimeras derived from MLA1 and MLA6 shed more light on the role of RAR1 and SGT1 in disease resistance. *AvrMla6*

specificity was successfully uncoupled from the *RAR1/SGT1* dependence in one chimera, suggesting that the reliance on RAR1/SGT1 is not absolute for a given *Mla* recognition specificity. One implication from this finding is that RAR1/SGT1 cannot have a role in processing or transport of *Bgh* AVR effectors.

7.3.1. The Ubiquitin/26S proteasome degradation pathway and MLA-mediated resistance are connected to RAR1/SGT1

The ubiquitin (Ub)/26S proteasome system is a proteolytic pathway that selectively targets proteins for degradation in eukaryotic cells to regulate the activity of crucial cellular regulators (Hershko and Ciechanover, 1998; Callis and Vierstra, 2000; Sullivan et al., 2003). In this pathway, targeted proteins are marked by ubiquitylation through an ubiquitin conjugation cascade, which involves three enzyme families, ubiquitin-activating (E1), -conjugating (E2), and -ligating (E3) enzymes (Glickman and Ciechanover, 2002). Subsequently, the polyubiquitinated proteins are recognized and degraded by the multi-subunit 26S proteasome that consists of a 20S core particle flanked by 19S regulatory complexes (Hershko and Ciechanover, 1998; Vierstra, 2003).

One type of complex E3 ubiquitin ligases is the SCF (SKP1, CDC53p/or Cullin1, F-box protein) complex, which consists of four subunits (RBX1/or ROC1/or HRT1 being the fourth subunit) (Deshaies, R.J., 1999). Specificity of the SCF complex is conferred by the F-box subunit that contains a protein-interaction motif at its C-terminus and an F-box motif at its N-terminus (Gagne et al., 2002). The F-box protein recognizes and recruits a specific substrate to the SCF complex for ubiquitylation (Craig and Tyers, 1999). There are also other types of E3 ubiquitin ligases described based on subunit composition and mechanism of action, for example, Ring/U box, HECT, VBC-Cul2 and APC (Vierstra, 2003).

Evidence obtained from mutant analysis has already pointed out that

the Ubiquitin/26S pathway might be linked to plant disease resistance. For example, two F-box proteins, CORONATINE INSENSITIVE1 (COI1) and SUPPRESSOR OF *nim1*-1 (SON1), have been identified and connected to disease resistance responses (Xie et al., 1998; Kim and Delaney, 2002). The *Arabidopsis coi1* mutant is unable to express the JA-inducible gene *PDF1.2* and is susceptible to insect herbivory and to fungal and bacterial pathogens (McConn et al., 1997; Thomma et al., 1998). COI1 co-immunoprecipitates with other SCF subunits (SKP1, CUL1 and RBX1), suggesting a SCF^{COI1} complex *in planta*. This complex is thought to positively regulate JA-mediated responses *in vivo* (Devoto et al., 2002; Xu et al., 2002). The *son1* mutant was isolated in a screen for suppressors of the susceptible *nim1-1* mutant (Kim and Delaney, 2002). Mutant *son1* plants exhibit constitutive resistance against virulent fungal and bacterial pathogens without accompanying constitutive expression of defence-related genes that are normally induced during systemic acquired resistance. Thus, the F-box protein SON1 regulates a novel induced defence response that is independent of both salicylic acid and SAR (Kim and Delaney, 2002).

Supporting evidence for a role of the Ubiquitin/26S proteasome pathway in plant immunity comes from the involvement of RAR1/SGT1 in a broad range of race-specific resistance reactions to diverse pathogens (Austin et al., 2002; Azevedo et al., 2002; Liu et al., 2002b; Peart et al., 2002; Tör et al., 2002). In barley and *N. benthamiana*, SGT1 was shown to co-immunoprecipitate with two SCF components, SKP1 and CULLIN1, (Azevedo et al., 2002; Liu et al., 2002b). The same authors could also show that RAR1 physically associates with SGT1 in both barley and *N. benthamiana* plants (Azevedo et al., 2002; Liu et al., 2002b). Further evidence supporting a link to the Ubiquitin/26S proteasome pathway is based on the observation that both RAR1 and SGT1 co-immunoprecipitate with the COP9 signalosome (CSN) subunits in barley and tobacco (Azevedo et al., 2002; Liu et al., 2002b). It is relevant that CSN has been shown to physically interact with the 26S proteasome *in vivo* (Peng et al., 2003) and can interact with the SCF complex

through both the CULLIN and RBX1 subunits (Lyapina et al., 2001; Schwechheimer et al., 2001; Cope et al., 2002). Therefore, one role of the CSN is presumed to regulate SCF activity by removing ubiquitin-like conjugates NEDD8 or RUB1 from the CULLIN subunit (Lyapina et al., 2001; Schwechheimer et al., 2001). These recent findings strongly suggest RAR1/SGT1 may connect the Ubiquitin/26S proteasome pathway to pathogen responses in plants.

As discussed above, the data obtained from genetic and biochemical analysis in barley collectively indicates that RAR1/SGT1 provides a link between the Ubiquitin/26S proteasome pathway and MLA-mediated disease resistance. However, questions that remain to be answered are: how does the ubiquitynation pathway play a role in *Mla*-mediated resistance to the powdery mildew pathogen? What are the targets for ubiquitynation and degradation in MLA-mediated resistance responses?

SCF complexes may target suppressors in the *Mla*-triggered disease resistance pathway for degradation via the 26S proteasome and thus positively regulate defence responses. Potential suppressors can be host factors that repress defence responses in the absence of pathogen. MLA-mediated direct or indirect recognition of the cognate AVR effectors may activate the ubiquitynation pathway to degrade the suppressors and hence activate the down stream signalling cascade. In this context, the ubiquitin/26 proteasome pathway participates in MLA-mediated defence response downstream of AVR recognition. As mentioned before, despite the unusually high sequence similarity of known MLA R proteins, the resistance responses can differ in their requirement for RAR1/SGT1. This might indicate that a subset of *Mla*-mediated disease resistance responses may either occur independently of ubiquitination or involves non-SCF-mediated ubiquitynation. In this regard, it may be relevant that a second pool of HvSGT1 was detected in barley leaf protein extracts, which associates with RAR1 and CSN subunits, but not with SCF subunits (Azevedo, et al, 2002).

It is also conceivable that the MLA protein themselves are targets of SCF complexes. Indirect evidence for this supposition comes from the rapid disappearance of the CC-NB-LRR protein RPM1 coincident with the onset of the HR (Boyes et al., 1998). This has been interpreted to restrict the extent of cell death and overall resistance response at the site of infection. In yeast it was shown that SGT1p physically interacts with another LRR-containing adenylyl cyclase (CYR1; Dubacq et al., 2002; Schadick et al., 2002).

In the present study, yeast two-hybrid experiments using the fusion bait containing the MLA1-LRR-CT identified HvSGT1 many times from the barley prey library. Similarly, by targeted interaction analysis it was found that the same bait interacts with both SGT1 isoforms present in *Arabidopsis* (AtSGT1a and AtSGT1b; Shirasu K., JIC, personal communication). These findings are consistent with the idea that MLA proteins are direct targets of SCF complexes. The LexA and full-length MLA fusion proteins appeared to be truncated at the C-terminal end in yeast (Fig. 10). For this reason it is at present not possible to assess whether full-length MLA associates with HvSGT1 in yeast.

7.3.2. RAR1/SGT1 may act as co-chaperones in MLA-mediated resistance

New evidence revealed that the cytosolic heat shock protein 90 (HSP90) family play a crucial role in plant R proteins triggered immunity (Hubert et al., 2003; Kanzaki et al., 2003; Liu et al., 2004; Lu et al., 2003; Takahashi et al., 2003; reviewed in Schulze-Lefert, 2004). A mutant screen for loss of *AvrRpm1* recognition identified mutations of single amino acid substitutions in the ATPase domain of HSP90.2, one of four HSP90 isoforms in *Arabidopsis* plants expressing the RPM1 protein (Hubert, et al., 2003). By assessing growth of various *Avr* containing *P. parasitica* strains on *Arabidopsis* T-DNA insertion lines, it was demonstrated that a different isoform, HSP90.1, is required for full *RPS2* resistance function (Takahashi et al., 2003). Enhanced susceptibility to the bacterial phytopathogen was also

seen upon injection of a known inhibitor of HSP90 activity, geldanamycin, into leaves of *RPS2* containing *Arabidopsis*. Virus-induce gene silencing (VIGS) targeted at least four related *NbHSP90* isoforms in *Nicotiana benthamiana* and resulted in a loss of function of three tested NB-LRR proteins: RX, N and PRF, mediating race-specific recognition of potato virus X (PVX), tobacco mosaic virus (TMV) and *P. syringae* (expressing *AvrPto* effector), respectively (Lu et al, 2003). Undoubtedly, the above findings demonstrate that the HSP90 family is essential for the function of multiple NB-LRR R proteins.

Heat-shock proteins are involved in folding and degradation of damaged or misfolded peptides and assist in the correct folding of assembly of protein complexes (Rutherford, 2003). Studies of HSP90 in animal and yeast suggest that they work in association with many co-chaperones to regulate activities of their substrates that are mainly involved in signalling (Picard, 2002). The findings described above suggest that NB-LRR R proteins might be HSP90 client proteins (Hubert et al., 2003; Takahashi et al., 2003; Lu et al., 2003; Schulze-Lefert, 2003). Direct evidence for this claim comes from the observation that HSP90 interacts with RPM1 *in vivo* and that steady-state levels of RPM1 and RX are greatly reduced in HSP90 mutant or VIGS induced HSP90 silenced plants, respectively (Hubert et al., 2003; Lu et al., 2003).

Since animal RAR1 and SGT1 share a structural motif, the CS domain, with a known co-chaperone, the p23 and HSP20/a-crystallin family, it has been proposed that either protein might serve a co-chaperone-like role together with HSP90 to regulate R protein activity and/or stability (Shirasu and Schulze-Lefert, 2003; Schulze-Lefert, 2004; Garcia-Ranea et al., 2002). Experimental evidence in support of this hypothesis is still limited: RAR1/SGT1 co-immunoprecipitate with HSP90 *in planta* or can bind to HSP90 *in vitro* and *in vivo* (Hubert et al., 2003; Takahashi et al., 2003; Liu et al., 2004). The proposed co-chaperone role of RAR1/SGT1 may explain the finding that RAR1 has a role in determining the steady-state levels of MLA

and RPM1 R proteins *in planta*. It may also explain that SGT1 physically associates with several regulatory protein complexes in yeast and in plants (Shirasu and Schulze-Lefert, 2003).

How can one explain the known differential engagement of RAR1/SGT1 in MLA-mediated resistance responses to *Bgh* if RAR1/SGT1 exert a co-chaperone-like activity in race-specific immunity? Recent biochemical evidence suggests that RAR1 increases protein abundance of both MLA1 and MLA6 in barley (Bieri et al., unpublished data). MLA proteins belong to the CC-NB-LRR type of intracellular R proteins and are highly sequence related, sharing >90% sequence identity. Bearing this in mind, it seems possible that RAR1 increases the abundance of all MLA proteins. In one scenario, RAR1 might cooperate with HSP90 in assisting MLA to achieve a proper conformation and to stabilize MLA protein by assembling it into a complex. The different abundance of MLA1 and MLA6 detected in *rar1* mutant plants may reflect a quantitative difference in individual MLA protein stability/or activity. By extrapolation from its role predicted in RPM1 and RX protein function, SGT1 may also play a co-chaperone role in MLA protein function although direct biochemical evidence is still lacking. However, the role of SGT1 might be different from RAR1 in MLA mediated resistance. An indication of this is the existence of a second pool of SGT1 containing SCF subunits but not RAR1 in barley leaf extract (Azevedo et al., 2002). Likewise, silencing of *NbSGT1* does not reduce RX protein abundance (Lu et al., 2003).

7.4. Direct *versus* indirect AVRMLA recognition

The ‘gene-for-gene’ model provides a genetic framework for the phenomenon of race-specific resistance in plants (Flor, 1971). The implication of gene-for-gene relationships in plant and pathogen is the capacity of plants to recognize pathogen-derived AVR effectors by *R* genes. However, several biochemical interpretations could explain the genetic observations. The ‘receptor-ligand model’ predicts that an R protein directly interacts with its cognate AVR effector (Hammond-Kosack and Jones, 1997). The ‘guard hypothesis’

postulates that R proteins function in the surveillance of other host factors, which in turn are targeted by AVR effectors (Dangl and Jones, 2001). Either direct or indirect recognition by R proteins will trigger disease resistance responses.

The *Mla* locus is unusually polymorphic, encoding about 30 resistance specificities against *Bgh* isolates containing cognate *AvrMla* genes. The experimentally validated intracellular localization of MLA proteins (Bieri S. and S. Mauch, unpublished data) suggests that AVRMLA recognition occurs in the cytoplasm. However, how MLA mediates AVRMLA recognition remains still unclear. Transgenic barley lines expressing fully functional MLA1- or MLA6-HA epitope tagged variants provide a powerful future tool to approach the problem using biochemical methods. Preliminary evidence obtained by size exclusion chromatography suggests that MLA assembles in a complex of about 600-800 KDa in non-challenged plants (Mauch S., unpublished). One possibility is that this complex contains other yet unknown host proteins. Yeast two-hybrid screens using multiple LexA MLA fusion baits have identified several barley proteins that are capable to physically associate with MLA domains (see Chapter 6). One of these was shown to be HvSGT1. Preliminary biochemical evidence suggest that this interaction occurs also *in planta* because full-length MLA1-HA or MLA6-HA co-immunoprecipitate with SGT1 (Mauch S., et al., MPIZ, unpublished). However, it remains to be shown whether HSP90 and/or RAR1 are in the same complex. The characterization of other potential components in presumed MLA complex(es) may provide valuable hints for potential AVRMLA targets *in planta*.

Genetic mapping of *Bgh AvrMla* genes in the powdery mildew genome revealed mainly dispersed and a few linked positions on multiple *Bgh* chromosomes (Brown and Jessop, 1995; Caffier et al., 1996; Pedersen et al., 2002). Recently, *AvrMla10* and *AvrMlk* (the cognate *Avr* gene of the barley *Mlk R* gene to *Bgh*) candidate genes were isolated from *Bgh* by map-based cloning (Ridout C., et al., JIC, unpublished data). Sequence analysis of the deduced AVRMLA10 and AVRMLK proteins reveals a conserved core region

and variable N- and C-termini. The availability of these Avr genes provides future opportunities to study the AVRMLA-MLA interaction in the *Bgh*-barley pathosystem in greater detail. Multiple AVR fusion baits are currently under construction and will be used to screen the barley cDNA prey library. Any interesting host factors interacting with the *Bgh* AVR proteins might contribute to reveal the exact molecular mechanism of MLA-mediated AVRMLA recognition.

A possible direct AVRMLA-MLA interaction can not be ruled out at this stage. It was postulated that a direct AVR-R interaction may occur after multiple folding switches of complexes containing R proteins (Schulze-Lefert, 2004). However, such folding switches might be transient and might not be detectable by current biochemical techniques. A reconstitution of AVR-R complexes in yeast and or *Arabidopsis* may provide a novel future tool to unravel the mechanics of AVRMLA recognition. Known components for R protein complex stability and/or function, e.g. HSP90, RAR1 and SGT1, might be essential for such reconstitution experiments.

8. Summary

More than 30 race-specific resistance specificities to the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) map to the barley *Mla* locus. This exceptionally polymorphic locus harbors multiple members of three distantly related gene families, each encoding homologs of intracellular disease resistance (R) proteins. Previously isolated *Mla1* and *Mla6* *R* genes to *Bgh* encode highly sequence-related proteins (>90% identity) of approximately 105 kD that contain an N-terminal coiled-coil (CC) structure, a central nucleotide binding (NB) site, a Leucine rich repeat (LRR) region, and a C-terminal non-LRR (CT) region. A subset of *Mla R* genes are known to require for their function *Rar1* and *Sgt1*. Intracellular RAR1 and SGT1 are known to physically associate and are thought to have co-chaperone-like activity.

It was hypothesized that other *Mla R* gene specificities might be closely related to *Mla1* and *Mla6*. A cosmid library was constructed from an *Mla12*-containing barley line and cosmids containing candidate *Mla12* genes were isolated using molecular probes derived from *Mla1*. Race-specific resistance activity to an *AvrMla12* containing *Bgh* isolate was detected following transient single cell expression of the *Mla12* candidate gene in detached barley leaves. This and the identification of point mutations in susceptible *mla12* mutants demonstrated that the candidate gene is *Mla12*. Over-expression of *Mla12* in the single cell assay shifted the slow *Mla12* resistance response in wild type plants to a rapid *Mla1*/*Mla6*-like resistance, terminating fungal growth at the cell wall penetration stage before haustorium development. This indicates that the cognate *AvrMla12* product must be secreted before the switch from surface to invasive fungal growth. Resistance mediated by *Mla12* over-expression retained the requirement for *Rar1*. Single cell dsRNAi gene silencing experiments revealed a requirement of *Mla12* resistance for *Sgt1*. A series of reciprocal domains swaps between MLA1 and MLA6 identified in

each protein regions required for the recognition of cognate *Bgh* effectors (encoded by *AvrMla1* and *AvrMla6*). These regions comprise different but overlapping LRR regions and the CT part. This is consistent with the finding that the LRR and CT encoding parts of *Mla1* and *Mla6* exhibit evidence for diversifying selection. Unexpectedly, MLA chimeras that confer *AvrMla6* recognition specificity exhibit markedly different dependence on *Rar1.* Furthermore, uncoupling of MLA6-specific resistance from RAR1 also uncoupled the resistance response from SGT1. These findings suggest that differences in the degree of RAR1 dependence of different MLA immune responses are determined by intrinsic properties of MLA variants and place RAR1/SGT1 activity downstream of and/or coincident with the action of presumptive resistance protein–containing recognition complexes.

Previous analysis showed that loss-of-function mutations in both barley and *Arabidopsis Rar1* severely impair accumulation of MLA and RPM1 NB-LRR proteins in healthy plants, respectively. In this study it was shown that co-expression of RAR1 and MLA1 or MLA6 in heterologous yeast is not sufficient to elevate MLA steady state levels. This suggests that RAR1 stabilizes MLA proteins indirectly, e.g. through other host factors that might be components of MLA containing recognition complex(es). Yeast two-hybrid screenings were used to identify host proteins directly interacting with MLA R proteins. Several MLA interactors were identified using distinct or overlapping domains from MLA1 or MLA6 proteins. Amongst those is the SGT1 protein that is genetically required for the function of many NB-LRR proteins in diverse plant species. Other MLA interactors exhibit high sequence relatedness to carbonic anhydrase (CA), formin homology (FH) proteins, and a WRKY transcription factor. Interestingly, tobacco carbonic anhydrase was previously shown to be required for the activation of an efficient cell death response triggered upon race-specific recognition of the *Pseudomonas syringae* effector AVRPTO by the *R* gene *Pto*. The functional significance of the identified interactors for *Mla*-mediated race-specific resistance to *Bgh* remains to be validated, e.g. by dsRNAi experiments in the single cell gene

expression assay.

ZUSAMMENFASSUNG

In Gerste existieren über 30 rassespezifische Resistenz-Spezifitäten gegen den Gerstenmehltau *Blumeria graminis* f. sp. *hordei*, die dem *Mla*-Locus zugeordnet werden. An diesem außergewöhnlich polymorphen Locus befindet sich eine Vielzahl von Genen aus drei entfernt verwandten Familien, die Sequenzen mit hoher Homolgie zu bekannten Resistenzgenen (Resistance gene homologs RGH) beinhalten.. Bisher isolierte *Mla*-Gene kodieren für Proteine mit einem ungefähren Gewicht von 105 kD, die eine bemerkenswert hohe Ähnlichkeit in Struktur und Sequenz (über 90% identische Aminosäuren) zeigen. Die Proteine besitzen eine N-terminale coiled-coil-Struktur (CC), eine zentrale Nukleotidbindestelle (NB), eine Leucine-rich repeat-Region (LRR) und eine C-terminale nicht-LRR Region (CT). Ein Teil der *Mla R*-Gene benötigt *Sgt1* und *Rar1*um zu funktionieren. Intrazelluläres SGT1 und RAR1 assoziieren physisch und haben möglicherweise eine co-chaperone-ähnliche Aktivität.

Es wurde vermutet, dass andere *Mla*-Resistenzspezifitäten, zu den bisher bekannten, *Mla1* und *Mla6*, eng verwandt sind. Eine Cosmidbibliothek, die von einer *Mla12* enthaltenden Gerstelinie erstellt worden war, wurde verwendet, um mit Hilfe von molekularen Sonden von *Mla1* Cosmide mit Kandidaten für *Mla12* zu isolieren. Rassenspezifische Resistenzaktivität der *Mla12*-Kandidaten gegen *AvrMla12*-enthaltendes *Bgh*-Isolat wurde durch Überexpression mittels transienter Einzelzellgenexpression in abgetrennten Blättern festgestellt. So und durch die Identifikation von Punktmutationen in anfälligen *mla12*-Mutanten, wurde demonstriert, dass der Kandidat tatsächlich *Mla12* ist. Überexpression von *Mla12* mittels transienter Einzelzellgenexpression zeigte, dass die langsame *Mla12*-Immunantwort zu

einer schnellen, *Mla1/6*-ähnlichen Resistenz umgewandelt werden kann und die Entwicklung des Pilzes vor der Ausbildung des Haustoriums, im Stadium der Zellwandpenetration gestoppt wird. Das deutet an, dass die Sekretion des korrespondierenden *AvrMla12*-Produkts vor dem, Umschalten von oberflächlichem zu invasivem Wachstum stattfinden muss. Resistenz, die durch Überexpression von *Mla12* vermittelt wird, bleibt *Rar1* abhängig. Eine Abhängigkeit der *Mla12*-Resistenz von *Sgt1* wurde durch transiente dsRNAi-silencing-Versuche aufgedeckt.

Eine Serie von wechselseitigen Domänenaustauschexperimenten zwischen MLA1 und MLA6 identifizierte in jedem Protein die Domäne, die für die Erkennung der zugehörigen Effektormoleküle aus Mehltau (kodiert von *AvrMla1* und *AvrMla6*) notwendig ist. Diese Domänen lagen in verschiedenen, aber überlappenden Teilen der LRR- und der CT-Region. Das stimmt mit Hinweisen überein, dass diversifizierende Selektion in der LRR- und CT-Region angreift. MLA-Chimären, die die spezifische Erkennung von AVRMLA6 vermitteln, zeigten unerwarteterweise eine ausgesprochen unterschiedliche Abhängigkeit von *Rar1*. Darüber hinaus wurde durch Entkopplung der MLA6-spezifische Erkennung von RAR1 auch die Abhängigkeit von SGT1 entkoppelt. Diese Ergebnisse legen nahe, dass der unterschiedliche Grad der RAR1 Abhängigkeit verschiedener MLA-Resistenzen, eine intrinsische Eigenschaft der verschiedenen MLA-Varianten ist und platziert die Aktivität von RAR1/SGT1 hinter und/oder zusammen mit mutmaßlichen R-Protein enthaltenden Komplexen.

Bisherige Studien haben gezeigt, dass *Rar1 loss-of-function* Mutanten von Gerste und *Arabidopsis* in der Akkumulierung von MLA1 beziehungsweise RPM1 NB-LRR Proteinen in gesunden Pflanzen in stark beeinträchtigt sind. In dieser Studie wurde gezeigt, dass die Co-Exprimierung von RAR1 und MLA1 oder MLA6 in Hefe nicht ausreicht, um stationäre Proteinmenge an MLA zu erhöhen. Dies lässt vermuten, dass RAR1 MLA Proteine indirekt stabilisiert, z.B. durch andere Wirtsfaktoren, die Teil eines/von MLA enthaltenden Erkennungskomplexes/n sein könnten. Yeast 2-

Hybrid Screening wurde für die Identifizierung von potentiellen Wirtsfaktoren eingesetzt, die an der MLA-vermittelten Resistenz beteiligt sind. Mehrere potentielle MLA-Interaktoren wurden für einzelne oder überlappende Domänen von MLA1 oder MLA6 Proteinen identifiziert. Unter diesen Interaktoren ist das SGT1-Protein, das für die Funktion von vielen NB-LRR-Proteinen in verschiedenen Pflanzenarten notwendig ist, gefunden worden. Andere MLA-Interaktoren zeigen hohe Sequenzverwandschaft zu Carboanhydrase (CA), zu Proteinen mit Homologie zu Formin (FH), HvSGT1 und zu einem WRKY Transkriptionsfaktor. Interessanterweise wurde zuvor in Tabak gezeigt, dass Carboanhydrase für die Aktivierung einer effizienten Zelltodantwort nach rassespezifischer Erkennung des *Pseudomonas syringae* Effektors AVRPTO durch das *R* Gene Pto notwendig ist. Die funktionelle Signifikanz der identifizierten Interaktoren für die *Mla*-vermittelte rassenspezifische Resistenz gegen *Bgh* muss noch bestätigt werden, z.B. durch dsRNAi-Experimente mittels transienter Einzelzellgenexpression.

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10. Appendix: Publications

The Plant Cell, Vol. 15, 732–744, March 2003, www.plantcell.org © 2003 American Society of Plant Biologists

Recognition Specificity and RAR1/SGT1 Dependence in Barley *Mla* Disease Resistance Genes to the Powdery Mildew Fungus

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A large number of resistance specificities to the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* map to the barley *Mla* locus. This complex locus harbors multiple members of three distantly related gene families that encode proteins that contain an N-terminal coiled-coil (CC) structure, a central nucleotide binding (NB) site, a Leu-rich repeat (LRR) region, and a C-terminal non-LRR (CT) region. We identified *Mla12*, which encodes a CC-NB-LRR-CT protein that shares 89 and 92% identical residues with the known proteins MLA1 and MLA6. Slow *Mla12*-triggered resistance was altered dramatically to a rapid response by overexpression of *Mla12*. A series of reciprocal domains swaps between MLA1 and MLA6 identified in each protein recognition domain for cognate powdery mildew fungus avirulence genes (*AvrMla1* and *AvrMla6*). These domains were within different but overlapping LRR regions and the CT part. Unexpectedly, MLA chimeras that confer *AvrMla6* recognition exhibited markedly different dependence on *Rar1*, a gene required for the function of some but not all *Mla* resistance specificities. Furthermore, uncoupling of MLA6-specific function from RAR1 also uncoupled the response from SGT1, a protein known to associate physically with RAR1. Our findings suggest that differences in the degree of RAR1 dependence of different MLA immunity responses are determined by intrinsic properties of MLA variants and place RAR1/SGT1 activity downstream of and/or coincident with the action of resistance protein–containing recognition complexes.

INTRODUCTION

Intraspecific genetic variation in the capacity of plants to combat microbial attack is confined mainly to disease resistance (*R*) loci. These can encode a single gene but frequently they are complex, harboring multiple similar and/or dissimilar *R* genes (reviewed by Ellis et al., 2000). A single *R* gene has the capacity to recognize one or very few normally unrelated strain-specific pathogen effector molecules (encoded by avirulence [*Avr*] genes) that are released during pathogenesis. Most *R* genes encode one of two groups of Leu-rich repeat (LRR)–containing proteins. An intracellular class shares a central nucleotide binding (NB) site and C-terminal LRRs with variable repeat numbers. This is the largest group of known R proteins and can be divided further into subfamilies containing either N-terminal sequences predicted to form a coiled-coil (CC) structure (CC-NB-LRR subfamily) or sequences that are related to the cytoplasmic domain of the *Drosophila* Toll and human Interleukin1 receptor (TIR-NB-LRR). A second R protein class is membrane-anchored by a single transmembrane helix, consists of variable repeat numbers of extracellular LRRs, and contains at least in one case an intracellular Ser/Thr kinase module (reviewed by Ellis et al., 2000).

Little is known about the molecular mechanics of the R-AVR recognition process. Recent studies suggest that members of the intracellular and membrane-anchored classes assemble in preformed heteromultimeric recognition complexes in the absence of pathogens (Leister and Katagiri, 2000; Holt et al., 2002; Mackey et al., 2002; Rivas et al., 2002a, 2002b). With one exception, there are no documented examples of direct interactions between LRR-containing R and AVR proteins (rice Pi-ta and AVR-Pita from *Magnaporthe grisea*; Jia et al., 2000). Thus, it seems possible that R proteins function indirectly in the recognition process, which involves the surveillance of a host protein or a complex that is targeted by *AVR* products (Dangl and Jones, 2001; Mackey et al., 2002).

Approximately 30 genetically characterized barley *Mla* variants have been described, each triggering immunity responses upon recognition of cognate isolate-specific powdery mildew fungus (*Blumeria graminis* f. sp. *hordei* [*Bgh*]) effector molecules (encoded by *AvrMla* genes) (Jørgensen, 1994). Some of these variants confer a rapid resistance response resulting in *Bgh* growth termination at an early stage during pathogenesis, whereas others trigger a delayed response that permits substantial growth of fungal hyphae on the leaf surface (Wise and Ellingboe, 1983; Boyd et al., 1995). Although none of the *Bgh AvrMla* genes has been isolated to date, their genetic mapping in the powdery mildew genome revealed mainly dispersed and a few linked positions on multiple *Bgh* chromosomes (Brown and Jessop, 1995; Caffier et al., 1996; Pedersen et al., 2002).

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.009258.

The complex *Mla* locus was located genetically and physically within an interval of ~250 kb (Wei et al., 1999). A contiguous DNA sequence of the interval in barley cv Morex revealed 32 predicted genes, of which 8 encode CC-NB-LRR resistance gene homologs (*RGHs*) (Wei et al., 2002). The *RGHs* belong to three dissimilar families sharing <43% amino acid sequence similarity between families (Wei et al., 1999, 2002). Because Morex lacks a known *Mla* resistance specificity, the first two identified *Mla* powdery mildew *R* genes, *Mla1* and *Mla6*, were isolated from other barley accessions (Halterman et al., 2001; Zhou et al., 2001). The deduced proteins share 91% identical residues and show highest overall similarity to the deduced Morex *RGH1bcd* family member (83 and 79% identity to MLA1 and MLA6, respectively) (Halterman et al., 2001; Wei et al., 2002).

Mutants of barley *Rar1* were isolated originally as suppressors of *Mla12* function, and wild-type *Rar1* was shown subsequently to be required for the function of a subset of *Mla* powdery mildew resistance specificities (e.g., *Mla6* and *Mla12* but not *Mla1*) (Torp and Jørgensen, 1986; Jørgensen, 1996). Homologs of *Rar1* in Arabidopsis and *Nicotiana benthamiana* play a conserved role in the function of a subset of NB-LRR R proteins that confer resistance to different pathogens (Liu et al., 2002a; Muskett et al., 2002; Tornero et al., 2002). The highly conserved zinc binding RAR1 proteins interact physically with another conserved protein, SGT1, which was demonstrated originally to function in ubiquitin-dependent cell cycle control in yeast (Kitagawa et al., 1999; Shirasu et al., 1999a; Azevedo et al., 2002; Liu et al., 2002b). Genetic evidence for a role of plant *SGT1* in *R* gene–triggered resistance was obtained from Arabidopsis *sgt1b* mutants and *SGT1* gene-silencing experiments in barley and *N. benthamiana* (Austin et al., 2002; Azevedo et al., 2002; Liu et al., 2002b; Peart et al., 2002; Tör et al., 2002). Barley and *N. benthamiana SGT1* associate physically with one or several SCF ubiquitin E3 ligase complexes and the COP9 signalosome (Azevedo et al., 2002; Liu et al., 2002b). Because gene silencing of the core SCF component, SKP1, or the COP9 signalosome compromised *R* gene–triggered resistance in *N. benthamiana*, it seems likely that ubiquitin-protein conjugation pathways play an important role in plant innate immunity responses (Liu et al., 2002b). However, it remains unclear whether ubiquitin-dependent processes occur upstream of, coincident with, or downstream of R protein–containing recognition complexes.

Here, we exploited a high sequence relatedness between identified (*Mla1* and *Mla6*) and other genetically characterized *Mla* specificities to clone *Mla12*. Although *Mla12* might be an allele of *Mla1* and *Mla6*, it differs from them by belonging to a subgroup of *Mla* variants that trigger delayed resistance responses (Freialdenhoven et al., 1994; Boyd et al., 1995). Using a single-cell transient gene expression assay (Shirasu et al., 1999b; Zhou et al., 2001), we demonstrate that *Mla12* overexpression shifts the slow *Mla12*-triggered response to a rapid *Mla1*/*Mla6*-like resistance. We gained insights into structure-function relationships of MLA proteins by analyzing a series of reciprocal domain swaps between MLA1 and MLA6. This analysis revealed a function for the MLA LRR-CT unit in specificity determination, whereas CC-NB and LRR sequences modulated RAR1 dependence. Moreover, we show that recognition specificity can be uncoupled from both RAR1 and SGT1 dependence. We discuss possible roles of RAR1/SGT1 in folding presumed MLA recognition complexes and in signaling downstream of activated recognition complexes.

RESULTS

Isolation of *Mla12* and Characterization of Susceptible Mutant Alleles

To isolate *Mla12*, we constructed a genomic cosmid library comprising five barley genome equivalents using DNA from cv Sultan 5 containing *Mla12* (see Methods). Sixteen cosmid clones were isolated from this library with a DNA probe corresponding to the LRR region of MLA1. Low-pass DNA sequencing of the cosmid clones revealed that all of them contain NB-LRR–type *RGHs*. Two clones, designated Sp14-1 and Sp14-4, contain identical *RGHs* showing ~90% sequence identity to *Mla1* and *Mla6* in deduced exon and intron sequences. A closer comparison of the *NB-LRR* gene in Sp14-4 with *Mla1* and *Mla6* revealed an identical 5′ untranslated small open reading frame of nine amino acids and the same intron-exon structure (Halterman et al., 2001; Zhou et al., 2001). These genes share a simple sequence repeat $(AT)_n$ in intron 3, although the exact numbers of the repeats differ (see Figure 6 below). Therefore, we considered the *RGH* in Sp14-4 a candidate *Mla12* gene that encodes a predicted CC-NB-LRR-CT protein of 108 kD sharing 89% identical residues with MLA1 and 92% identical residues with MLA6 (Figure 1).

To obtain evidence for the function of the candidate *R* gene, we took advantage of chemically induced susceptible mutants that were isolated previously from a mutagenized barley M_2 population of Sultan 5 harboring *Mla12* (Torp and Jørgensen, 1986; Jørgensen, 1988). Genetic analysis indicated that susceptibility in some of the mutants (e.g., mutants M66 and M86) is likely attributable to mutations in *Mla12*, whereas susceptibility in three other lines (M22, M82, and M100) resulted from extragenic suppressor mutations of *Mla12* function. Mutant lines M82 and M100 were demonstrated to contain recessive mutations in *Rar1* (*rar1-1* and *rar1-2*, respectively) (Shirasu et al., 1999a), and genetic analysis of mutant M22 suggested another gene required for *Mla12* function, designated *Rar2* (Jørgensen, 1988, 1996; Freialdenhoven et al., 1994). DNA sequence analysis of the candidate *Mla12* in the susceptible mutants M66 and M86 revealed in each a single nucleotide substitution compared with the wild-type gene. The substitutions replace amino acid Leu-631 with Arg in the second LRR of the deduced candidate MLA12 protein in M66 and amino acid Glu-866 with Lys in the CT region in M86, respectively (Figure 1). Thus, we concluded that the Sp14-4–derived candidate gene probably is *Mla12*.

DNA marker–based mapping of susceptibility conferred by the M22 mutant revealed its location on chromosome 1H at the *Mla* locus between restriction fragment length polymorphism markers MWG036 and MWG068 (Schüller et al., 1992; our unpublished data). This finding suggested that susceptibility might be caused by a mutation in *Mla12* or in a tightly linked gene. DNA sequence analysis of the candidate *Mla12* in M22 plants

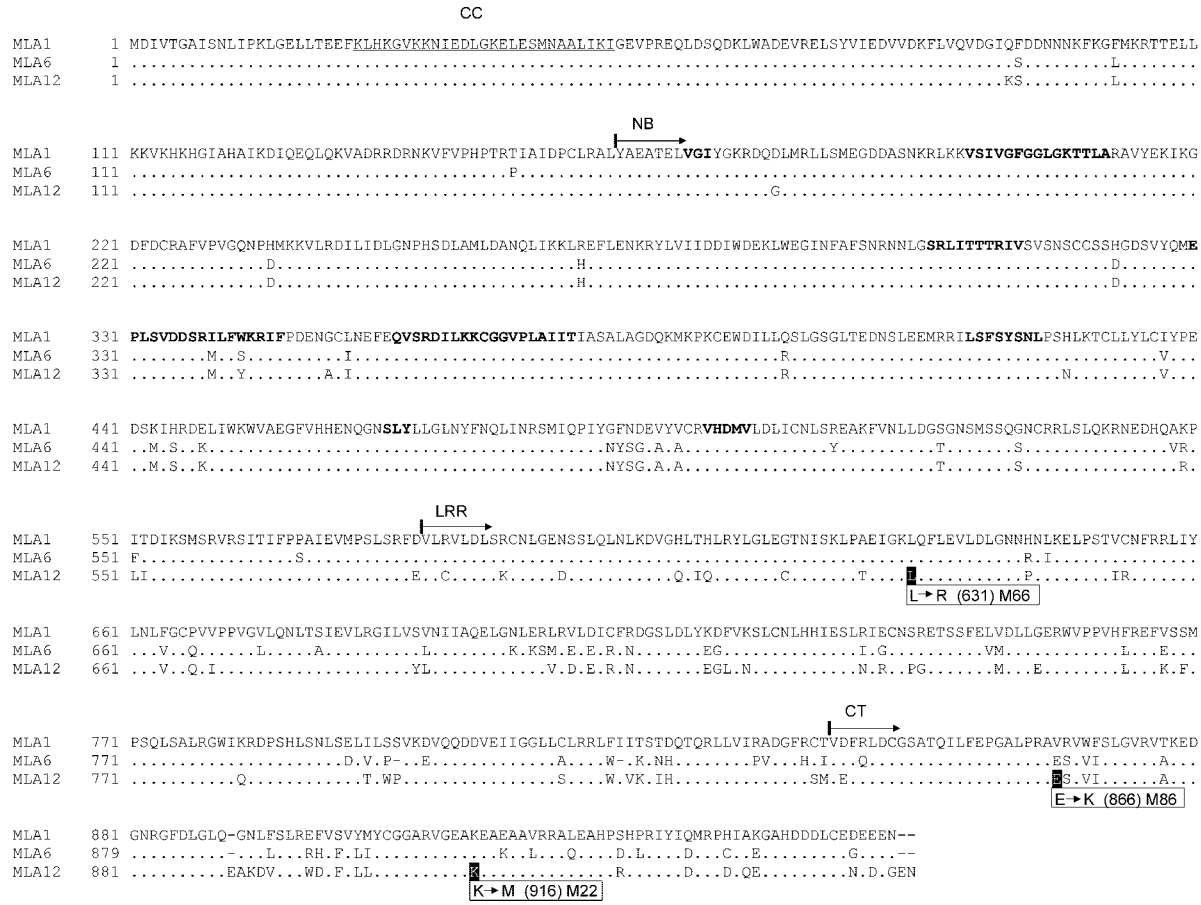

Figure 1. Amino Acid Sequence Alignment of Deduced Products of the *Mla1*, *Mla6*, and *Mla12* Genes.

Residues identical to those in MLA1 are shown as dots, and deletions are shown as hyphens. A predicted CC structure is underlined. The starts of the NB, LRR, and CT regions are indicated with arrows and are operational according to Zhou et al. (2001). Boldface letters in the NB domain indicate amino acid motifs conserved among known NB-LRR proteins. Boxes indicate amino acid exchanges identified in three susceptible *Mla12* mutants, and affected residues are shaded in black.

revealed a single nucleotide substitution that replaces amino acid Lys-916 with Met in the CT region (Figure 1). This finding suggests that M22, like M66 and M86, likely is a mutant allele of *Mla12* (see below).

Overexpression of *Mla12* Alters the Resistance Kinetics but Retains *Rar1* Dependence

To test directly the function of the candidate *Mla12* gene, Sp14-4 DNA was delivered into epidermal cells of detached barley leaves by particle bombardment (Shirasu et al., 1999b). Transformed cells were tested for their ability to activate race-specific powdery mildew resistance upon inoculation with *Bgh* conidiospores of isolates expressing or lacking *AvrMla12* (isolate A6 harboring *AvrMla6* and *AvrMla12* and isolate K1 harboring *AvrMla1*) (Zhou et al., 2001). Infection phenotypes of transgene-expressing epidermal cells were microscopically inspected at 48 h after inoculation by scoring the presence or absence of intracellular *Bgh* haustoria at single interaction sites. Unlike control bombardments with cosmid DNA harboring *Mla1* or *Mla6*, which are known to mediate race-specific resistance in the transient gene expression assay (Halterman et al., 2001; Zhou et al., 2001), delivery of Sp14-4 DNA failed to trigger detectable resistance upon inoculation with *Bgh* strains A6 and K1 (data not shown). This effect may be caused by insufficient 5′ flanking regulatory sequences (~400 bp upstream of the transcription start) in cosmid Sp14-4, driving expression of the candidate *Mla12*, or delayed activation of *Mla12* compared with *Mla1* and *Mla6* resistance (see Discussion) (Freialdenhoven et al., 1994; Boyd et al., 1995).

To examine this possibility further, we subcloned the coding region of the *Mla12* candidate under the control of the strong maize ubiquitin promoter and the nopaline synthase (*Nos*) terminator. DNA of this overexpression construct and two similar control overexpression plasmids harboring *Mla1* or *Mla6* were delivered into leaf epidermal cells of barley cv Ingrid lacking

Mla12 and *Mlo* (Figure 2A). Delivery of each plasmid DNA together with an *Mlo*-expressing construct resulted in a haustorium index of 2 to 5% upon challenge with the *Bgh* isolate containing the cognate *Avr* genes, whereas the control compatible interactions showed an index of ~80%. Note that the very high level of haustorium incidence found in the compatible interactions likely is the result of cobombardment of the race-nonspecific defense modulator *Mlo*, which renders transformed epidermal cells supersusceptible to the fungus (Kim et al., 2002). These data provided evidence that the candidate *Mla12* gene subcloned from cosmid Sp14-4 triggered *AvrMla12*-dependent *Bgh* growth termination. Interestingly, bombardments with empty vector DNA into epidermal cells of Sultan 5, which contains *Mla12*, resulted in a high haustorium index of 45% when inoculated with the incompatible isolate *Bgh* A6 (Figure 2B). This finding suggests that *Mla12* resistance is not effective before haustorium development, consistent with a previous quantitative inspection of single interaction sites in resistant *Mla12* wild-type and susceptible mutant leaves (Freialdenhoven et al., 1994). However, when the putative *Mla12* was overexpressed in Sultan 5 using the single cell expression assay, the haustorium index was reduced to ~2%, similar to the level conferred by *Mla6* (Figure 2B). Apparently, overexpression of the candidate *Mla12* shifted the resistance response from posthaustorium growth arrest to an abortion of fungal development before haustorium formation.

To corroborate the function of *Mla12*, we bombarded the overexpression construct in epidermal cells of mutant lines M66, M22, and M100 (the latter contains the severely defective *rar1-2* allele) (Shirasu et al., 1999a) (Figure 2C). In these experiments, full *AvrMla12*-dependent resistance was restored in both M66 and M22 plants, demonstrating that the mutant phenotypes were complemented by the candidate *Mla12*. By contrast, neither overexpression of *Mla6* nor overexpression of the candidate *Mla12* restored full resistance in the *rar1-2* mutant line M100. The *Mla12* overexpression phenotype was affected more strongly than the *Mla6* response in the *rar1* mutant background. Together, these data strongly support our claim that the *RGH* in cosmid Sp14-4 is *Mla12*.

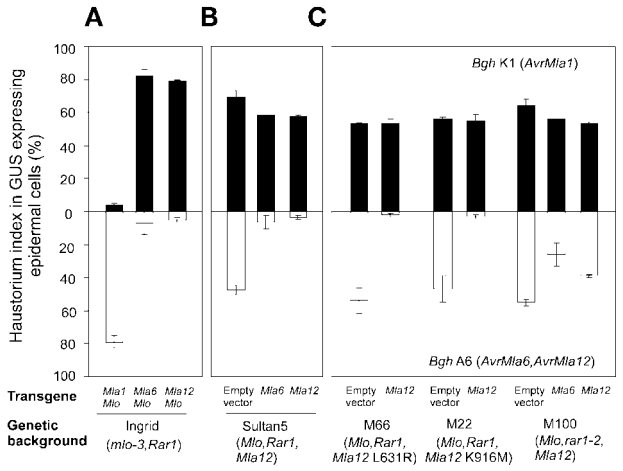

Figure 2. Complementation of Susceptible *Mla12* Mutants by Overexpression of *Mla12* Resistance.

Relative single cell resistance/susceptibility upon delivery of various *Mla* transgenes at 48 h after spore inoculation is indicated by haustorium indices of attacked β-glucuronidase (GUS)–expressing cells (%). Data shown were obtained by bombardment of plasmid DNAs into epidermal cells of detached barley leaves (described by Shirasu et al., 1999b; Zhou et al., 2001). A β-glucuronidase reporter gene was used to identify transformed cells.

(A) The indicated transgenes were tested in detached leaves of barley cv Ingrid harboring *mlo-3 Rar1*. In this line, broad-spectrum *mlo-3* resistance was complemented by cobombardment with a plasmid expressing wild-type *Mlo*; this renders cells supersusceptible to all tested *Bgh* isolates (Zhou et al., 2001; Kim et al., 2002). Results obtained with the *Bgh* isolate K1 (*AvrMla1*) are shown by closed columns, and results obtained with isolate A6 (*AvrMla6* and *AvrMla12*) are shown by open columns in downward orientation. The data shown are means of at least three independent experiments (SD indicated). Each experiment involved light microscopic examination of at least 100 interaction sites between a single *Bgh* sporeling and an attacked epidermal cell.

(B) The indicated transgenes and an empty vector control were delivered into epidermal cells of Sultan 5 containing *Mla12 Mlo Rar1*. Experimental conditions and symbols are identical to those in **(A)**.

(C) Transgene *Mla12* or an empty vector control was delivered into epidermal cells of two susceptible *Mla12* mutant lines (M66 and M22). Transgene *Mla6* or *Mla12* or an empty vector control also was delivered into the *rar1-2* mutant line M100. Experimental conditions and symbols are identical to those in **(A)**.

Context-Dependent Function of Conserved MLA Residues Leu-631 and Lys-916

We noted that amino acid substitutions in the susceptible *Mla12* mutants M66 (L631R) and M22 (K916M) affect residues that are conserved in MLA1 and MLA6, whereas the substitution in mutant M86 (E866K) changes a nonconserved residue (Figure 1). To investigate the importance of Leu-631 and Lys-916 in *Mla1*- and *Mla6*-triggered resistance, the same amino acid substitutions were introduced into *Mla1* and *Mla6* under the control of the ubiquitin promoter and were reintroduced into *Mla12* for comparison and confirmation. Wild-type and mutant variant plants were tested in the transient gene expression system. This analysis showed that *Mla12* mutant variant L631R impaired *AvrMla12*-dependent resistance fully (84%) and K916M impaired it partially (31%), indicating that the MLA12 (K916M) variant protein retains residual activity (Figure 3). This observation is consistent with the fully compromised and partially impaired *Mla12* resistance reported for M66 and M22 mutant plants (infection types 4 and 2/3, respectively) (Torp and Jørgensen, 1986) and validates the usefulness of the single-cell assay to evaluate *Mla12* activity using the strong ubiquitin promoter. The weakly susceptible infection phenotype of M22 mutant plants likely complicated the scoring of infection phenotypes in progeny derived from M22 test crosses and may explain the apparent misinterpretation of the mutant line as an extragenic suppressor of *Mla12* resistance (Jørgensen, 1988, 1996). Surprisingly, despite an overall sequence relatedness of 90% between the tested MLA proteins, none of the amino acid replacements in MLA6 or MLA1 resulted in a detectable change

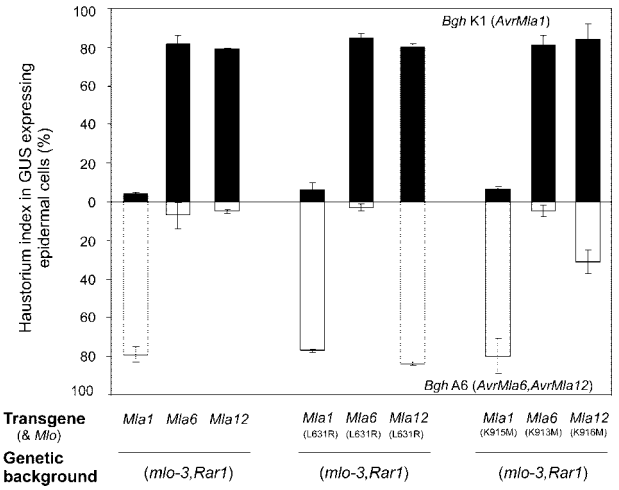

Figure 3. Context-Dependent Functions of Conserved MLA Residues Leu-631 and Lys-916.

Mean values of single cell resistance/susceptibility (%) are shown at left after delivery of *Mla1*, *Mla6*, or *Mla12* into the genetic background of cv Ingrid (*mlo-3 Rar1*). Results obtained with L631R variants of *Mla1*, *Mla6*, and *Mla12* are shown in the middle. Results obtained with *Mla1*, *Mla6*, and *Mla12* variants each containing a K to M substitution at the indicated positions are shown at right. Experimental conditions and designations are identical to those in Figure 2. GUS, β-glucuronidase.

of resistance activity compared with that in the respective wild-type genes (Figure 3). Thus, it is possible that other regions are critical for R protein function in MLA1 and MLA6 (see below). Alternatively, other residues that are absent or polymorphic in MLA12 might compensate for the functional contributions of Leu-631 and Lys-916 in the MLA1/MLA6 substitution mutants.

Recognition Specificity Is Determined by the LRR-CT Unit

For further analysis of regions that are critical for MLA function, we constructed a series of reciprocal domain swaps between *Mla1* and *Mla6* (Figure 4A). These two *R* genes recognize different *AvrMla* genes and have different requirements for *Rar1* and *Sgt1* (Halterman et al., 2001; Zhou et al., 2001; Azevedo et al., 2002). The maize ubiquitin promoter drove the expression of each chimeric gene, and their function was tested after bombardment into leaf epidermal cells by spore inoculation with *Bgh* isolates K1 (*AvrMla1*) and A6 (*AvrMla6*) at 15 h after delivery. Recognition specificity and activity of the chimeras were compared with those of the respective *Mla1* and *Mla6* wild-type genes whose expression was driven by either native regulatory 5′ sequences or the strong ubiquitin promoter (Figure 4B). No significantly different activity was seen using constructs driven by the native or the strong ubiquitin promoter. Full *AvrMla6*-dependent recognition specificity was retained in chimeras containing the complete MLA1-derived CC-NB domains and in chimeras containing progressively more MLA1-derived N-terminal LRR repeats (constructs 16666, 11666, and 11166; Figure 4B). Activities mediated by chimeras containing only MLA6-derived LRRs 3 to 11 (11661) or only the MLA6-derived C terminus (11116) were inactive or severely impaired, respectively. These data suggest that MLA6 LRRs 9 to 11 act together with the cognate C-terminal domain to confer *AvrMla6* recognition specificity.

Reciprocal domain swaps showed that *AvrMla1*-dependent activity was retained upon replacement of the entire MLA1 CC-NB domain only and upon additional replacement of LRRs 1 and 2 (constructs 61111 and 66111). Interestingly, longer substitutions up to LRR 8 rendered the 66611 chimera fully inactive, although the reciprocal construct 11166 fully retained *AvrMla6*-dependent activity. Substitutions containing LRRs 3 to 11 (construct 11661) also compromised *AvrMla1* recognition specificity. Because chimeras containing only MLA1-derived LRRs 3 to 11 (66116) or only the MLA1-derived C terminus (66661) were inactive, we conclude that MLA1-derived LRRs 3 to 11 together with the cognate C-terminal domain are required for MLA1 recognition specificity.

Uncoupling MLA6 Recognition Specificity from RAR1 Dependence

Barley *Rar1* is required for the function of *Mla6* but not *Mla1* (Jørgensen, 1996; Halterman et al., 2001; Zhou et al., 2001). This fact prompted us to examine the activities of wild-type MLA1 and MLA6 and the MLA chimeras in the *rar1-2* genetic background (Figure 4C). The *rar1-2* mutation leads to a transcript-splicing defect, and a RAR1 antiserum fails to detect RAR1 signals on protein gel blots (Azevedo et al., 2002). Delivery of wild-type MLA1 or MLA6 plasmid DNA in *rar1-2* leaf epidermal cells led to fully retained or partially compromised resistance (4 and 39% haustorium index, respectively) (Figure 4C). No significant differences were found between wild-type constructs driven by the native and strong ubiquitin promoters. Thus, *Mla6* function is compromised partially by the *rar1-2* mutation compared with bombardments of the same constructs in the *Rar1* background (Figure 4B). Remarkably, delivery of the three chimeras conferring *AvrMla6*-dependent resistance in *Rar1* plants (16666, 11666, and 11166) displayed either full RAR1 dependence (constructs 16666 and 11666, each showing 80% haustorium index) or uncoupled RAR1 dependence from recognition specificity (construct 11166, showing 10% haustorium index) in the *rar1-2* background. Neither of the two chimeras that retained *AvrMla1*-dependent resistance activity (61111 and 66111) was impaired functionally upon delivery in *rar1-2* mutant plants. Unless MLA6 accumulation is self-limited, we conclude that RAR1 dependence cannot be overcome by *Mla6* overexpression and appears to be modulated by both the CC-NB and LRR regions. Because it was reported that an Arabidopsis *rar1* mutant line failed to accumulate a CC-NB-LRR protein, RPM1 (Tornero et al. 2002), we also tested whether MLA6 becomes unstable in the *rar1-2* mutant background. At 96 h after delivery, MLA6 remained as active as at 15 h after delivery (39% haustorium index), suggesting that the stability of MLA6 remained unchanged in *rar1-2* plants (see below for examples of unstable MLA variants 16666 and 11666).

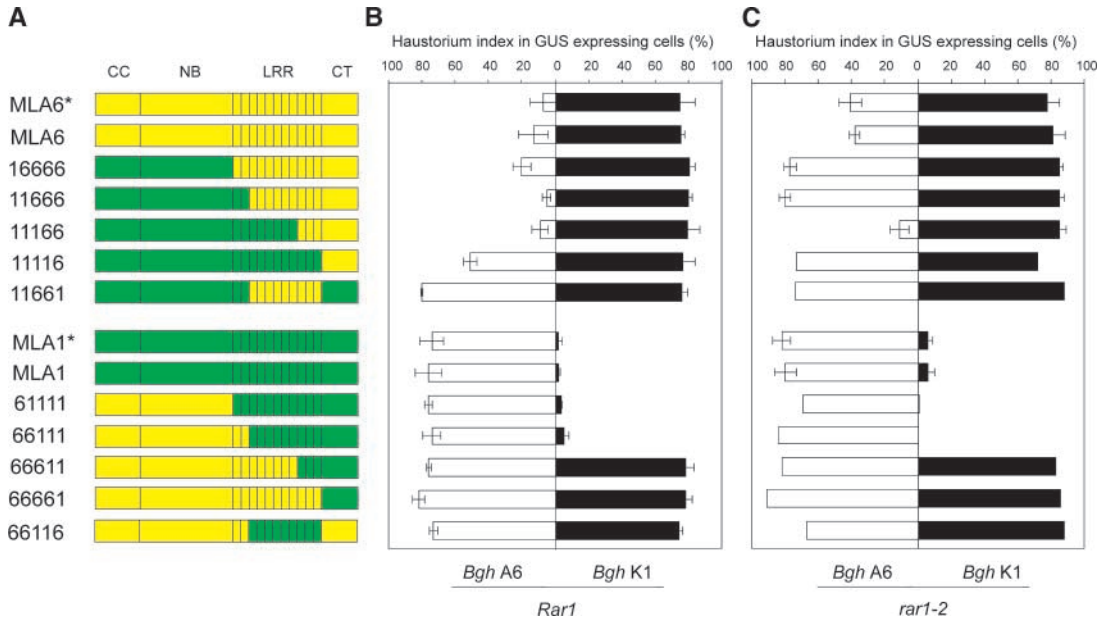

Figure 4. Domain Swaps between MLA1 and MLA6 Reveal Determinants for Recognition Specificity and RAR1 Dependence.

(A) Schemes of MLA6 (yellow), MLA1 (green), and 10 chimeras are shown. The relative positions of the CC, NB, LRR, and CT parts are indicated at top, and acronyms for each chimera are shown at left. The stars indicate gene expression driven by native 5′ flanking sequences; the strong ubiquitin promoter drove the expression of all other genes.

(B) All genes shown in **(A)** were expressed in the *Rar1* wild-type background, and mean values of single cell resistance/susceptibility were scored microscopically upon challenge inoculation with *Bgh* isolates A6 or K1. Experimental conditions and designations are identical to those in Figure 2. GUS, β-glucuronidase.

(C) All genes shown in **(A)** were expressed in the *rar1-2* mutant background, and mean values of single cell resistance/susceptibility were scored microscopically upon challenge inoculation with *Bgh* isolates A6 or K1. Experimental conditions and designations are identical to those in Figure 2.

Requirement of *Sgt1* for MLA-Mediated Resistance

Barley *Sgt1* (*HvSgt1*) was shown to be required for *Mla6*- but not *Mla1*-mediated resistance using double-stranded RNA interference (dsRNAi) gene silencing of *HvSgt1* in a single-cell expression system (Azevedo et al., 2002). This technique was used to examine in the *Rar1* wild-type background the SGT1 requirement of MLA chimeras that retain MLA6 recognition specificity (constructs 16666, 11666, and 11166 in Figure 5). In these experiments, *Bgh* spore inoculations were performed at 48 or 96 h after delivery, and the leaf tissue was fixed for microscopic analysis 48 h after spore inoculation. Cobombardment of SGT1 dsRNAi DNA with a plasmid driving wild-type *Mla6* from the ubiquitin promoter resulted in a small but significantly increased haustorium index (19% at 96 h after delivery) compared with delivery of an empty dsRNAi vector control (2%). This finding is consistent with previous data (Azevedo et al., 2002). Unexpectedly, the functioning of chimeras 16666 and 11666 was partially impaired at 48 h after delivery in cobombardment experiments with the empty vector dsRNAi control. This phenomenon was time dependent in that the chimeras were almost completely inactive at 96 h after delivery. This finding may indicate that the two chimeric MLA proteins are less stable or that fewer or less active recognition complexes are formed compared with complex formation in the MLA6 wild-type protein. Nevertheless, at 48 h after delivery, cobombardment of plasmids 16666 and 11666 with SGT1 dsRNAi DNA significantly enhanced the haustorium index compared with that in empty vector controls ($P < 0.05$), indicating at least a partial requirement of the chimeras for *Sgt1*. By contrast, the 11166 chimeric protein retained full activity upon cobombardment with the empty dsRNAi plasmid control, and its function remained unaffected by *Sgt1* silencing even at 96 h after delivery (Figure 5). Unlike wild-type *Mla6*, *AvrMla6*-dependent resistance conferred by the 11166 variant appears to be uncoupled from both *Rar1* and *Sgt1* dependence (Figures 4C and 5).

DISCUSSION

Allelic Variants Encode MLA Powdery Mildew R Proteins

Eight *NB-LRR* genes are present in a 260-kb interval comprising the *Mla* locus in barley cv Morex and were classified into three dissimilar families (*RGH1*, *RGH2*, and *RGH3*) with <43% amino acid sequence similarity between families (Wei et al., 2002). Computational analysis of the Morex 260-kb sequence contig suggested that a progenitor *Mla* locus harbored at >8 million years before the present one member of each *RGH* family (*RGH1bcd*, *RGH2a*, and *RGH3a*) (Wei et al., 2002). Each of

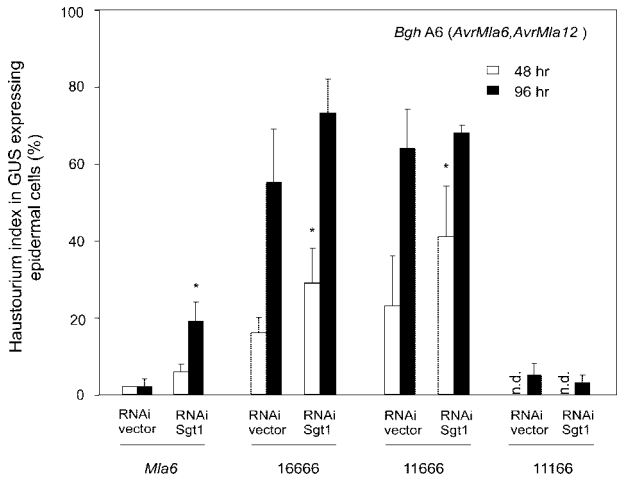

Figure 5. Single Cell Silencing of Sgt1 by dsRNAi.

Wild-type *Mla6* or chimeras retaining *AvrMla6*-dependent recognition specificity were coexpressed with a *HvSgt1* dsRNAi-silencing plasmid (Azevedo et al., 2002) in the *Rar1* wild-type background using a modified single cell transient gene expression assay (Azevedo et al., 2002). After delivery of plasmid DNAs into epidermal cells, detached barley leaves were incubated for 48 h (open bars) or 96 h (closed bars). Subsequently, leaves were inoculated with spores of *Bgh* isolate A6 (*AvrMla6*) and incubated for another 48 h. Microscopic scoring of single interaction sites was identical to that described for Figure 2. Asterisks indicate haustorium indices that are significantly different ($P < 0.05$) from bombardments using empty dsRNAi vector controls. GUS, β-glucuronidase; n.d., not determined.

the *Mla* powdery mildew *R* genes identified to date shows highest overall sequence similarity to Morex *RGH1bcd* in coding regions and shares the same exon/intron structure (Figure 6) (Wei et al., 2002). Unlike *RGH1bcd*, however, *Mla1/6/12* each contains a 5′ untranslated open reading frame and, within intron 3, an $(AT)_n$ simple sequence repeat consisting of different repeat numbers (Figure 6). Also, Morex *RGH1bcd* contains a *BARE1* solo long terminal repeat in intron 3 that is absent in *Mla1/6/12*, and the presence of a 29-bp deletion in the LRR region, resulting in a premature stop codon, suggests that it is nonfunctional (Figure 6). Because Morex lacks known *Mla* powdery mildew resistance specificity, it has been inferred that *RGH1bcd* is a naturally inactive allele of *Mla1* and *Mla6* that may have served as a progenitor for the other Morex *RGH1* family members (*RGH1a*, *RGH1e*, and *RGH1f*) (Wei et al., 2002). Closer examination of all possible pair-wise sequence comparisons of the four Morex *RGH1* variants and the identified *Mla* resistance specificities revealed for exon 4 sequences a common cluster that includes genes *Mla1/6/12* and *RGH1bcd*. However, sequences of *RGH1bcd* exon 3 and intron 3 cluster together with the other *RGH1* gene sequences, whereas the identified *Mla* resistance specificities form a second group (even after the exclusion of the *BARE1* long terminal repeat in intron 3 of *RGH1bcd*; data not shown). Therefore, it is possible that *RGH1bcd* is the product of a recombination between an ancestral Morex allele of *Mla1/6/12* and another more divergent *RGH*.

DNA gel blot analysis and preliminary sequence information obtained from nearly isogenic barley lines containing other *Mla* powdery mildew resistance specificities indicate for each line the presence of one candidate gene with high sequence relatedness to MLA1/6/12 (data not shown). Thus, it is possible that many genetically characterized powdery mildew *R* genes at *Mla* are variants of the same ancestral *RGH1* family member. The presence of the $(AT)_n$ microsatellite in all *Mla R* genes in *Bgh* identified to date and its absence in currently available *Mla RGHs* are consistent with our hypothesis, because recent findings indicate that most microsatellites reside in regions predating recent genome expansion in plants (Morgante et al., 2002).

The very high level of DNA sequence conservation in exon and intron sequences of identified *Mla R* genes (average overall identity of 94 and 93%, respectively) may be indicative of selective constraints acting on both coding and noncoding regions. By contrast, inspection of flanking regions revealed evidence for extensive intralocus recombination events that reshuffled both genes and intergenic regions (Figure 6). For example, a *HORPIA2* element was found in the same direction immediately 3′ of *RGH1bcd* and 3′ of *Mla1*, whereas 3.7 kb of 3′ flanking sequence of *Mla6* showed no significant relatedness to any stretch in the 260-kb *Mla* Morex contig. Sequences located immediately 3′ of *Mla12* were found 5.5 kb downstream of *RGH1bcd*, indicating an extensive intralocus insertion/deletion event. Morex *RGH1f/e* exhibited highest sequence relatedness to the *Mla1* paralog *Mla1-2*; their altered relative orientation to *RGH1bcd* and *Mla1*, respectively, suggests the occurrence of an intralocus inversion event (Figure 6).

Altering Resistance Response Kinetics by *Mla* Dosage

Different *Mla* resistance genes to *Bgh* show characteristic infection phenotypes that are macroscopically visible by different infection types (Boyd et al., 1995). A quantitative analysis of single interaction sites in nearly isogenic lines containing different *Mla* genes revealed for *Mla1* and *Mla6* early termination of *Bgh* growth coincident with haustorium differentiation (Boyd et al., 1995). By contrast, *Mla3* and *Mla7* mediated cessation of fungal growth at a later stage of the infection process, permitting the growth of elongating secondary hyphae on the leaf surface in addition to haustorium differentiation. These *Mla* gene-specific differences correlated with the timing of a cell death response that was either rapid, involving attacked epidermal cells, or slower, including epidermal and subtending mesophyll cells (Boyd et al., 1995). Similarly, delayed cell death–associated resistance is characteristic for lines carrying *Mla12*, permitting indistinguishable fungal growth for up to 36 h after *Bgh* spore inoculation and a high haustorium index of ~60% on both *Mla12*-resistant and *Mla12*-susceptible mutant plants (Freialdenhoven et al., 1994). It is possible that differences in the speed of *Mla* resistance responses are the indirect consequence of different infection stage–specific delivery systems for particular *Bgh* AVRMLA effector proteins (e.g., delivery of AVRMLA12 only after or coincident with haustorium differen-tiation).

Precedence for this idea is found in the expression of *Cladosporium fulvum AVR9*, which is induced strongly upon a switch from surface to intercellular growth of the fungus in leaves,

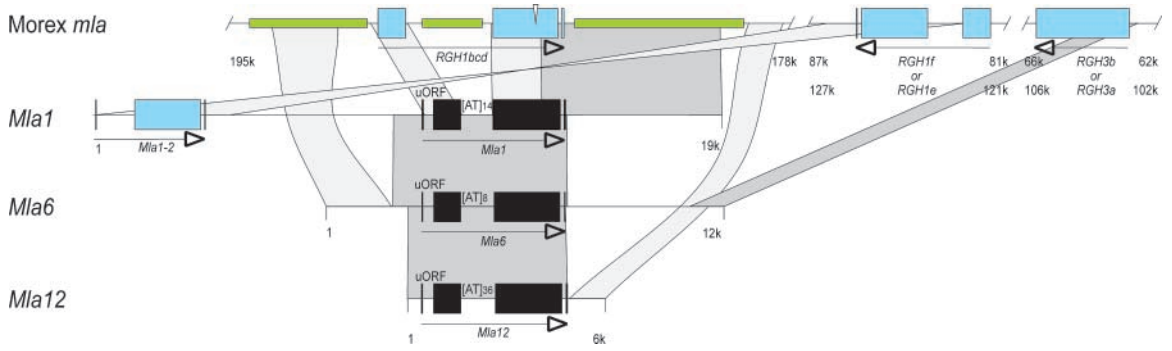

Figure 6. Schemes of the Morex *Mla* Locus and Genomic Regions Containing Identified *Mla* Resistance Genes.

DNA sequences encompassing the Morex *Mla* locus (261 kb, in reverse orientation) (Wei et al., 2002) are represented schematically and drawn to scale in the top line (relevant sequences only). Available genomic sequences of *Mla1*, *Mla6*, and *Mla12* and flanking regions are shown below. Coding sequences of functional *Mla R* genes and *RGHs* are boxed and highlighted in black and blue, respectively. A conserved upstream open reading frame (uORF) and a simple $[AT]_n$ microsatellite are shared among functional *Mla R* genes. Green boxes denote retrotransposon sequences: a *BARE1* solo LTR in intron 3 of *RGH1bcd*, *HORPIA2* immediately 3′ of *RGH1bcd*, and *ALEXANDRA* 5′ of *RGH1bcd*. Dark gray areas denote sequences showing >90% identity, and light gray areas denote sequences showing >75% identity. A possible inversion event could explain the altered relative orientations of homologous genes *Mla1-2* and *RGH1f* as indicated. Note that *RGH1e*/*f* and *RGH3a*/*b* are extremely similar and located within a 40-kb duplicated region (Wei et al., 2002). For this reason, the indicated homologies exist between *RGH1e* and *RGH1f* and between *RGH3a* and *RGH3b*. Arrows indicate the relative orientations of genes (5′ to 3′). Borders of Morex sequences are indicated in kb according to accession AF427791.

which may be cued by fungal nitrogen starvation (Van Kan et al., 1991; Perez-Garcia et al., 2001). Here, we have shown that slow *Mla12*-triggered resistance was altered dramatically to a rapid response by *Mla12* overexpression, leading to almost complete abortion of *Bgh* attack before haustorium differentiation (Figure 2). Because the rapid response retained AVRMLA12 dependence, the *Bgh* effector protein must be, like AVRMLA1 and AVRMLA6 (Halterman et al., 2001; Zhou et al., 2001) (Figure 2), delivered before or during the switch from surface to invasive fungal growth. The rapid *Mla12* overexpression response suggests that cellular amounts of MLA12 or protein complexes containing MLA12 are rate limiting for the onset or speed of the resistance. This finding is consistent with previous results demonstrating markedly reduced resistance in plants that are heterozygous for *Mla12* (Torp and Jørgensen, 1986). In addition, the retained *Rar1* dependence of the *Mla12* overexpression phenotype corroborates this as an authentic response. Assuming that expression levels of different *Mla* genes are similar and sustain comparable protein abundance, it remains possible that the gene-specific infection types reflect differences in the activities of presumed MLA-containing recognition complexes or different intrinsic activities of AVRMLA proteins.

Determinants of MLA Recognition Specificity

Functional analysis of reciprocal domain-swap constructs between *Mla1* and *Mla6* revealed an essential role of the LRR-CT unit in specificity determination (Figure 4B). We found that distinct regions in the LRRs of MLA1 and MLA6 (LRRs 3 to 11 and 9 to 11, respectively) were necessary for cognate AVRMLA perception. This finding is in agreement with LRRs representing the most variable part of MLA and other characterized NB-LRR–type R proteins (Botella et al., 1998; McDowell et al., 1998; Meyers et al., 1998; Ellis et al., 1999; Halterman et al., 2001). It also is consistent with the finding that potentially solvent-exposed residues in MLA LRRs and those of other NB-LRR R proteins are subject to diversifying selection (Botella et al., 1998; McDowell et al., 1998; Meyers et al., 1998; Halterman et al., 2001). One interpretation of these data is that the diversified regions are involved in ligand-specific recognition.

LRRs have been demonstrated to function as specificity determinants of membrane-anchored R proteins (Van der Hoorn et al., 2001; Wulff et al., 2001). Successful domain-swap experiments have been reported only for intracellular TIR-NB-LRR–encoding resistance alleles at the *L* locus in flax to the flax rust fungus (Ellis et al., 1999; Luck et al., 2000). Both MLA and L proteins exhibit comparable average polymorphisms in corresponding domains (based on four MLA variants, including MLA13 [Halterman et al., 2003], and 11 L variants from flax). Unlike our study involving CC-NB-LRR proteins, the analysis of L chimera functions suggested that both TIR-NB and LRR regions can determine specificity differences (Ellis et al., 1999; Luck et al., 2000). Although it is possible that the CC-NB domain is irrelevant for specificity determination, more diverged CC-NB domains from other MLA proteins must be tested before we can generalize from the observations based on MLA1 and MLA6 chimeras.

Reciprocal swaps of the CT domains between MLA1 and MLA6 resulted in nonfunctional chimeras (11116 and 66661; Figure 4B). Our interpretation that cognate LRR-CT units are required for MLA specificity determination was supported by the finding that two of three single–amino acid replacements in mutant MLA12 variants affect CT amino acids and the third affects an LRR residue (Figure 1). Additional evidence for a role of the MLA CT in specificity determination comes from the identification of a hypervariable region in the middle of this domain

(residues 893 to 945 in MLA1). This hypervariable region shows an increased ratio of nonsynonymous (k_a = 15.4) to synonymous (k_s = 9.6) nucleotide substitutions (based on *Mla1*, *Mla6*, *Mla12*, and *Mla13* sequences [Halterman et al., 2003]; significant at $P < 0.1\%$), which is indicative of the operation of diversifying selection. This is like the C-terminal non-LRR domain of *P* locus genes that encode flax TIR-NB-LRR proteins, which also was found to contain a region that is subject to diversifying selection and might contribute to specificity determination (Dodds et al., 2001).

RAR1/SGT1 May Act Downstream of Presumptive MLA Recognition Complexes

There is strong evidence suggesting a conserved role for *RAR1* in *R* gene–mediated resistance to different pathogen classes and in different plant clades (Shirasu et al., 1999a; Liu et al., 2002b; Muskett et al., 2002; Tornero et al., 2002). RAR1 has been implicated in ubiquitin-protein conjugation pathway(s) together with SGT1 (Azevedo et al., 2002; Liu et al., 2002b). Ubiquitination targets have not been identified to date, and it remains unclear whether RAR1/SGT1 acts upstream of, coincident with, or downstream of R protein recognition complexes. The variation in *Rar1* requirement for the function of different *Mla* resistance specificities (Jørgensen, 1996) is unique with regard to their potential allelism and unusual sequence relatedness. Despite a dramatic shift to a rapid resistance response resulting from the overexpression of *Mla12*, its *Rar1* dependence remained unaltered (Figure 2). Likewise, the partial *Rar1* requirement for *Mla6* function and the *Rar1*-independent *Mla1* activity remained unchanged upon the expression of both *R* genes from either the strong ubiquitin promoter or native 5′ flanking regulatory sequences (Figure 4). Thus, RAR1 dependence appears to be conditioned by subtle intrinsic properties of MLA proteins but not by dosage. Consistent with this finding, replacement of MLA6 domains with the corresponding MLA1 parts generated variants conferring *AvrMla6*-specific immunity that was either fully dependent on or independent of *Rar1* (Figure 4C). We were unable to examine this using the reciprocal chimeras because these were either nonfunctional (66611) or mediated *Rar1*-independent resistance activity (61111 and 66111).

A role for RAR1 in the assembly of preformed R protein–containing recognition complexes may be inferred from the finding that a nonchallenged Arabidopsis *rar1* mutant line failed to accumulate the RPM1 CC-NB-LRR protein to *Pseudomonas syringae* (Tornero et al., 2002). Our study demonstrates that the reliance on RAR1 and SGT1 is not absolute for a given *Mla* recognition specificity. Successful uncoupling of *AvrMla6* recognition from *Rar1*/*Sgt1* dependence implies that RAR1 cannot be required for processes that occur "upstream" from recognition (e.g., in planta processing or transport of AVRMLA6) (see chimera 11166 in Figures 4C and 5). Also, the uncoupling excludes the possibility that MLA6 "guards" RAR1 or SGT1 in presumed MLA-containing recognition complexes. It is possible that the MLA6 CC-NB domain and the LRRs exert antagonistic roles, the former inhibiting and the latter enhancing RAR1-dependent R protein function (cf. constructs 16666, 11166, and wild-type MLA6 in Figure 4C). The observed partial impairment of *Mla6* wild-type function in *rar1* plants probably is not the result of MLA6 destabilization, because the activity was time independent (unchanged at 15 and 96 h after DNA delivery). This result is consistent with the finding that *Mla6* overexpression in the *rar1* mutant background did not increase resistance (i.e., the amount of functional recognition complexes) (Figure 4C). Thus, it seems possible that RAR1/SGT1 exerts a function downstream from activated MLA recognition complexes in resistance signaling. Therefore, the observed variation in *Rar1*/*Sgt1* reliance on the function of different MLA wild type or MLA chimeras may be attributable to variation in signal flux set by intrinsic activities of MLA variants in AVRMLA-activated recognition complexes (e.g., by different half-lives of active complexes).

Do MLA Chimeras Affect Folding of MLA Recognition Complexes?

The SGT1 binding function of plant RAR1 proteins has been conserved in monocots and dicots (Azevedo et al., 2002; Liu et al., 2002b). Our data obtained from *Sgt1*-silencing experiments in cells expressing MLA chimeras that retain AVRMLA6 recognition suggest that RAR1/SGT1 functions in MLA6 resistance are closely linked (Figure 5). For example, the RAR1-independent function of chimera 11166 retained full activity upon *Sgt1* silencing; inversely, chimeras showing full RAR1 dependence also retained SGT1 dependence. In addition, the function of *Mla12*, which requires *Rar1*, was compromised significantly in *Sgt1*-silencing experiments (data not shown). Recent experiments using *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe sgt1* mutant strains indicate a potential role of the wild-type protein as a co-chaperone or an assembly factor of diverse regulatory multiprotein complexes, including SCF-type E3 ubiquitin ligases, the structurally related CBF3 kinetochore complex, and the Cyr1p adenylyl cyclase complex (Kitagawa et al., 1999, Dubacq et al., 2002; Garcia-Ranea et al., 2002; Schadick et al., 2002). In this context, it is notable that either of two Arabidopsis *SGT1* genes was shown to complement *S. cerevisiae sgt1* mutant strains (Azevedo et al., 2002). A central conserved part in SGT1 proteins likely adopts a fold similar to that of the p23 co-chaperone, which is known to interact with the heat-shock protein hsp90 chaperone and participates in the folding of different regulatory proteins (Dubacq et al., 2002; Garcia-Ranea et al., 2002). Therefore, it is possible that the observed variation in RAR1/SGT1 dependence for the function of different *Mla* resistance specificities or MLA chimeras reflects differences in the degree of folding/activation assistance needed for presumed MLA-containing recognition complexes. In this scenario, the signal flux in downstream signaling pathways might be similar for both RAR1/SGT1-dependent and -independent resistance.

METHODS

Plant and Fungal Material

Sultan 5 is a chromosome-doubled haploid barley (*Hordeum vulgare*) cultivar containing *Mla12*. *Mla12* mutants (M66 and M86), a *rar1-2* mutant

allele (M100), and the *rar2* mutant (M22) were generated by chemical mutagenesis of Sultan 5 seeds (Torp and Jørgensen, 1986). Ingrid (*mlo-3 Rar1*) was generated by seven backcrosses with cv Ingrid, and the *mlo-29 rar1-2* double mutant was isolated originally from a remutagenized *rar1-2* M2 population. The latter line was used to test the *Rar1* dependence of MLA chimeras (Figure 4). All barley seedlings were grown at 20°C with 16 h of light and 8 h of darkness. The barley powdery mildew (*Blumeria graminis* f. sp. *hordei* [*Bgh*]) isolate A6 (*AvrMla6*, *AvrMla12*, *virMla1*) was maintained on P01, a nearly isogenic line from cv Pallas containing *Mla1*. Isolate K1 (*AvrMla1 virMla6 virMla12*) was maintained on I10, a nearly isogenic line from cv Ingrid containing *Mla12*. Plants or detached leaves were kept at 18°C and 60% RH with 16 h of light and 8 h of darkness after inoculation with Bgh spores.

Genomic Library Construction and Screening for MLA12-Containing Cosmids

High molecular mass genomic DNA was isolated from Sultan 5 containing *Mla12* and partially digested with Sau3AI to produce DNA fragments of 30 to 60 kb. After dephosphorylation, the fragments were ligated to the XbaI-BamHI–linearized SuperCos cosmid vector according to the manufacturer's instructions (Stratagene). A total of 240 pools averaging 4000 clones each were made and kept frozen as glycerol stocks. The library had an average insert size of 25 kb (ranging from 15 to 40 kb) and represents approximately five genome equivalents. DNA preparations were made using the R.E.A.L Prep 96 Plasmid Kit (Qiagen, Valencia, CA) from all pools. For library screening, the plasmid DNA of each pool was digested with HindIII or EcoRI, resolved by 0.8% agarose gel electrophoresis, and blotted onto Hybond-N^+ membranes (Amersham Pharmacia Biotech). To identify positive pools containing the *Mla12* candidate gene, the DNA gel blots were hybridized with a ^{32}P-labeled probe, which was derived from the Leu-rich repeat region of *Mla1* (covering exon 4 of *Mla1*) (Zhou et al., 2001). Approximately 15,000 colonies of each positive pool were screened by hybridization with the same probe to obtain purified clones. Positive clones were fingerprinted using various restriction enzymes.

Sequencing and Gene Characterization

Plasmid DNA of *Mla12*-containing clones was isolated using the Midi-Plasmid-DNA Prep Kit (Qiagen), subcloned, and sequenced as described (Zhou et al., 2001). Construction of sequence contigs was performed using the GCG9 and STADEN software packages (University of Wisconsin Genetics Computer Group, Madison). Sequence alignment was performed using a World Wide Web–based program (http://prodes.toulouse.inra.fr/multalin/multalin.html).

Sequencing of *Mla12* Mutant Alleles

Genomic DNA was isolated from the *Mla12* mutants M86 and M66 and the *rar2* mutant M22. The DNA was used as a template for PCR amplification of the respective *Mla12* mutant alleles. *Mla12*-specific primers were designed based on the sequence alignment of *Mla12*, *Mla1*, *Mla6*, *Mla1-2*, and *RGH1a* (primer sequences are listed in Table 1). PCR products were purified using the Qiagen PCR Product Purification Kit and then sequenced directly. Mutations were identified by aligning the sequences of PCR products to *Mla12* and confirmed by three additional independent PCR procedures and sequencing of plus and minus strands of the mutated region.

Construction of *Mla*-Containing Plasmid Expression Vectors

pUbi-GFP-Nos [maize ubiquitin1 promoter-GFP-Nos poly(A) signal] (Shirasu et al., 1999b) was used as a backbone to subclone *Mla1*, *Mla6*, and *Mla12*. The green fluorescent protein open reading frame was deleted using restriction enzymes PstI and SacI and replaced by an adaptor with a suitable multiple cloning site for *Mla* genes. The 5′ untranslated region of *Mla1* was amplified by PCR using primer pairs MlapstIs1 and MlaAgelas1, and the product was cloned into pGEM-T vector (Promega) and confirmed by sequencing. The 5′ untranslated regions were subcloned into the pUbi-Adaptor-Nos vector using enzymes HindIII and AgeI. The 3′ untranslated region of *Mla1* was amplified with primers MlaEcoRIas1 and MlaBsrDIs1 and cloned into pGEM-T vector. After sequence confirmation, the 3′ untranslated region was subcloned into the pUbi-Adaptor-Nos vector using BsrDI and NotI. The plasmid vector then was linearized with AgeI and BsrDI, and coding regions of *Mla1*, *Mla6*, and *Mla12*, including introns 3 and 4, were inserted. The resulting overexpression plasmids were designated pUbiMla1Nos, pUbiMla6Nos, and pUbiMla12Nos. They served as backbones to generate domain-swap constructs between *Mla1* and *Mla6* and *Mla* mutant variant constructs (see below). Plasmids driven by native 5′ flanking *Mla* promoter sequences were generated by subcloning an 8-kb SacII-XhoI fragment from *Mla1* containing cosmid p6-49-15, or an AvrII-PciI fragment of *Mla6* containing cosmid 9589-5a, into pBluescript II KS− (Stratagene).

Plasmids 16666 and 61111 were generated by exchanging BbsI-NotI fragments, which were derived from pUbiHEMla1Nos and pUbiHEMla6Nos, respectively. Likewise, plasmids 11666 and 66111 were generated by exchanging Bsu36I-NotI fragments. Plasmids 11166 and 66611 were generated by splicing by overlap extension (SOE) using the forward primer MlaBbSIs, the reverse primer NotIas, and the overlapping primers P10s and P10as. The Bsu36I-NotI enzyme pair was used to digest the SOE products that were inserted into pUbiMla1Nos or pUbiMla6Nos digested with the same enzyme pair, respectively. Plasmids 11661 and 66116 also were generated by SOE with primers P5s/P5as and P12s/P12as covering the swap sites and the flanking primers MlaBbSIs and Mla1EcoRIas1. The BbsI-NotI–digested fragments of the SOE products were inserted into pUbiMla1Nos and pUbiMla6Nos, respectively. Plasmids 11116 and 66661 were generated by subcloning Bsu36I-NotI fragments of plasmids 66116 and 11661 into pUbiMla1Nos and pUbiMla6Nos, respectively.

Table 1. *Mla12*-Specific Primer Sequences

Primer	Sequence	Position in *Mla12*
Mla12S1a	5′-CACCTCACCTTCTGTCTCTCTC-3′	−488
Mla12S1b	5′-GCATCTTTCTTGCTATTCTGCTC-3′	−328
Mla12S1c	5′-TGCCATTTCCAACCTGATTCCC-3′	12
Mla12AS1a	5′-CCTTGTTCCTGTCACGCCTATC-3′	34
Mla12AS1b	5′-CCTTTAATCTTCTCGTATACCGCTC-3′	658
Mla12AS1c	5′-TGTTTAGTGTGAACTGCTTATGCC-3′	945
Mla12As1d	5′-TCTCCCTCTTTCCTTCCTCTCC-3′	1228
Mla12S2a	5′-GATGCTTAATGAGAGTAAGATTATCGAG-3′	1705
Mla12S2b	5′-GGCATCAACTTTGCTTTCTCCAATAG-3′	1913
Mla12AS2b	5′-CGACGACAATTACTCTGTGAAGAC-3′	2652
Mla12AS2a	5′-GAAGGGACAAACGACGACAATTACT-3′	2663
Mla12S3a	5′-TAACAGTTTAGAGGAGATGCGG-3′	2366
Mla12S3b	5′-CTCCCGACTGAGATAGGAAAAC-3′	2915
Mla12S3c	5′-TTGTTGTCCCTTCGTCGTCTCTGG-3′	3586
Mla12AS3b	5′-CACAATAGAGAAGAACAAAGACATC-3′	3775
Mla12AS3c	5′-TGTGCGCCAAAAATCAGTTCTCAC-3′	4057
Mla12AS3a	5′-ATGGAGAAAGGAAGGTAGGTGG-3′	4139

For the construction of plasmids pUbiMla1(K915M), pUbiMla6 (K913M), and pUbiMla12(K916M), a single amino acid exchange was introduced by SOE using a template of pUbiMla1Nos, pUbiMla6Nos, and pUbiMla12Nos, respectively. Likewise, variants pUbiMla1(L631R), pUbiMla6(L631R), and pUbiMla12(L631R) were generated by SOE reactions using the same template DNAs. Primers used for these reactions are listed in Table 2. For site-directed mutagenesis of the codon leading to the replacement of Lys with Met, two common primers, MLABbSIs and MLABsrDIas1 (for *Mla1* and *Mla6*) and MLA12BsrDIas1 (for *Mla12*), were used in combination with overlapping primers MLA12DNas2 and Mla12DNs1. The BbSI-BsrDI enzyme pair was used to digest the SOE products, and the resulting fragments were inserted into pUbiMla1Nos, pUbiMla6Nos, and pUbiMla12Nos. For site-directed mutagenesis of the codon leading to the replacement of Leu with Arg, four common primers, P2s, M66-as, M66-s, and Exon-5as, were used for SOE reactions. The SOE products were digested with Bsu36I-SbfI (for *Mla1* and *Mla12*) or Bsu36I-BspEI (for *Mla6*), and fragments were inserted into pUbiMla1 Nos, pUbiMla6Nos, and pUbiMla12Nos digested with the same enzyme pair, respectively.

Single-Cell Transient Expression Assay

The single-cell transient expression assay was performed essentially according to Shirasu et al. (1999b). Reporter plasmids containing *Mlo* and β-glucuronidase (*GUS*) genes (*GUS* alone in the case of the *Mlo* genetic background) and the respective effector plasmids were mixed before coating of the particles (molar ratio of 2:1; 5 μg of total DNA). The bombarded leaves were transferred to 1% agar plates supplemented with 85 μM benzimidazole and incubated at 18°C for 15 h before high-density inoculation with *Bgh* spores. Leaves were stained for GUS, and single leaf epidermal cells attacked by *Bgh* germlings were evaluated microscopically at 48 h after spore inoculation. In the double-stranded RNA interference single-cell silencing experiments, particles were co-coated with a construct encoding an intron-spliced double-stranded RNA interference construct targeting *HvRAR1* or *HvSGT1* according to Azevedo et al. (2002) (molar ratio of 1:1:1; 5 μg of total DNA). Note that in the gene-silencing experiments, the bombarded leaves were inoculated at 18°C for 48 or 96 h before high-density inoculation to allow the turnover of preformed RAR1 or SGT1.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Table 2. Primers Used in SOE Reactions

Primer	Sequence
Exon-5as	5′-AATCGTCATCATGAGCACCTT-3′
M66-as	5′-CCAACACCTCCAAAAACTGCCGTTTTCCT-3′
M66-s	5′-CTGAGATAGGAAAACGGCAGTTT-3′
Mla12BsrDIas1	5′-CTGATGCAATGTGAATCCTTGTG-3′
Mla12BsrDIs1	5′-ACATTGCATCAGATGTGCTCTG-3′
Mla12DNas2	5′-GCTTCCATTGCCTCCCCAACCCT-3′
Mla12DNs1	5′-GAGCGAGGGTTGGGGAGGCAATG-3′
Mla1EcoRIas1	5′-AAGCGGCCGCGAATTCTAATACTACTAGGA-CTCG-3′
MlaAgeIas1	5′-TGGCACCGGTGACAATATCCAT-3′
MlaBbSIs	5′-TGGGAATAGCATGTCTTCACAG-3′
MlaBsrDIas1	5′-TGATGCAATGTGAGTCGCTCTGG-3′
MlaBsrDIs1	5′-CTGATCCAGAGCGACTCACATTGC-3′
MlaPstIs1	5′-CTTCTGCAGACTGAGTCATCGGCACCTTGC-3′
NotIas	5′-GCAAGACCGGCAACAGGATTCAA-3′
P10as	5′-TCGCAGTGCAGAGAGTTGGCT-3′
P10s	5′-AGCCAACTCTCTGCACTGCGA-3′
P12as	5′-TCAAACAATATCTGCGTGGCA-3′
P12s	5′-TGCCACGCAGATATTGTTTGA-3′
P2s	5′-GCTCGGATTAAATTACTTCAACC-3′
P5as	5′-CAAGATCCAACACCTCCAAAAACT-3′
P5s	5′-AGTTTTTGGAGGTGTTGGATCTT-3′

Accession Number

The GenBank accession number for the *Mla12* genomic sequence is AY196347.

ACKNOWLEDGMENTS

We thank Roger Wise and Dennis Halterman for the gift of C.I. 16151 cosmid clone 9589-5a containing *Mla6* and for sharing associated sequence data before publication. We thank Jane Parker for providing valuable comments on the manuscript. This work was supported by the Max Planck Society and the Gatsby Charitable Organization.

Received November 14, 2002; accepted December 29, 2002.

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11. Acknowledgements

I find I owe a debt to many people when I reflect on all the way of pursuing my Ph.D study. However, it is more appropriate to acknowledge those I know have directly or indirectly contributed to this work.

There is one person who turned the track of my 'academic life' - although it is still too early to use these words - by allowing me working on MLA and the way of his superb guidance. My sincere gratitude goes to Prof. Dr. Paul Schulze-Lefert, my supervisor, for his excellent advices, unlimited encouragement and support throughout my study. It was difficult to know what is 'Consistency and Precision' for scientific work, however, it will become easier to apply them in my future career.

There are some other people whose help was so precious. I particularly thank Dr. Staphane Bieri for his valuable support when I moved to Köln and his discussions that proved to be fruitful. I would like also to thank Dr. Fasong Zhou for his help when I started my doctorate training in Sainsbury Laboratory, John Innes Centre, England in 2001. I am also grateful to Dr. Ken Shirasu for hosting me in Sainsbury Lab and for extending his generous assistance upto the present time. I would like to thank other MLA members, Stefan Mauch, Francesca Ceron and Simone Pajonk. Thanks for the nice time working together and helping each other. I have been also benefited a lot from other colleagues, Drs. Stephan Bau, Riyaz Bhat, Judith Müller and Ralph Panstruga, as well as Chiara Consonni, Jan Dittgen, Häweker Heidrun, Marco Mikilis, and Anja Reinstädler. They are so helpful in the lab or during the preparation of this thesis. I also appreciate the help from other colleagues in MPIZ, special thanks to Drs. Laurant Deslandes and Joachim Uhrig for helped me on yeast work.

I realize that it would have been impossible to take care of both my work and myself without the unbelievable support from my wife, for this she deserves all credits that I deserve for the completion of the study.

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Ein Teil dieser Arbeit wurde bereits veröffentlicht:

> **Shen, Q.-H., Zhou, F.S., Bieri, S., Haizel, T., Shirasu, K., and Schulze-Lefert, P.** (2003). Recognition specificity and RAR1/SGT1 dependence in barley *Mla* disease resistance genes to the powdery mildew fungus. **Plant Cell 15,** 732-744.

Ein Teil dieser Arbeit wird zur Veröffentlichung vorbereitet:

> **Bieri, S., Shen, Q.-H., Mauch, S., and Schulze-Lefert, P.** Barley MLA protein abundance is controlled by RAR1 and is rate-limiting for effieient resistance to the powdery mildew fungus. Manuscript in preparation.

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