The Plant Cell, Vol. 16, 3480–3495, December 2004, www.plantcell.org 

# RAR1 Positively Controls Steady State Levels of Barley MLA Resistance Proteins and Enables Sufficient MLA6 Accumulation for Effective Resistance

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**The polymorphic barley (*Hordeum vulgare*) *Mla* locus harbors allelic race-specific resistance (*R*) genes to the powdery mildew fungus *Blumeria graminis* f sp *hordei*. The highly sequence-related MLA proteins contain an N-terminal coiled-coil structure, a central nucleotide binding (NB) site, a Leu-rich repeat (LRR) region, and a C-terminal non-LRR region. Using transgenic barley lines expressing epitope-tagged MLA1 and MLA6 derivatives driven by native regulatory sequences, we show a reversible and salt concentration–dependent distribution of the intracellular MLA proteins in soluble and membrane-associated pools. A posttranscriptional process directs fourfold greater accumulation of MLA1 over MLA6. Unexpectedly, in *rar1* mutant plants that are compromised for MLA6 but not MLA1 resistance, the steady state level of both MLA isoforms is reduced. Furthermore, differential steady state levels of MLA1/MLA6 hybrid proteins correlate with their requirement for RAR1; the RAR1-independent hybrid protein accumulates to higher levels and the RAR1-dependent one to lower levels. Interestingly, yeast two-hybrid studies reveal that the LRR domains of RAR1-independent but not RAR1-dependent MLA isoforms interact with SGT1, a RAR1 interacting protein required for the function of many NB-LRR type R proteins. Our findings implicate the existence of a conserved mechanism to reach minimal NB-LRR R protein thresholds that are needed to trigger effective resistance responses.**

## INTRODUCTION

Plants recognize pathogen-derived effectors that are either conserved amongst several microbial species (pathogen-associated molecular patterns) or are polymorphic among isolates of a single pathogen species. Recognition of pathogen-associated molecular patterns is mediated by membrane-resident receptors, such as *Arabidopsis thaliana* FLS2 (Gomez-Gomez et al., 2001; Zipfel et al., 2004). Perception of isolate-specific pathogen effectors involves either membrane-resident or intracellular race-specific resistance (R) proteins (Ellis et al., 2000; Dangl and Jones, 2001). Most known *R* genes encode intracellular nucleotide binding site Leu-rich repeat (NB-LRR)–type proteins that form two subclasses, containing at the N terminus either a coiled-coil (CC) structure or a domain sharing sequence similarity with *Drosophila melanogaster* TOLL and human interleukin 1 receptors (CC-NB-LRR and TIR-NB-LRR subfamilies, respectively). R proteins recognize isolate-specific effectors that are encoded by so-called avirulence (*Avr*) genes and trigger a resistance response that is frequently linked to a rapid cell death (hypersensitive response [HR]) of host cells at attempted invasion sites.

Mutational analyses uncovered several genes required for *R* gene function in several plant–microbe interactions (Freialdenhoven et al., 1994; Hammond-Kosack et al., 1994; Parker et al., 1996; Warren et al., 1999; Tornero et al., 2002a). Among these was the barley (*Hordeum vulgare*) *Rar1* gene (Rar, required for Mla12 resistance), which is essential for the function of a subset of *Mla R* gene specificities (Torp and Jørgensen, 1986; Freialdenhoven et al., 1994; Shen et al., 2003). *Rar1* encodes an intracellular $Zn^{2+}$ binding protein (Shirasu et al., 1999), and subsequent analyses have shown that RAR1 is required for several *R* gene–mediated resistance responses in monocotyledonous and dicotyledonous plant species against different pathogen classes (Shirasu and Schulze-Lefert, 2003). RAR1 exerts a critical role for the function of both subtypes of NB-LRR R proteins, TIR-NB-LRR and CC-NB-LRR–type proteins. This is in contrast with other identified components required for *R* gene function, such as *EDS1* and *NDR1*, which appear to be preferentially engaged by either the TIR-NB-LRR or the CC-NB-LRR subclass (Century et al., 1997; Falk et al., 1999). Barley RAR1 interacts with SGT1 (for suppressor of G-two allele of skp1) in the yeast two-hybrid assay and in planta. SGT1 is another intracellular protein

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.104.026682.

engaged by a subset of NB-LRR R proteins (Austin et al., 2002; Azevedo et al., 2002). Genetically, some *R* genes require *Rar1* or *Sgt1*, whereas others require both or none (reviewed in Shirasu and Schulze-Lefert, 2003).

A central region of human SGT1, called the CS domain, adopts a tertiary structure that is related to the cochaperone p23 and binds to cytosolic heat shock protein 90 (HSP90) (Lee et al., 2004). Consistent with this, conserved interactions were found between SGT1 and cytosolic HSP90 in plants (Hubert et al., 2003; Takahashi et al., 2003; Liu et al., 2004). Plant cytosolic HSP90 also interacts with RAR1 and has been shown to play a critical role for the function of the NB-LRR R proteins Arabidopsis RPM1, RPS2, potato (*Solanum tuberosum*) Rx, and tobacco (*Nicotiana tabacum*) N (Hubert et al., 2003; Lu et al., 2003; Takahashi et al., 2003; Liu et al., 2004). A common feature of HSP90 proteins is that they bind their target proteins in nearly mature conformations, retaining and releasing them in an activity cycle driven by ATP hydrolysis and regulated by binding of cochaperones. These findings and the presence of the CS domain at the C terminus of animal RAR1 homologs (Shirasu et al., 1999) led to the idea that RAR1 and SGT1 might exert cochaperone-like activities. In plants, vertebrates, and yeast, SGT1 also associates with SCF-type E3 ubiquitin-ligase complexes and the 26S proteasome, which is indicative of a link to ubiquitination-dependent processes (Azevedo et al., 2002; Liu et al., 2002). However, neither genetic nor biochemical analysis could position RAR1 or SGT1 in the process leading to race-specific resistance, and the exact biochemical functions of both proteins in NB-LRR–mediated resistance are not understood.

The polymorphic barley *Mla* (for mildew-resistance locus A) *R* locus encodes more than 28 characterized race-specific resistance specificities to *Blumeria graminis* f sp *hordei* (*Bgh*) isolates (Jørgensen, 1994; Wei et al., 2002). Molecular isolation of *Mla1*, *Mla6*, *Mla7*, *Mla10*, *Mla12*, and *Mla13* suggest that these might be alleles of one of several NB-LRR *R* gene homologs at the complex locus (Halterman et al., 2001, 2003; Zhou et al., 2001; Wei et al., 2002; Shen et al., 2003; Halterman and Wise, 2004). Analysis of *Mla1*/*Mla6* hybrid genes revealed that recognition specificity is determined by different but overlapping LRR regions and the C-terminal (CT) part (Shen et al., 2003). The reported differential engagement of RAR1 and SGT1 in *Bgh* resistance triggered by different *Mla R* specificities is remarkable because of the unusually high sequence identity (>90%) of the deduced MLA proteins (Jørgensen, 1996). Functional MLA chimeras have been generated in which *AvrMla6* recognition specificity became uncoupled from RAR1 dependence (Shen et al., 2003). A single amino acid substitution in the LRR domain, which resides within the region contributing to RAR1 engagement in MLA1/MLA6 hybrid proteins, alleviates RAR1 dependence of *Mla6* and *Mla13* resistance (Halterman and Wise, 2004). Both reports implicate the LRRs of MLA as one determinant for RAR1 engagement.

Further insights in *Mla*-mediated resistance require the development of tools for the investigation of MLA proteins. In this study, we have generated transgenic barley lines expressing functional epitope-tagged MLA1 and MLA6. Biochemical fractionation experiments revealed the existence of at least two intracellular MLA pools in healthy plants. We used systematic yeast two-hybrid analysis to show direct interactions between MLA and SGT1 as well as cytosolic HSP90. A decisive role for RAR1 in the control of R protein steady state levels was revealed upon analysis of MLA1, MLA6, and MLA chimeras in transgenic lines that exhibit a differential genetic RAR1 requirement. Our findings indicate that RAR1 functions in R protein resistance pathways where RAR1 involvement was not revealed by previous genetic studies. An analogous analysis of RAR1-dependent steady state levels of the Rx R protein in *N. benthamiana* and RAR1-independent control of the abundance of the LRR containing F-box protein COI1 in Arabidopsis lead us to propose a general role for RAR1 to reach minimum NB-LRR R protein levels that are needed to trigger effective resistance responses.

## RESULTS

### Epitope-Tagged MLA1 and MLA6 Are Functional

We generated various epitope-tagged *Mla1* and *Mla6* derivatives. The epitope tags were introduced as N- or C-terminal fusions into genomic clones of *Mla1* and *Mla6*, each comprising ~2-kb native 5′ and 1-kb 3′ regulatory sequences (Figure 1A). In addition, we generated C-terminal fusions with the 30-kD green fluorescent protein (GFP). To find out whether the resulting fusion proteins were able to trigger race-specific resistance upon recognition of cognate avirulence gene products (AVRMLA1 and AVRMLA6, respectively), we delivered the plasmid DNA constructs into barley leaf epidermal cells using particle bombardment and scored MLA1 or MLA6 activity in single cells after challenge with *Bgh* spores (Figure 1B; three component assay, Zhou et al., 2001). During compatible interactions, delivery of wild-type MLA1 or MLA6 constructs permitted invasive fungal growth of isolates A6 (*AvrMla6*) and K1 (*AvrMla1*), respectively, in ~75% of transformed cells (percentage haustorium index). During incompatible interactions, delivery of *Mla1* or *Mla6* constructs reduced the haustorium index to 2 and 8%, respectively, which is comparable to previously published data (Zhou et al., 2001). N-terminal fusions of small c-myc or hemagglutinin (HA) epitopes rendered both MLA1 and MLA6 derivatives partially or fully nonfunctional (Figure 1B). By contrast, when the same epitope tags were fused to the C termini of MLA1 and MLA6, the fusion proteins exhibited race-specific resistance activity similar to the wild-type level. Likewise, MLA activities were only slightly impaired or not affected upon C-terminal fusions to myc-HA or triple HA (3xHA) tags, whereas both MLA1-TAP and MLA6-TAP (for tandem affinity purification tag) fusion proteins were essentially nonfunctional. Because C-terminal fusions with the large GFP reporter (30 kD) to MLA1 and MLA6 are functional, unknown TAP-tag specific features but not its large size (20 kD) must render the MLA-TAP fusion proteins inactive or unstable. Collectively the same epitope affected MLA1 and MLA6 activity to a similar degree, and C-terminal fusions retained resistance activities in a tag-dependent manner.

We generated transgenic barley lines expressing MLA1 and MLA6 C-terminal fusions to c-myc or HA epitopes for subsequent studies. A minimum of five independent single-copy insertion lines were generated for each construct using *Agrobacterium tumefaciens*–mediated transformation. Transgenic

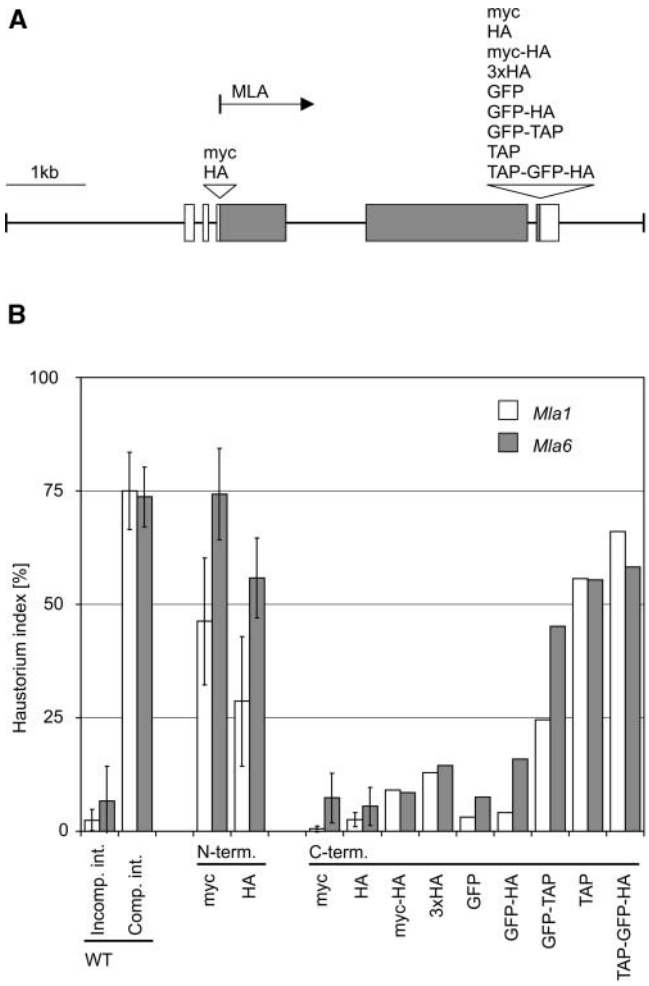


**Figure 1.** Single-Cell Transient Gene Expression Analysis of Epitope-Tagged *Mla* Variants.

**(A)** Schematic representation of 8-kb genomic fragment harboring *Mla1* and the sites and nature of sequences inserted in the *Mla1* derivatives. A corresponding 8-kb genomic fragment was used for expression of *Mla6*. Shaded boxes represent coding sequences, and open boxes denote noncoding transcribed sequences. All elements are drawn to scale. Epitope tags were inserted as N- or C-terminal translational fusions upstream or downstream of the coding sequence. The tags tested were as follows: myc, peptide derived from human c-myc protein; HA, peptide derived from the influenza hemagglutinin protein; 3xHA, triple HA tag; GFP, green fluorescent protein; TAP, tandem affinity purification tag. Various combinations of these tags were also tested as indicated. The arrow indicates the predicted translation start.

**(B)** Functional analysis of epitope-tagged *Mla1* and *Mla6* derivatives. Relative single cell resistance/susceptibility is shown upon biolistic delivery of wild-type *Mla1, Mla6*, or epitope-tagged transgenes at 48 h after *Bgh* spore inoculation. Relative activities mediated by epitope-tagged MLA1 and MLA6 derivatives were recorded in incompatible interactions after challenge with *Bgh* isolates K1 (expressing *AvrMla1*) and A6 (expressing *AvrMla6*), respectively. Relative activities of the wild-type transgenes *Mla1* and *Mla6* were recorded during both compatible and incompatible interactions upon challenge with K1 or A6 isolates. Data were obtained from three (SD indicated) or two independent experiments, each involving light microscopic examination of at least 100 interaction sites.

plants expressing the tagged MLA derivatives as well as lines expressing untagged *Mla1* or *Mla6* triggered effective race-specific resistance toward the cognate *Bgh* strains (K1 and A6, respectively). The observed infection phenotypes were indistinguishable from nontransgenic barley containing *Mla1* or *Mla6* (data not shown). Transgenic lines expressing the N-terminal myc-MLA1 fusion were found to be fully susceptible upon inoculation with the cognate *AvrMla1* expressing K1 isolate. Taken together, the activities of MLA derivatives were comparable in transgenic plants and in the transient single-cell gene expression assay.

### Tissue-Specific Distribution of MLA in Leaf Tissues Does Not Coincide with Its Physiological Site of Action

We examined the accumulation of epitope-tagged MLA1-HA fusion protein in various plant tissues including root, stem, the first leaf, and young (growing) as well as fully developed leaves of 6-week-old plants in homozygous transgenic lines (Figure 2A). Protein gel blot analysis of total protein extracts using $\alpha$-HA antiserum detected a signal at $\sim$110 kD in all tissues tested, which is in accordance with the molecular mass predicted for MLA1 (Figure 2A). The presence of MLA1-HA in roots is remarkable because powdery mildew fungi are known to attack only aerial epidermal tissue. To examine a potential preferential accumulation of MLA in the epidermis, the physiological target tissue of powdery mildews, we collected abaxial leaf epidermis that can be readily peeled away from detached first leaves. Surprisingly, the presence of MLA1 could not be demonstrated in epidermal tissue by the protein gel blot detection system used in these experiments. We estimate that MLA1 accumulates to at least 20-fold lower levels in epidermal tissue as opposed to total leaf protein if equal protein amounts are compared (Figure 2B; see Discussion; data not shown). This distribution contrasts with ROR2 syntaxin, a component of broad-spectrum penetration resistance to *Bgh*, which is known to preferentially accumulate in the leaf epidermis (Figure 2B; Collins et al., 2003). These data suggest that the distribution of MLA1 in leaves does not correlate with its main physiologic site of action. Because MLA1 protein was not detectable in the epidermis, all following experiments were based on whole leaf protein extracts.

### Soluble and Membrane-Associated MLA Pools

NB-LRR R proteins are predicted intracellular proteins, but this has only been experimentally tested to our knowledge in two cases using *R* gene constructs driven by native regulatory sequences (Boyes et al., 1998; Axtell and Staskawicz, 2003). We performed basic cellular fractionation experiments to identify compartments containing MLA. After removal of cell debris at 16,000*g*, MLA1-HA was found in both soluble and in 100,000*g* microsomal fractions at 150 mM near-physiological salt concentration (Figure 2C). The relative MLA abundance in these two fractions was strongly dependent on salt concentrations of the extraction buffer. At 200 and 500 mM NaCl, increasing amounts of soluble MLA were found, whereas decreasing levels were seen in the membrane-associated fractions. Unlike this, two other tested intracellular proteins, cytosolic HSP90 and SGT1, were

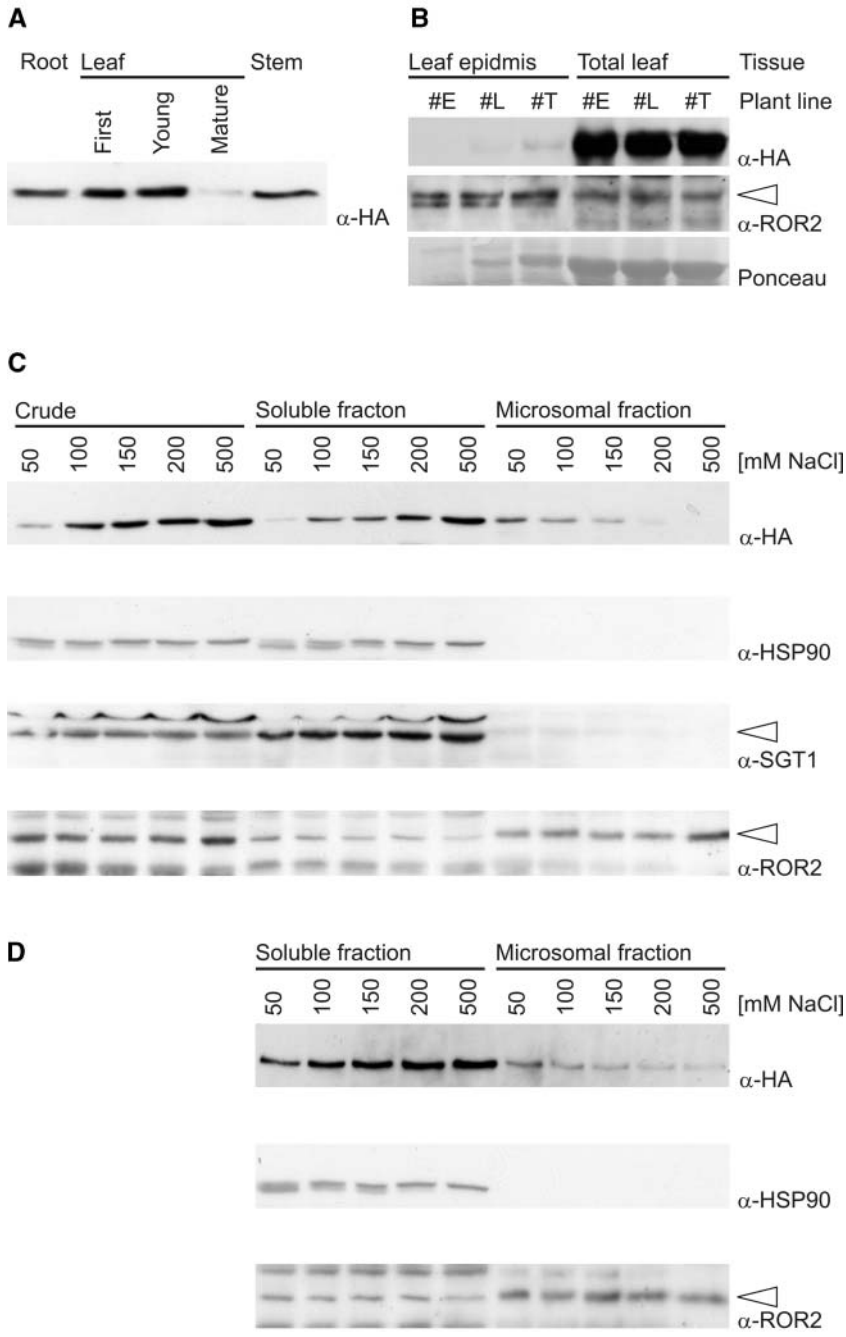


**Figure 2.** Abundance of MLA1-HA in Plant Organs and Subcellular Fractionation.

**(A)** Protein gel blot analysis of protein extracts derived from the indicated tissues/organs of a transgenic line expressing a single copy *Mla1-HA*. Forty micrograms of total protein were loaded in each lane. The size of the detected protein is consistent with a predicted MLA1 molecular mass of 109 kD.
**(B)** MLA1-HA abundance in leaf epidermis and total leaves (first fully expanded leaf) of 7-d-old seedlings. #E, #L, and #T denote three independent transgenic lines. Equal amounts of protein were loaded for protein gel blot analysis. Duplicate blots were probed with anti-HA and anti-ROR2 antibodies. A Ponceau stain of the membrane shows the most abundant protein species (large subunit of ribulose bisphosphate carboxylase) present in each lane. Note low-level contamination of leaf epidermal peels with mesophyll cells as indicated by detectable amounts of the large subunit of ribulose bisphosphate carboxylase in lanes #L and #T.
**(C)** Crude extract and soluble and microsomal fractions of an *Mla1-HA* containing transgenic line were tested using antisera against HA (MLA protein), HSP90, SGT1, and ROR2 by immunoblotting. Total leaf protein was obtained using extraction buffers of different ionic strength (indicated by different NaCl concentrations), and crude extract was recovered after removal of cell debris at 16,000*g* (designated crude). The crude extract was adjusted to

found at all tested salt concentrations only in the soluble fraction. ROR2 syntaxin, an intracellular protein containing a predicted single tansmembrane helix, was preferentially recovered in the microsomal fractions. These data suggest the existence of soluble and membrane-associated MLA pools. Because the salt concentration of the extraction buffer also influenced the total amount of MLA extractable from leaf tissue samples such that at higher salt concentrations significantly more MLA was recovered from equal amounts of tissue (Figure 2C, crude extract), we modified our experimental setup to find out whether MLA can reversibly change its location from one pool to the other. Crude extracts prepared at 150 mM NaCl were readjusted after removal of cellular debris at 16,000*g* to higher or lower salt concentrations, allowed to equilibrate, and then separated into soluble and microsomal fractions (Figure 2D). At higher salt concentrations, more MLA was found in the soluble fraction, whereas at lower concentrations, increasing amounts associated with the microsomal fraction. These data indicate loose and reversible associations of soluble MLA with membrane fractions, possibly through ionic interactions.

### MLA1 and MLA6 Steady State Levels Are Different

We compared the abundance of MLA1-HA relative to MLA6-HA in nonchallenged leaves (Figure 3A). MLA6-HA accumulated to about fourfold lower levels in comparison with MLA1-HA in each of four tested independent transgenic lines. Semiquantitative RT-PCR experiments were performed to detect potential differences in *Mla1* and *Mla6* gene expression and showed indistinguishable amounts of RT-PCR products (see below and Figure 6D). This is consistent with the fact that the 5′ regulatory sequences driving the expression of *Mla1* and *Mla6* are highly similar (95% identity in a 750-bp contiguous region immediately upstream of the transcription start site). The difference between *Mla* gene expression levels and protein accumulation suggests the existence of a posttranscriptional regulatory mechanism.

*Mla R* specificities to *Bgh* appear to be alleles of one of three highly sequence-diverged NB-LRR *R* gene homologs at the complex *Mla* locus (Wei et al., 2002; Shen et al., 2003; Halterman and Wise, 2004). This makes it impossible to generate lines expressing two or more homozygous *Mla R* specificities by crossings between naturally polymorphic *Mla* accessions. We crossed *Mla1-myc* and *Mla6-HA* expressing transgenic lines and selected homozygous F3 individuals coexpressing both or expressing each transgene separately. Inoculation experiments with powdery mildew isolates expressing *AvrMla1* or *AvrMla6* revealed efficient *Mla1*- and *Mla6*-specific resistance in lines coexpressing both transgenes, thereby demonstrating that two *Mla R* gene specificities can be functional in a single plant when both specificities were either heterozygous (F1) or homozygous (F3). Interestingly, the isoform-specific MLA abundance remained unchanged in F3 individuals coexpressing MLA1-HA and MLA6-myc in comparison with F3 individuals expressing either transgene alone (Figure 3B). This demonstrates lack of potential allelic interference and shows that the isoform-specific accumulation differences are attributable to intrinsic properties of MLA1 and MLA6.

### *Rar1* Is Involved in MLA1 and MLA6 Abundance in Nonchallenged Plants

Transgenic barley plants expressing epitope-tagged *Mla1-HA* or *Mla6-HA* were crossed with a *rar1*-2 mutant line lacking detectable RAR1 protein (Freialdenhoven et al., 1994). F2 seedlings were selected containing each at least one *Mla* transgene copy and homozygous wild-type or mutant *rar1* alleles. Analysis of F3 families derived from F2 individuals after selfing enabled us to identify plants that were homozygous at both the transgene and the *Rar1* locus. Lines containing *Mla1-HA* triggered an effective *AvrMla1*-dependent immune response without macroscopically visible HR irrespective of the presence or absence of wild-type *Rar1* (Figure 4F). Unlike this, effective *Mla6-HA* triggered resistance was clearly impaired in a *rar1* mutant background upon challenge with the *AvrMla6* containing *Bgh* isolate. In *MLA6-HA rar1* plants, frequent necrotic patches were visible underneath fungal colonies, and the colony size was smaller compared with fully compatible interactions, which is indicative of ineffective resistance lagging behind the fungal infection process (trailing HR; data not shown). This differential impairment of *Mla1* and *Mla6* resistance in the *rar1-2* mutant background is consistent with previous studies using nontransgenic barley (Jørgensen, 1996). Unexpectedly, we found a fourfold reduction of both MLA1-HA and MLA6-HA steady state levels in *rar1* mutant backgrounds in nonchallenged plants (Figures 4A and 4D). This is surprising to us because previous genetic studies implied that RAR1 is not involved in the MLA1 resistance pathway (Jørgensen, 1996; Zhou et al., 2001). The decrease of MLA abundance in *rar1* backgrounds is not accompanied by a reduction of *Mla* gene expression (Figure 4B; *Mla1-HA* not shown).

We also identified F3 individuals that were homozygous for *MLA6-HA* and contained one *Rar1* wild-type copy (*Rar1 rar1* plants). In these heterozygous *Rar1 rar1* lines, MLA6-HA accumulation was indistinguishable in comparison with homozygous *Rar1* lines, revealing that *Rar1* gene dosage is not important for the control of MLA6 accumulation (Figures 4C and 4E). This is consistent with indistinguishable resistant infection phenotypes

**Figure 2.** (continued).

similar protein concentrations, and equal volumes were then separated in soluble and microsomal fractions at 100,000*g*. The microsomal fraction was resolubilized (in the original volume), and equal volumes were used for immunoblotting.

**(D)** Immunoblotting of soluble and microsomal fractions of an *Mla1-HA* containing transgenic line using antisera against HA (MLA protein), HSP90, and ROR2. Unlike in **(A)**, crude extract was obtained with extraction buffer at the near-physiological salt concentration of 150 mM NaCl. Aliquots of the crude extract were then adjusted to the indicated NaCl concentrations and allowed to equilibrate for 2 h and subsequently separated in soluble and microsomal fractions at 100,000*g*.

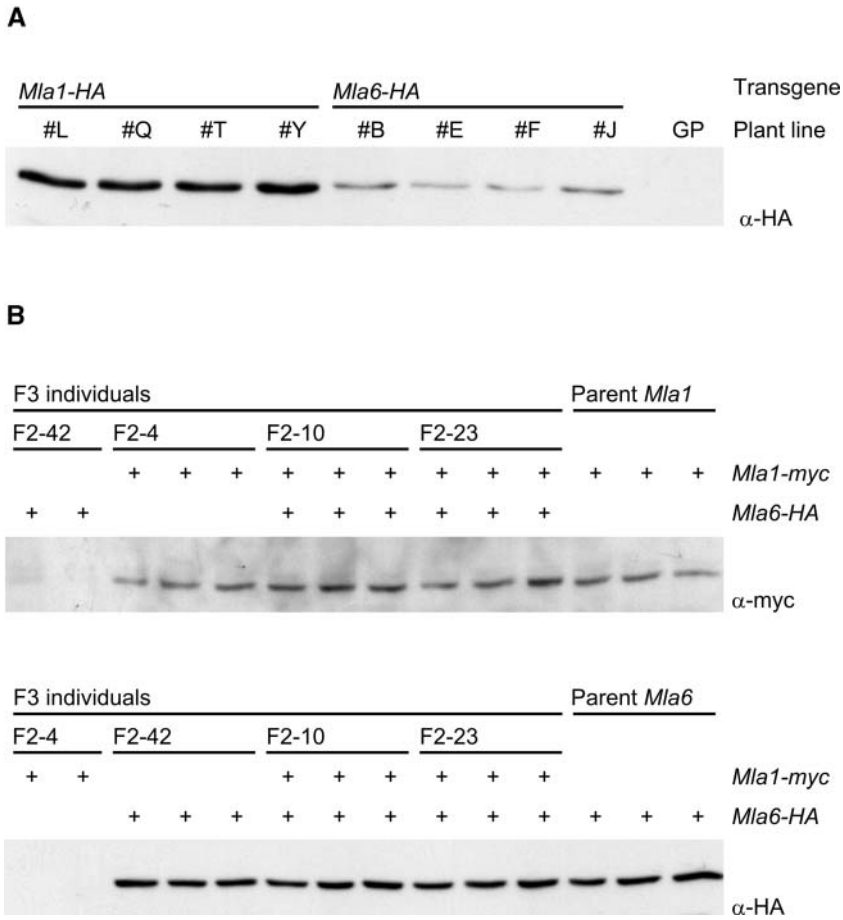


**Figure 3.** Abundance of MLA1-HA and MLA6-HA in Transgenic Barley Lines.

**(A)** MLA1-HA and MLA6-HA accumulation in single copy transgenic lines. Forty micrograms of total leaf protein extracts of eight independent single copy transgenic lines (denoted by #) were tested by immunoblotting with the anti-HA antibody. The barley cultivar Golden Promise (GP) was used for the generation of the stable transgenic lines.

**(B)** Coexpression of MLA1 and MLA6 in F3 progeny derived from a cross between a line expressing *Mla1-myc* and *Mla6-HA*. Total leaf protein extracts were analyzed by immunoblotting using anti-myc antiserum (top panel) and anti-HA antiserum (bottom panel). Tested homozygous F3 progeny were shown by PCR analysis to contain only *Mla1-myc* or *Mla6-HA* or both transgenes. Two or three F3 progeny derived from four F2 individuals (F2-4, F2-10, F2-23, and F2-42) were examined. Note that lines coexpressing both transgenes retain MLA1 and MLA6 accumulation levels seen in lines expressing either transgene alone.

found in homozygous *Mla6-HA* containing lines differing for the presence of one or two *Rar1* copies (data not shown).

In contrast with *Rar1* gene dosage insensitivity, *Mla1* copy number does affect the resistance phenotype. However, *Mla1* dosage sensitivity can be detected macroscopically only in the absence of RAR1 (Figure 4F). Plants containing two *Mla1* copies are fully resistant in a *rar1* mutant background, whereas one *Mla1* dosage in this background confers attenuated resistance leading to large necrotic patches beneath small fungal colonies as well as permitting occasional conidiospore formation (indicative of a trailing HR; Figure 4F). This demonstrates that *Mla1* gene dosage becomes rate limiting for resistance in the absence of *Rar1*. Consistent with this, MLA1-HA abundance in transgenic plants carrying a single transgene copy were about half the level seen in plants containing two *Mla1-HA* copies, in both *Rar1* and *rar1* backgrounds (data not shown). We conclude that *Mla1* functions independently of *Rar1* only where MLA1 abundance exceeds a threshold level.

### RAR1 May Specifically Control the Abundance of NB-LRR–Type R Proteins

To find out whether RAR1 interferes with the accumulation of other NB-LRR R proteins than MLA, we tested the abundance of Rx in *N. benthamiana* in the presence and absence of RAR1. Rx is a CC-NB-LRR type R protein that confers resistance to the potato virus X (PVX) and requires SGT1 for its function (Peart et al., 2002). We used transgenic *N. benthamiana* lines expressing

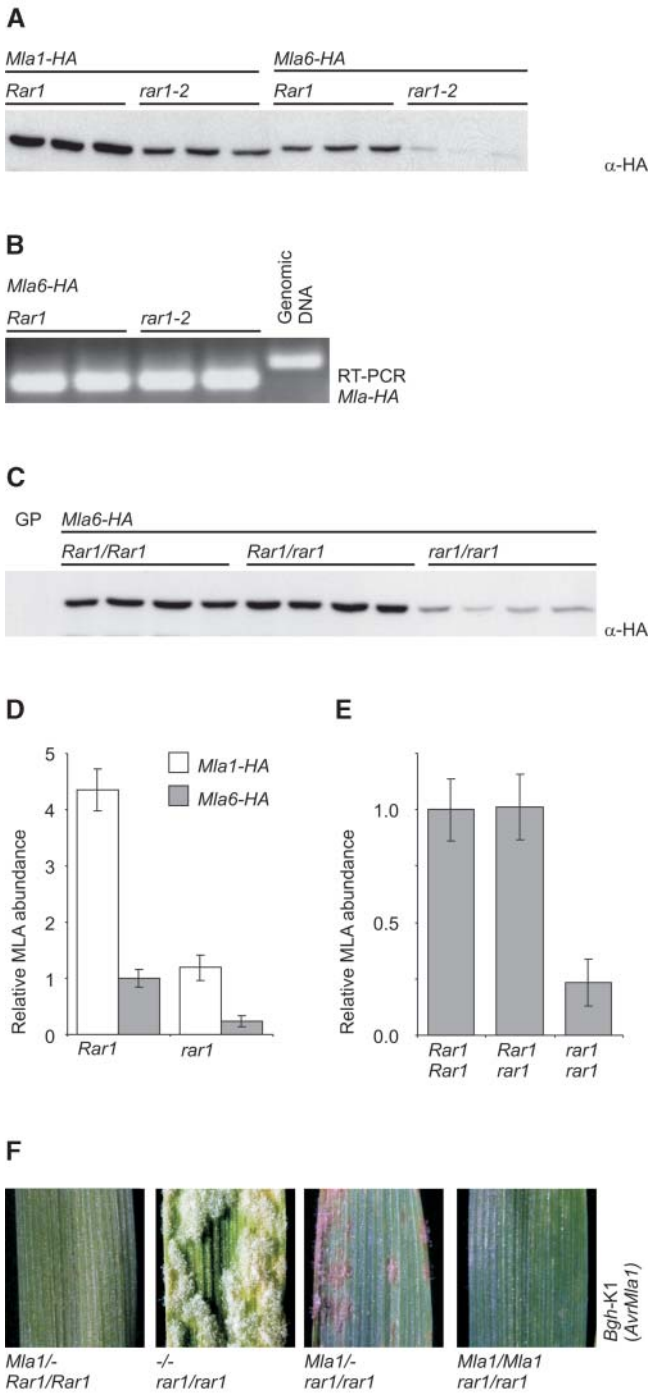


**Figure 4.** RAR1 Elevates MLA1 and MLA6 Abundance.

**(A)** Protein gel blot analysis of 40 μg of total leaf protein extracts from barley plants homozygous for the transgene *Mla1-HA* or *Mla6-HA* in *Rar1* or homozygous *rar1-2* backgrounds. Three individuals of each genotype were tested.

**(B)** *Mla6* transcript abundance was tested by semiquantitative RT-PCR using total RNA extracted from plants expressing the transgene *Mla6-HA* in *Rar1* or homozygous *rar1-2* backgrounds. The primer combination used for the RT-PCR spans *Mla6* intron 4 and amplifies a larger fragment in the presence of genomic DNA template. Results from two individuals of each genotype are presented.

**(C)** MLA6 abundance detected by protein gel blot analysis of total leaf protein extracts from plants homozygous for the transgene *Mla6-HA* expressed in *Rar1* wild-type, heterozygous *Rar1 rar1-2*, or homozygous *rar1-2* background. Four individuals per genotype are shown. GP, cultivar Golden Promise lacking transgenes.

**(D)** Luminometric quantification of chemiluminescence signals obtained by protein gel blot experiments shown in **(A)**. Error bars indicate one standard deviation of signals recorded from three individuals per genotype.

**(E)** Luminometric quantification of chemiluminescence signals obtained by protein gel blot experiments shown in **(C)**.

**(F)** Infection phenotypes observed in *rar1-2* seedlings segregating for the transgene *Mla1-HA*. Seven-day-old seedlings were inoculated with spores of the incompatible *Bgh* isolate K1. Photographs were taken 7 d postinoculation.

Rx-HA under the control of native regulatory sequences (Lu et al., 2003). Gene silencing of *RAR1* by virus-induced gene silencing strongly reduced both RAR1 and Rx abundance (Figure 5A). However, silencing of *RAR1* did not impair the resistance response to PVX (data not shown). This demonstrates that the residual Rx protein in RAR1-silenced plants is still sufficient to trigger an effective resistance response.

To find out whether RAR1 elevates protein abundance of all intracellular LRR containing proteins, we examined the Arabidopsis F-box protein COI1, containing C-terminal LRRs, in the wild type, *rar1, sgt1b*, and the double mutant. COI1 is known to play an essential role in the signaling of jasmonate-dependent resistance responses (Xie et al., 1998). Unaltered COI1 steady state levels in wild-type and mutant backgrounds (Figure 5B) might indicate that RAR1 selectively interferes with NB-LRR R protein abundance.

### MLA Abundance Is Inversely Correlated to RAR1 Requirement for Resistance

To obtain further insights into the link between RAR1 and MLA abundance in nonchallenged plants, we used two barley MLA chimeras, designated 11666 and 11166, whose functionality was previously examined by transient single-cell gene expression studies using the strong ubiquitin promoter (Shen et al., 2003). The CC and NB domains of these two chimeras are derived from MLA1, the LRRs are hybrids between MLA1 and MLA6, and the CT region is derived from MLA6 (Figure 6A). Both MLA chimeras confer *AvrMla6*-dependent resistance but differ in their requirement for *Rar1* (resistance triggered by 11666 but not 11166 needs the presence of *Rar1*; 11666-mediated resistance is disrupted in the absence of *Rar1*, whereas wild-type *Mla6* plants show intermediate resistance in *rar1* mutants with a haustorium index of 40%; Shen et al., 2003). We generated transgenic barley containing HA epitope-tagged genomic clones of both MLA chimeras whose expression is driven by native *Mla6* regulatory sequences. Wild-type *Rar1* plants expressing chimera 11166-HA exhibited *AvrMla6*-dependent resistance (Figure 6E). The RAR1-dependent chimera 11666-HA permitted limited growth of the isolate A6 expressing *AvrMla6* and unrestricted growth of the isolate K1 lacking *AvrMla6*, suggesting activation of an inefficient

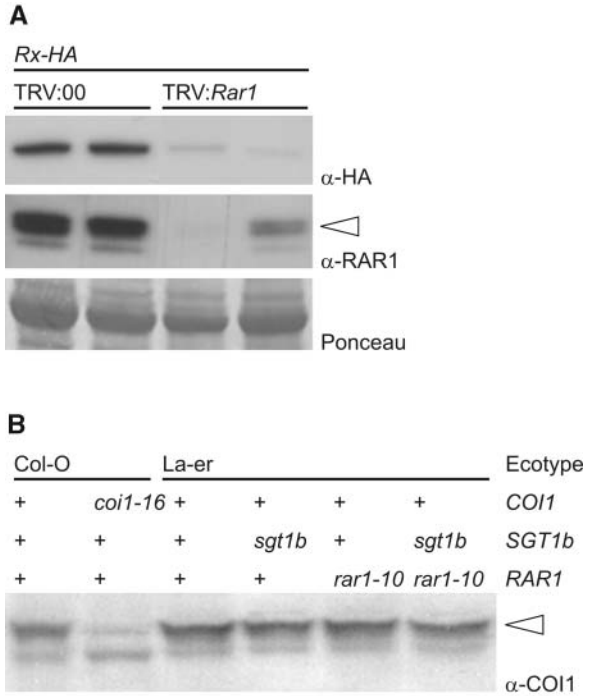


**Figure 5.** RAR1 Elevates Rx Protein Level in *N. benthamiana* and Does Not Interfere with Arabidopsis COI1 Abundance.

**(A)** Rx-HA and RAR1 steady state levels were examined by protein gel blot analysis using leaf protein extracts from transgenic *N. benthamiana* plants expressing *Rx-HA* after gene silencing of *Rar1*. Gene silencing was triggered by inoculation with a tobacco rattle virus (TRV) derivative expressing *NbRar1* in antisense orientation (*TRV:Rar1*). TRV:00, empty vector control. For each treatment, results from two independent experiments are shown. Equal amounts of total protein were loaded and validated by Ponceau staining.

**(B)** Protein gel blot analysis of total leaf protein extracts of Arabidopsis wild type, *rar1*, *sgt1b*, and the double mutant. The abundance of the Arabidopsis F-box protein COI1 containing a C-terminal LRR was detected using a COI1-specific antiserum.

*AvrMla6*-triggered resistance. Necrotic patches beneath A6 colonies were indicative of a trailing HR and were absent beneath K1 colonies (Figure 6E). Protein gel blot analysis of leaf protein extracts derived from multiple independent single-copy transgenic lines expressing chimeras 11666-HA or 11166-HA revealed markedly higher steady state levels of the RAR1-independent 11166-HA chimera (Figure 6B). The abundance of chimera 11166-HA was found to be indistinguishable from wild-type MLA1-HA–expressing lines, whereas the 11666-HA protein was barely detectable (Figures 6B and 6C). These dramatic differences in chimera abundance are not the result of differential *Mla* gene expression levels (Figure 6D). The findings suggest an inverse correlation between MLA abundance and RAR1 requirement for resistance. It seems possible that *AvrMla6*-dependent 11166 resistance functions in the absence of RAR1 because the chimera accumulates to a level that is indistinguishable from wild-type MLA1. Conversely, chimera 11666-triggered resistance might exhibit enhanced dependence on RAR1 relative to wild-type MLA6 because of the diminished in planta accumulation.

### MLA Protein Abundance Is Temperature Sensitive

We accidentally discovered that MLA proteins had disappeared in leaves harvested during hot summer days. To examine a potential temperature-sensitive MLA accumulation, we grew *Mla1-HA* and *MLA6-HA* transgenic seedlings for 7 d at 18°C and transferred them subsequently to 37°C (which is still below a typical heat shock for barley plants). We observed a marked and rapid decrease of both MLA1 and MLA6 abundance upon elevation of the ambient temperature. Interestingly, the reduction of MLA6 abundance was faster (detectable within 30 min) than of MLA1 (2 h), and both proteins remained at low levels at the end of the 8 h interval tested (Figure 7). Unlike this, exposure of the seedlings to the elevated temperature had no significant effect on RAR1, SGT1, and cytosolic HSP90 abundance, suggesting that MLA proteins might be intrinsically temperature sensitive. Unfortunately, we are unable to test whether the reduction of MLA abundance at elevated temperature impairs resistance to *B. graminis* because the fungus is unable to colonize barley at temperatures above 30°C (Aust, 1974; Jenkyn and Bainbridge, 1978).

### MLA Isoform-Dependent Interactions with HvSGT1 and Isoform-Independent Interactions with HvHSP90

We searched for proteins that interact with MLA1 and MLA6 using the yeast two-hybrid method (Gyuris et al., 1993). For this purpose, we employed various domains or full-length MLA1 and MLA6 as baits and screened a barley prey cDNA library derived from leaf epidermal tissue (see Methods). We included in these screens baits comprising the N-terminal 46 amino acids (designated CC), the CC and nucleotide binding motifs (designated CC-NB), only the NB motifs (NB), or the LRRs and the C terminus (LRR-CT, Figure 8A). The most frequent MLA interactor identified in the prey library was barley SGT1 (26 independently isolated clones). This interaction was restricted to the MLA1-derived bait encompassing the LRR-CT region. The identified prey clones encoded either the complete coding sequence of SGT1 or three different truncations in the TPR domain, as well as one truncation immediately N-terminal to the SGS domain (Figure 8B). No prey clones were recovered encoding the CS plus SGS domains alone.

All MLA baits were tested in parallel in a directed screen for interactions with RAR1, SGT1, and cytosolic HSP90 together with further baits expressing the LRR-CT portions of MLA chimeras 11166 and 11666. No association was detected between any tested MLA bait and barley RAR1. The LRR-CT portion of MLA1 and 11166, but not of MLA6 and 11666, was found to interact with full-length barley SGT1 as well as with both full-length Arabidopsis SGT1 isoforms, designated AtSGT1a and AtSGT1b (Figure 8C; Austin et al., 2002; Azevedo et al., 2002). By contrast, cytosolic HvHSP90 interacted with the LRR-CT of all tested MLA variants. Baits encoding full-length MLA1 or MLA6 did not interact with either SGT1 or HSP90, although the LexA fusion baits were detectable in yeast cells (data not shown). This

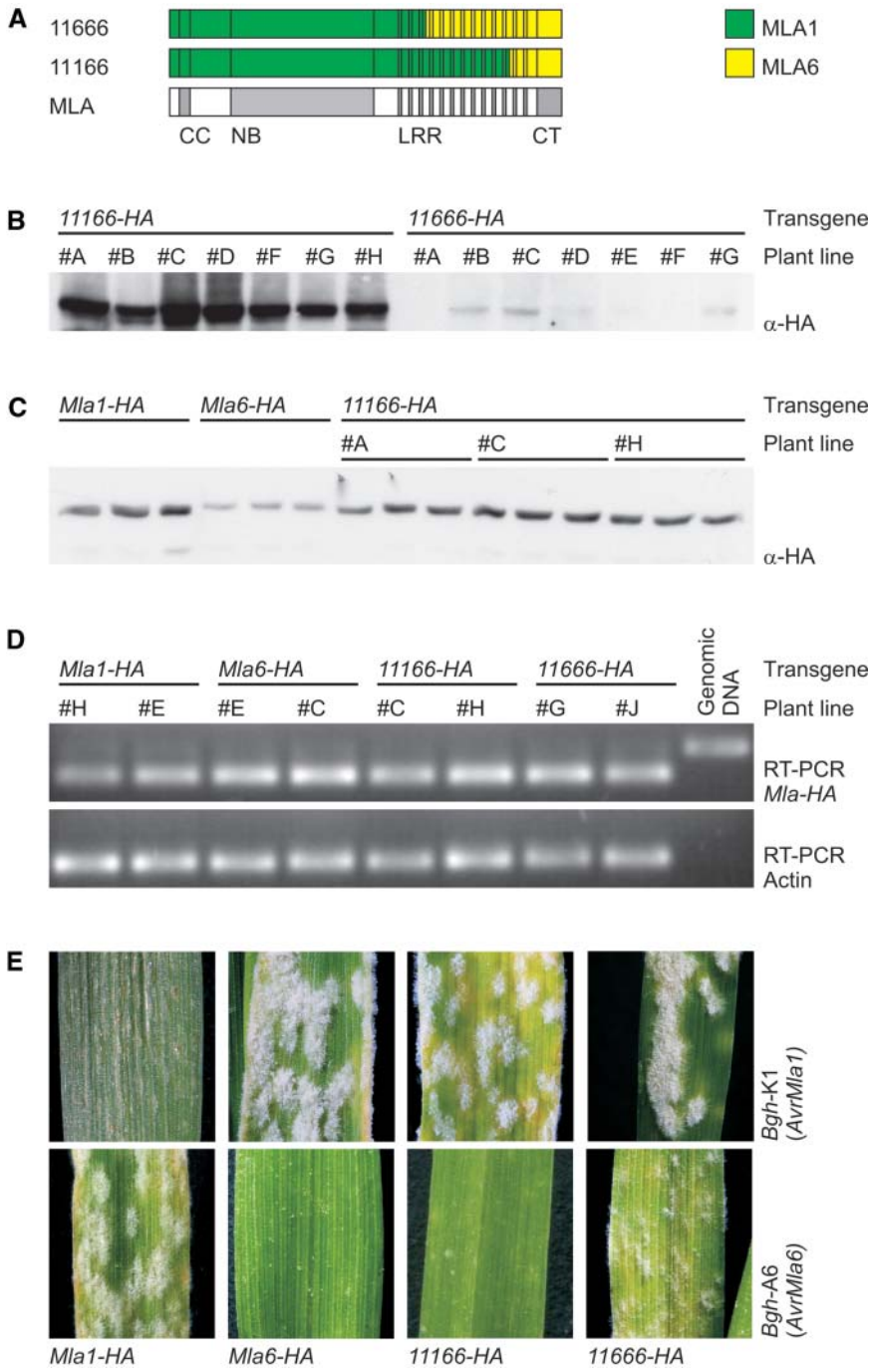


**Figure 6.** Infection Phenotypes Mediated by MLA Chimeras Are Linked to R Protein Steady State Levels.

**(A)** Schematic representation of the domain structure and swap sites of two *Mla* chimeras, 11166 and 11666.
**(B)** Abundance of MLA protein revealed by protein gel blot analysis of total leaf protein extracts from T0 plants expressing the tested *Mla* chimeras driven by native *Mla6*-derived regulatory sequences. The results obtained from seven independent single copy transgenic lines of each chimera are shown.
**(C)** Abundance of MLA protein in total leaf protein extracts from homozygous plants expressing *Mla1-HA*, *Mla6-HA*, and chimera *11166-HA.* Three individuals per genotype were tested by protein gel blot analysis using anti-HA anitserum. #A, #C, and #H denote independent transgenic lines expressing *11166-HA*.
**(D)** *Mla* transcript abundance was tested by semiquantitative RT-PCR using total RNA extracted from plants expressing the transgenes *Mla1-HA*, MLa6-HA, chimera *11166-HA*, or *11666-HA*. The primer combination used for the RT-PCR spans intron 4 and amplifies a larger fragment in the presence of genomic DNA template. Results from two independent lines of each genotype are shown.
**(E)** Representative infection phenotypes of plants homozygous for the respective transgene 7 d postinoculation with *Bgh* isolates expressing *AvrMla1* or *AvrMla6*.

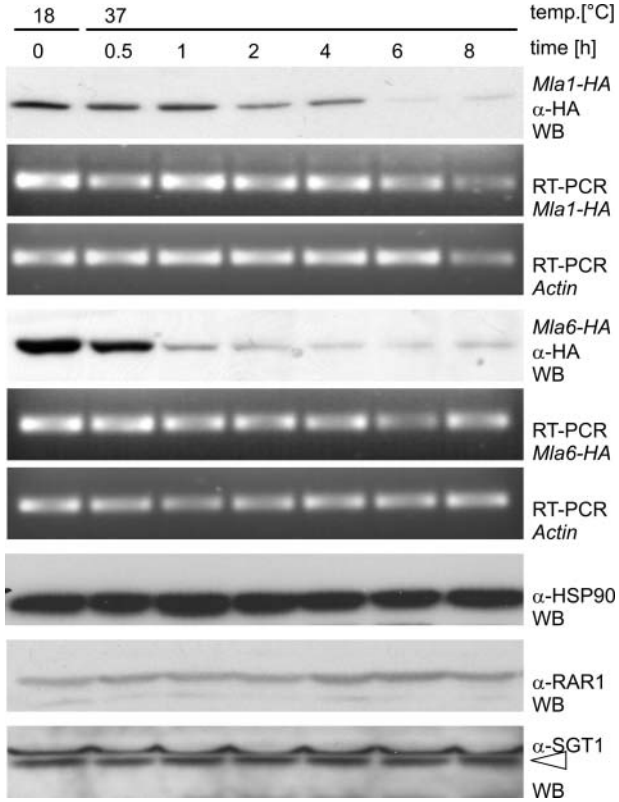


**Figure 7.** MLA Abundance Is Temperature Sensitive.

Barley seedlings expressing *Mla1-HA* or *Mla6-HA* were grown at 18°C. The temperature was shifted to 37°C, and samples were taken to examine abundance of MLA, HSP90, RAR1, and SGT1 by protein gel blot analysis. Transcript accumulation of the transgenes was tested as described in Figure 6D using semiquantitative RT-PCR analysis. Note the earlier reduction of MLA6-HA steady state levels in comparison with MLA1-HA.

might be indicative of inhibitory intramolecular MLA domain interactions (see Discussion).

## DISCUSSION

Here, we used epitope-tagged *Mla* variants, whose expression is driven by native regulatory sequences, to gain insights in *Mla*-mediated resistance. N-terminal fusions were generally found to inactivate MLA1 and MLA6, whereas most C-terminal fusions were functional. It is striking that the N-terminal sequences, including the CC, are invariant among nine available MLA R specificities to *Bgh*, whereas considerable divergence in sequence and length is found at the C terminus (Halterman et al., 2001, 2003; Zhou et al., 2001; Shen et al., 2003; Halterman and Wise, 2004; S. Pajonk and P. Schulze-Lefert, unpublished data). The extreme sequence conservation of the N-terminal region of MLA proteins suggests a marked sequence constraint of a common MLA subfunction. It is possible that fusions of epitopes to the invariant N terminus obstruct this subfunction.

### Subcellular Localization and Tissue-Specific Abundance of MLA Proteins Might Be Governed by Interacting Partners

Little is known about the subcellular localization of predicted intracellular NB-LRR R proteins expressed at native levels. Detectable Arabidopsis RPS2 was found to be entirely plasma membrane associated as well as the majority of Arabidopsis RPM1 (Boyes et al., 1998; Axtell and Staskawicz, 2003). Unlike this, at near-physiological salt concentrations, the bulk of MLA appears soluble, and a second pool associates with membranes

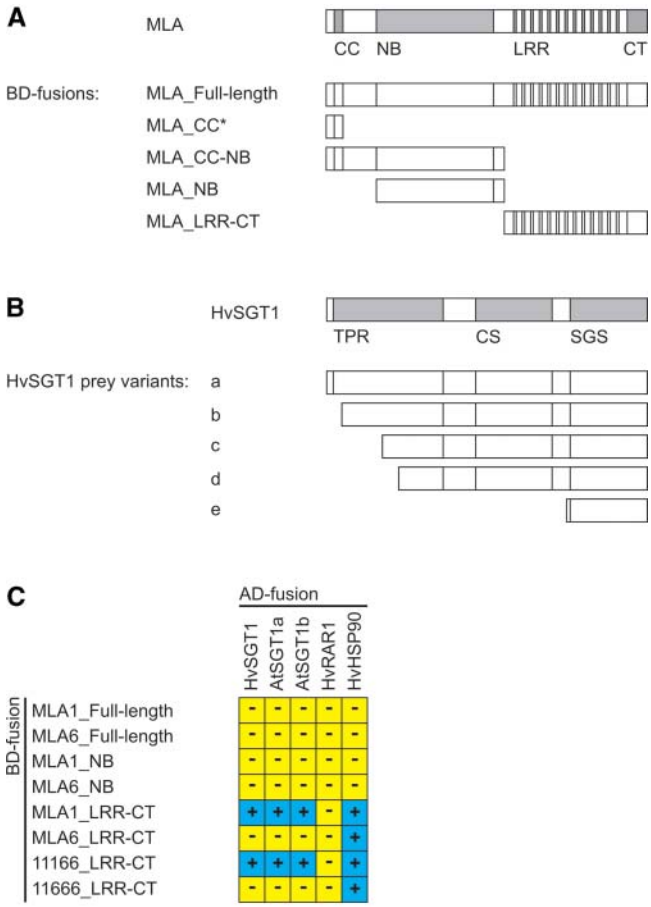


**Figure 8.** MLA1 but Not MLA6 Interacts with SGT1.

**(A)** Schematic representation of MLA1 and MLA6 domains used as baits in yeast two-hybrid experiments. The asterisk denotes a bait containing a sequence stretch identical in *Mla1* and *Mla6*.

**(B)** Yeast two-hybrid analysis of the interaction between MLA1 and HvSGT1. The domain architecture of SGT1 is shown in the top line. Deduced truncated SGT1 forms encoded by independently isolated prey clones interacting with the LRR-CT bait of MLA1 are indicated below. TPR, tetratricopeptide repeat domain; CS, motif present in CHORD and SGT1 proteins; SGS, SGT1-specific motif.

**(C)** Directed yeast two-hybrid experiments using the indicated MLA1-, MLA6-, and MLA chimera-derived fragments as binding domain (BD) fusions. Candidate interactors HvSGT1, AtSGT1a, AtSGT1b, HvRAR1, and HvHSP90 were expressed as fusion proteins to the activation domain (AD). Detected interactions are indicated by blue boxes (+) and a lack of interaction by yellow boxes (−).

(Figures 2C and 2D). Because the association with microsomal membrane fractions was reversible and dependent on ionic strength, the subcellular localization of MLA might be governed by charge interactions with integral or peripheral membrane proteins. For example, the RPM1 and RPS2 interactor RIN4 colocalizes with these two NB-LRR proteins at the plasma membrane (Axtell and Staskawicz, 2003; Mackey et al., 2003). RIN4 was also shown to be required for the accumulation of RPM1. Thus, it is conceivable that the tissue-specific difference in MLA abundance seen in epidermis and total leaf reflects an unequal tissue distribution of an MLA1 interactor.

The presence of high MLA levels in total leaf and undetectable MLA protein in the epidermis is counterintuitive because host cell invasion by powdery mildew fungi is restricted to epidermal cells (Figure 2B). Because epidermal cell monolayers prepared from partially dissected coleoptiles of *Mla1* genotypes were shown to mount a race-specific resistance response (including single cell HR; Bushnell and Liu, 1994; Schiffer et al., 1997), epidermal cells must be competent to detect the cognate fungal effector and thus contain presumably very low levels of MLA1.

### A Universal Role for RAR1 in Folding Preactivated NB-LRR–Type R Proteins?

The function of RAR1 in race-specific resistance is conserved in plant species, including Arabidopsis, tobacco, and barley, and its genetic requirement is limited to subsets of both CC-NB-LRR and TIR-NB-LRR–type R proteins (Shirasu and Schulze-Lefert, 2003). By crossing epitope-tagged *Mla6* or *Mla1* transgenic lines into the *rar1* mutant background, we have demonstrated that the steady state levels of both isoforms are equally dependent on RAR1, although *Mla1* function is genetically independent of *Rar1* (Jørgensen, 1996). This suggests that the involvement of RAR1 in *R* gene–triggered resistance is broader than indicated by previous genetic studies. Consistent with this, Rx resistance in *N. benthamiana* against PVX virus was found to be genetically independent of *Rar1*, but Rx abundance was dramatically reduced in *Rar1* silenced plants (Figure 5A). In Arabidopsis, the abundance of RPM1, which recognizes *Pseudomonas syringae* AvrRpm1, is also reduced in nonchallenged *rar1* mutant plants. However, similar to MLA6, this reduced R protein accumulation is accompanied by an impaired immune response (Tornero et al., 2002b). Collectively, a reduction of NB-LRR protein steady state levels in nonchallenged plants appears to be a common feature of plants lacking RAR1 irrespective of whether RAR1 is genetically essential for the resistance response.

To further examine the differential genetic requirement and general biochemical involvement of RAR1 in NB-LRR–mediated resistance, we used MLA chimeras that were generated by domain swap experiments between MLA1 and MLA6. The two chimeras tested, each recognizing *AvrMla6*, showed in resistance assays either an enhanced requirement for or uncoupling from *Rar1* in comparison to wild-type *Mla6* (Shen et al., 2003). The abundance of these two chimeras differed and inversely correlated with their genetic dependence for *Rar1.* Furthermore, a reduction of *Mla1* gene dosage from two to one resulted in compromised *Mla1* resistance in *rar1* mutant plants (i.e., *Mla1* function became haploinsufficient in the absence of RAR1) (Figure 4F). Taken together, these findings implicate the existence of a threshold for R protein–derived signal to trigger effective resistance and a decisive function of RAR1 in this process. The new data suggest an activity for RAR1 that is distinct from its previously inferred role in the signaling of resistance responses (Jørgensen, 1996; Shirasu et al., 1999; Halterman and Wise, 2004; Liu et al., 2004).

If RAR1 exerts a cochaperone-like activity (Shirasu and Schulze-Lefert, 2003), its role in elevating NB-LRR protein abundance might be in folding of R proteins into a form competent for effector recognition and activation of the resistance response. If so, plant cells might contain both recognition inactive and recognition competent (preactivated) NB-LRR protein forms (Schulze-Lefert, 2004). However, RAR1 is unlikely to stabilize R proteins through direct physical associations because we failed to identify a direct interaction with MLA baits in our yeast two-hybrid studies and because coexpression of MLA and RAR1 in yeast was insufficient to alter R protein abundance (data not shown). This implies the need for additional plant component(s) that act together with RAR1 to elevate MLA steady state levels.

### Direct and Isoform-Dependent Interactions between the MLA LRR-CT Region and SGT1

RAR1 physically interacts with SGT1 through the C-terminal CHORD-II of RAR1 and the central CS domain of SGT1 (Azevedo et al., 2002). Both proteins also interact with cytosolic HSP90 (Hubert et al., 2003; Takahashi et al., 2003). Our yeast two-hybrid studies showed that barley or Arabidopsis SGT1 interacts with the LRR-CT of MLA1 and chimera 11166 but not with MLA6 or chimera 11666, indicating MLA isoform-dependent associations. These interactions cannot involve heterologous RAR1 because yeast lacks a RAR1 homolog. Similarly, because cytosolic HSP90 was found to interact with all four tested MLA LRR-CT variants, highly sequence-related heterologous yeast HSP90 might participate in but cannot serve as determinant of MLA isoform-specific assemblies with SGT1. We conclude that subtle sequence-specific differences in the LRR are critical for associations with SGT1. This is consistent with the observation that a thermosensitive single amino acid substitution in the LRR of yeast adenylate cyclase, Cyr1p, abolished in vivo interactions with yeast SGT1 (Dubacq et al., 2002).

Halterman and Wise (2004) identified a single amino acid substitution in the MLA6 LRR (G721D) that alleviated the resistance from *Rar1* dependence. Based on our data, we predict that this mutant form of MLA6 accumulates more than the wild-type protein. However, uncoupling of RAR1 dependence in MLA6 (G721D) may not merely reflect altered SGT1 binding because we failed to detect interactions between SGT1 and the LRR-CTs of wild-type MLA6 and MLA6 (G721D) in yeast two-hybrid experiments (data not shown).

MLA variants interacting with SGT1 (MLA1 and chimera 11166) seemingly do not require SGT1 for function, whereas resistance triggered by MLA6 or chimera 11666 was significantly compromised upon transient *Sgt1* single cell silencing (Azevedo et al., 2002; Shen et al., 2003). However, it is conceivable that, in analogy to RAR1, all MLA variants engage SGT1. Because it is likely that the depletion of SGT1 was incomplete in cells

undergoing silencing, reduced SGT1 activity might still be sufficient for MLA isoforms that interact strongly with SGT1, but not for others with weaker interaction. A differential *Sgt1* dependence might therefore reflect variation in binding between the LRR-CT and SGT1.

We note that the associations involving both HSP90 and SGT1 with the C-terminal half of MLA proteins require the absence of the CC-NB, which is strongly indicative of intramolecular R protein domain interactions. Such interactions have been shown for Rx in planta, involving the LRR and NB, as well as the CC and NB-LRR. These intramolecular interactions are thought to become sequentially disrupted upon Rx activation (Moffett et al., 2002). Thus, isoform-specific in planta interactions between MLA and SGT1 are expected to be transient or absent before activation of the R proteins. Consistent with this, we failed to coimmunoprecipitate SGT1 in extracts derived from nonchallenged MLA1-HA or MLa6-HA expressing barley lines with anti-HA antibodies (data not shown), but refined future experiments will need to clarify a role for SGT1-MLA interactions in the formation of recognition-competent R forms and/or in the signaling of resistance responses after pathogen recognition.

In yeast, SGT1 is required for proper functioning of multiple nuclear and cytoplasmic targets, including the nuclear kinetochore complex, the SCF ubiquitination complex, and the LRR containing adenylate cyclase Cyr1p (Kitagawa et al., 1999; Dubacq et al., 2002; Schadick et al., 2002). In Arabidopsis, absence of SGT1b enhances defects in auxin signaling in plants containing a partially inactive TIR1 F-box protein, indicating an SGT1 function unrelated to disease resistance (Gray et al., 2003). Activation of cell death by membrane-resident Cf-4 or Cf-9 R proteins, containing extracellular LRRs, using cognate *Cladosporium fulvum* avirulence peptides in *N. benthamiana* requires intracellular SGT1, thereby revealing a SGT1 subfunction in disease resistance that must occur independently from a direct interaction with LRRs of the R protein (Peart et al., 2002). The previously reported impaired resistance upon silencing of *SGT1* without detectable changes of Rx abundance in healthy *N. benthamiana* plants (Lu et al., 2003) and the dramatic reduction in Rx steady state levels observed in this study upon silencing of *RAR1* without accompanying suppression of the resistance (Figure 5A), favors distinct roles for RAR1 and SGT1 in the formation of recognition-competent Rx forms and in resistance signaling after pathogen effector recognition.

Why do steady state levels of highly sequence-related MLA1 and MLA6 proteins differ fourfold in the presence and absence of RAR1 in nonchallenged plants? Retained isoform-specific abundance in plants coexpressing both R proteins suggests that this is directed by intrinsic R protein properties rather than by the existence of a rate-limiting common factor(s) that might be a constituent(s) of preactivated MLA complexes. The more rapid turnover of MLA6 compared with MLA1 upon exposure of the plants to elevated temperature is indicative of inherent differences in folding processes and/or stability of protein–protein interactions. Likewise, the differential steady state levels of MLA1 and chimera 11166 compared with MLA6 and 11666 could reflect a cochaperone-like activity of SGT1 in concert with HSP90. In this scenario, one of several SGT1 activities would include the formation of recognition-competent MLA.

Decay of both MLA1 and MLA6 (but not of RAR1, SGT1, and HSP90) upon exposure to elevated temperature indicates that rapid turnover of these R proteins presumably is the default. It is conceivable that RAR1, SGT1, and HSP90 rescue R proteins from default decay and enable regulated abundance sufficient for effective resistance but below levels that could result in autoactivation of resistance responses as observed upon overexpression of Rx or Arabidopsis RPS2 (Tao et al., 2000; Bendahmane et al., 2002).

Plants have relatively few NB-LRR genes available to recognize a large number of rapidly evolving pathogen effector molecules (Meyers et al., 2003). Consequently, diversifying selection acts on some of these plant *R* genes to persist in the arms race with the pathogen (Dodds et al., 2004). However, diversified recognition capability might be possible only at the cost of structural instability. Interestingly, HSP90 proteins have been shown in animals and plants to buffer phenotypic variation (Rutherford, 2003). Thus, it is conceivable that HSP90 together with RAR1 and SGT1 might buffer such structural instability and widen the conformational limits of NB-LRR proteins to allow more functional variants and, therefore, more recognition specificities.

## METHODS

### Plant Material, Fungal Isolates, and Plant Transformation

Transgenic barley lines were generated by *Agrobacterium tumefaciens*–mediated transformation of immature embryos derived from *Hordeum vulgare* ssp *vulgare* (barley) cultivar Golden Promise essentially as described (Wang et al., 2001; S. Schulze and H. Steinbiß, unpublished data). Transgenic plants were analyzed by DNA gel blots: DNA was extracted from young leaf material with the Nucleon PhytoPure Genomic DNA extraction kit (Amersham Biosciences, Piscataway, NJ), 20 μg of DNA was digested with *Hind*III (New England Biolabs, Beverly, MA), resolved in 0.7% TEA agarose gels, and blotted on nylon membranes (Roche, Penzberg, Germany) using SSC as described (Maniatis et al., 1982). Membranes were hybridized with probes matching the 5′ part of *Mla1* intron 3 (primers 5′-CATTATATTTCCATGCATGCC-3′ and 5′-ACACATCAGAAAGCTGAGGG-3′) and *Hpt* (primers 5′-CCTCGGACGAGTGCTGG-3′ and 5′-ATTTCGTGTGTCTATGATGATG-3′) using the DIG system (Roche) according to the manufacturer's protocol. The three-component transient single-cell transformation assay to test epitope-tagged *Mla* variants was performed as described (Zhou et al., 2001). The vector V26-UMUG was used to express the *Mlo* and β-glucuronidase genes, both driven by the maize (*Zea mays*) ubiquitin promoter, in barley cultivar Ingrid *mlo3* or Ingrid *mlo5* seedling. *Mla1* function was tested with *Blumeria graminis* f. sp *hordei* isolate K1 (*AvrMla1*, *AvrMla3*, *AvrMla7*, *AvrMla13*, and *AvrMla22*) and *Mla6* function with isolate A6 (*AvrMla3*, *AvrMla6*, *AvrMla7*, *AvrMla9*, *AvrMla10*, *AvrMla12*, and *AvrMla13*). The resistance phenotype of the different genotypes was assayed by infecting 7-d-old seedlings with low density of *Bgh* spores of the compatible or incompatible fungal isolate. The resistance response was scored 7 d postinoculation.

### Plasmid Construction

A pBluescript II KS− derivative with *Asc*I and *Not*I sites flanking the polycloning site (pBS+AN) was used to subclone ~8-kb fragments containing *Mla1* (pBS-Mla1; with *Sac*II-*Xho*I derived from cosmid p6-49-2; Zhou et al., 2001) or *Mla6* (pBS-Mla6; with *Avr*II-*Pci*I derived from

cosmid 9589-5a; Halterman et al., 2001). N-terminal epitope tags were introduced by PCR with primers 5′-GATTGAAGCGACCCTCAC-3′ (*Mla1* exon 2) or 5′-ATAAGTGCTTTTGAGTACTTGC-3′ (*Mla6* exon 1) and 5′-TACCGACCGGTGACAATATCGGCTAAATCTTCCTCACTTATTAATT-TTTGTTC**CAT**GAGAGCAGGAGGAC-3′ (*Age*I site underlined, start codon bold) to introduce the c-*myc* epitope or 5′-TACCGACCGGTGA-CAATATCGGCAGCATAATCTGGAACATCGTATGGATA**CAT**GAGAGCA-GGAGGAC-3′ for the HA epitope. The *Hind*III-*Age*I (*Mla1*) or *Afl*II-*Age*I (*Mla6*) fragment, respectively, of these PCR products was ligated into pBS-Mla1 or pBS-Mla6.

To generate the C-terminal epitope-tagged variants, overlapping PCR products (PCR a and b) were extended with a final PCR reaction, cut *Sbf*I-*Eco*RI or *Bsp*EI-*Xho*I and ligated into pBS-Mla1 or pBS-Mla6, respectively. Primers 5′ and 3′ of the stop codon of *Mla1* (PCR a forward, 5′-GTAAGAGTTTGGTTCAGCC-3′, and PCR b reverse, 5′-TACAATCAA-CACCCCTAGG-3′) or *Mla6* (PCR a forward, 5′-ATAAGTGCTTTTGAGT-ACTTGC-3′, and PCR b reverse, 5′-CTTTTCGGTAAAACAACACTCC-3′) were used together with internal primers to generate two PCR products that cover the c-*myc* epitope (*Mla1* PCR a, 5′-TAAATCTTCCTCA-CTTATTAATTTTTGTTCAGCGTTCTCCTCCTCGTCCTC-3′; *Mla6* PCR a, 5′-TAAATCTTCCTCACTTATTAATTTTTGTTCAGCGTTCTCCTCCTCGC-CCTC-3′; PCR b, 5′-GAACAAAAATTAATAAGTGAGGAAGATTTA**TGA**-TTTCTGATCCAGAGCG-3′; epitope underlined, stop codon in bold), the HA epitope (*Mla1* PCR a, 5′-AGCATAATCTGGAACATCGTATGGA-TAAGCGTTCTCCTCCTCGTCCTC-3′; *Mla6* PCR a, 5′-AGCATAATCTG-GAACATCGTATGGATAAGCGTTCTCCTCCTCGCCCTC-3′; PCR b, 5′-GCTTATCCATACGATGTTCCAGATTATGCT**TGA**TTTCTGATCCAGAG-CG-3′), or a *Nco*I-*Bam*HI-*Hind*III adapter to insert other tags (*Mla1* PCR a, 5′-ATAAGCTTTTGGATCCGCCGCCCATGGCGTTCTCC-TCCTCGTCCTC-3′; *Mla6* PCR a, 5′-ATAAGCTTTTGGATCCGCCGCC-CATGGCGTTCTCCTCCTCGCCCTC-3′; PCR b, 5′-GAGGAGGAGAAC-GCCATGGGCGGCGGATCCAAAAGCTTA**TGA**TTTCTGATCCAGAGCG-ACTC-3′; restriction sites underlined). The *Nco*I-*Bam*HI-*Hind*III adapter was used to introduce the myc-HA tag (5′-CATGGGCGAGCAGAAG-CTCATCTCCGAGGAGGACCTCGGCTACCCGTACGACGTGCCGGAC-TACGCCT-3′ and 5′-GATCAGGCGTAGTCCGGCACGTCGTACGGGTA-GCCGAGGTCCTCCTCGGAGATGAGCTTCTGCTCGCC-3′ were hybridized and cloned into *Nco*I-*Bam*HI; epitope sequences underlined), the triple-HA tag (5′-CCATGGCCTACCCGTACGACGTGCCGGACTACG-CCGGTGGTTACCCGTATGATGTACCCGATTATGCG-3′ and 5′-GGAT-CCTTAGGCGTAGTCCGGCACGTCGTACGGGTAACCACCCGCATAAT-CGGGTACATCAT-3′ were hybridized, extended with polymerase, cut, and ligated with *Nco*I-*Bam*HI), GFP (*Nco*I-*Bam*HI fragment from pCAT-GFP, a gift from J. Müller, MPI Cologne, Germany), GFP-HA (PCR with primers 5′-GATAGCCATGGGTAAAGGAGAAG-3′ and 5′-CGCGCGG-ATCCTTAGGCGTAGTCCGGCACGTCGTACGGGTAGGCTTTGTATAGT-TCATCCATGCCA-3′ on pCAT-GFP, inserted as *Nco*I-*Bam*HI fragment), the TAP-tag (a *Nco*I-*Hind*III fragment was subcloned from plasmid pBS1539 provided by EMBL, Heidelberg, Germany; Rigaut et al., 1999), GFP-TAP-tag (overlapping PCR products a with primers 5′-GATAGC-CATGGGTAAAGGAGAAG-3′ and 5′-CTTTTTCCATCTTCTCTTTTCCAT-TTTGTATAGTTCATCCATGCC-3′ on plasmid pCAT-GFP and b with primers 5′-GGCATGGATGAACTATACAAAATGGAAAAGAGAAGATGG-AAAAAG-3′ and 5′-GGTCCGGATCCTCAGGTTGACTTCCCCGCGGAG-TTCGCGTCTAC-3′ on pBS1539 were hybridized, extended in a final PCR reaction and ligated as *Nco*I-*Bam*HI fragment), and TAP-GFP-HA (overlapping PCR products a with primers 5′-ACGATCCATGGAAAAGAGAA-GA-3′ and 5′-ACGATCCATGGAAAAGAGAAGA-3′ on plasmid pBS1539 and b with primers 5′-TCCGCGGGGAAGTCAACGATGGGTAAAGGA-GAAGAACTTTTC-3′ and 5′-CGCGCGGATCCTTAGGCGTAGTCCGGC-ACGTCGTACGGGTAGGCTTTGTATAGTTCATCCATGCCA-3′ on plasmid pCAT-GFP were hybridized, extended in a final PCR reaction and ligated as *Nco*I-*Bam*HI fragment). *Mla* chimeras 11166 and 11666 were placed under *Mla6* native regulatory sequences and fused to a C-terminal HA epitope by subcloning *Age*I-*Bsp*EI fragments from plasmids pUbi-11166 and pUbi-11666 (Shen et al., 2003) into the C-terminal HA epitope tag derivative of pBS-Mla6 (see above). *Mla1*, *Mla6*, their epitope-tagged derivatives, as well as the epitope-tagged chimeras were subcloned as *Asc*I-*Not*I fragments into the Agrobacterium binary vector pWBVec8+A, which contains an additional unique *Asc*I site in proximity to the unique *Not*I site.

### Genotyping and Semiquantitative RT-PCR

Genomic DNA was extracted from leaf material (Cone, 1989) and subjected to PCR to test the presence of *Mla1* or *Mla6* with a C-terminal epitope (5′-GGATGGTAACCGTGGCTTC-3′ with 5′-ATAATCTGGAACA-TCGTATGG-3′ for the HA epitope and 5′-TTCCTCACTTATTAATTTTTG-TTC-3′ for the myc epitope). A cleaved-amplified polymorphic sequence marker was used to detect the *rar1-2* mutation: PCR amplification with 5′-AGGCAATCCCAAATTCGA-3′ and 5′-CCACTGCCTGCCATGGGA-3′ followed by digest with *Alw*NI (New England Biolabs) leads to a cleaved product for the wild-type gene that is uncleaved in *rar1-2* genotypes.

For RT-PCR, total RNA was extracted using the Trizol reagent according to the provider's instructions (Invitrogen, Carlsbad, CA). Five micrograms of RNA was subjected to reverse transcription (SuperScript II; Invitrogen) with a polyT primer and PCR with MLA-HA specific primers spanning intron 4 (see above). PCR products were resolved in agarose gels after 20, 25, 30, and 35 cycles. The control PCR on actin was performed with primers 5′-TGGCACCCGAGGAGCACC-3′ and 5′-GTAACC-TCTCTCGGTGAG-3′.

### SDS-PAGE and Immunoblotting

Crude protein extract was produced from total primary leaf tissue of 10-d-old seedlings or from tissues as indicated. Samples were ground in liquid nitrogen, thawed in two volumes of extraction buffer (50 mM Hepes, pH 7.5, 150 mM NaCl or as indicated, 10 mM EDTA, 5 mM L-ascorbic acid, 5 mM 1,4-dithiothreitol, and 1× complete protease inhibitor cocktail from Roche) and cleared from debris by two centrifugation steps at 16,000*g* for 5 min. The separation into soluble and microsomal fractions included ultracentrifugation at 100,000*g* for 1 h and the subsequent resolubilization of the pellet in the original volume of extraction buffer using an ultrasonic bath (in ice water). Protein concentration was determined with the Bio-Rad protein assay (Hercules, CA). Proteins were separated in a 7% SDS polyacrylamide gel (12% for experiments to detect RAR1, SGT1, or ROR2) and blotted to nitrocellulose membrane (Hybond ECL; Amersham Biosciences), both using the Mini-Protean II system (Bio-Rad). Blots were incubated with primary antibodies against the HA epitope (rat monoclonal, clone 3F10, dilution 1:5000; Roche), c-myc epitope (rabbit polyclonal, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), RAR1 (rat polyclonal, 1:2000; Takahashi et al., 2003), HSP90 (rat polyclonal, 1:10000; Takahashi et al., 2003), SGT1 (rabbit polyclonal, 1:10000; Takahashi et al., 2003), ROR2 (rabbit polyclonal, 1:250; Collins et al., 2003), and COI1 (rabbit polyclonal, 1:100; Devoto et al., 2002) followed by anti-rat/rabbit IgG (whole molecule)-peroxidase (Sigma, St. Louis, MO), and signals were detected with the ECL chemiluminescent protein gel blotting detection reagents (Amersham Biosciences). Protein gel blot signals were quantified with a CCD camera (Lumi-Imager and Lumi-Analyst 3.0 software; Boehringer Mannheim/Roche).

### Yeast Two-Hybrid Screening

Fusion baits were constructed using *Mla1*, *Mla6*, or chimeric full-length cDNA or partial cDNA fragments fused to the LexA DNA binding domain in

the pLexA bait vector (Azevedo et al., 2002). Using the LiAc transformation method (Gietz and Woods, 1998), 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 used bait plasmids did not activate the reporter gene by themselves. A barley prey library was created using cDNA synthesized from poly(A)$^+$ RNA isolated from mixed leaf tissue samples of barley cultivar Sultan5. Directional, poly(dT)$_{17}$-primed cDNA with *Eco*RI/*Xho*I adapters (Stratagene, La Jolla, CA) was ligated into the corresponding sites of the prey vector pB42AD (CLONTECH, Palo Alto, CA) according to the manufacturer (P. Piffanelli and P. Schulze-Lefert, unpublished data). High purity library DNA was obtained and used to transform the YM4271 strain (mating type, *MAT*a). In total, $\sim 2 \times 10^6$ independent yeast transformants were obtained from SD/-Trp selection plates. Glycerol stocks of this library were kept as individual aliquots and used for subsequently mating type protein–protein interaction screens as described (Kolonin et al., 2000). More details about the screen and other potential interactors recovered will be described elsewhere. The prey vectors expressing HvRAR1, HvSGT1, AtSGT1a, AtSGT1b, and HvHSP90 have been described previously (Azevedo et al., 2002; Takahashi et al., 2003).

### TRV-Mediated Gene Silencing in *Nicotiana benthamiana*

The Rx-4HA transgenic *N. benthamiana* plants are described elsewhere (Lu et al., 2003). The TRV:*RAR1* construct and virus-induced gene silencing initiation is as described elsewhere except, importantly, plants were sampled at 13 to16 d postinoculation rather than 21 d postinoculation (Schornack et al., 2004). Protein extracts were prepared by grinding 0.5 g of leaf tissue in 1 mL of extraction buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, and 5 mM DTT). Samples were run and blotted using NuPAGE gels (Invitrogen) according to the manufacturer's guidelines. Rx levels were determined by detecting the HA epitope tag using 3F10 antibody (Roche) as previously described (Moffett et al., 2002). RAR1 levels were determined using custom antibody EP031453 (Eurogentec, Seraing, Belgium) generated using the peptide antigen GCKTGKHTTEKPVLAKS that corresponds to the CHORD I domain of AtRAR1.

## ACKNOWLEDGMENTS

We thank Jane Parker for the Arabidopsis *rar1* and *sgt1b* mutants, Ming-Bo Wang and Peter Waterhouse (CSIRO Plant Industry, Canberra, Australia) for plasmid pWBVec8 and the Agrobacterium strain AGL1, EMBL (Heidelberg, Germany) for plasmid pBS1539, Dennis Halterman and Roger Wise (Iowa State University) for cosmid 9589-5a, Akira Takahashi for antisera against RAR1, SGT1, and HSP90, and Caren Dawidson for maintenance of the plants in the greenhouse. This work was supported by the Max Planck Society, by the Biotechnology and Biological Science Research Council and the Gatsby Foundation to K.S., and by a Marie Curie training fellowship to S.B. by the Swiss National Science Foundation.

Received August 6, 2004; accepted October 4, 2004.

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