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Barley *Rom1* Reveals a Potential Link Between Race-Specific and Nonhost Resistance Responses to Powdery Mildew Fungi

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The *Rar1* gene, identified in the context of race-specific powdery mildew resistance mediated by the *Hordeum vulgare* (barley) resistance (*R*) gene *Mla12*, is required for the function of many *R*-mediated defense responses in mono- and dicotyledonous plant species. *Mla* resistance is associated with an oxidative burst and a subsequent cell death reaction of attacked cells. *Rar1* mutants are impaired in these responses and, to identify genetic elements which negatively regulate the *Mla12*-triggered response, we have screened mutagenized *Mla12 rar1* mutant populations for restoration of the resistance response. Here we describe the restoration of *Mla12*-specified resistance (*rom1*) mutant that restores features of disease resistance to a *Blumeria graminis* f. sp. *hordei* isolate expressing the avirulence gene *AvrMla12* and retains susceptibility to an isolate lacking *AvrMla12*. Histochemical analyses show that, in *rom1* mutant plants, a whole-cell oxidative burst and cell death response in attacked epidermal cells is restored in the incompatible interaction. Defense responses against tested inappropriate powdery mildews, *B. graminis* f. sp. *tritici* and *Golovinomyces orontii*, were diminished in *rar1* mutant plants and enhanced in *rom1* mutant plants relative to the wild type. These findings indicate antagonistic activities of *Rar1* and *Rom1* and reveal their contribution to nonhost and race-specific resistance responses.

Additional keywords: negative regulator, pathogen resistance, suppressor screen.

Plants have evolved different levels of resistance to defend themselves against pathogen attack (Dangl and Jones 2001). The most common defense renders an entire plant species resistant to a specific pathogen and is known as nonhost resistance (Mysore and Ryu 2004). Nonhost resistance is the most durable form of resistance and is thought to be genetically complex, involving preformed and inducible defenses. Insights into its genetic components were laid by the cloning of genes *NHO1* and *PEN1* in *Arabidopsis thaliana* involved in resistance to the bacterium *Pseudomonas syringae* and the grass powdery mildew *Blumeria graminis* f. sp. *hordei*, respectively (Collins et al. 2003; Kang et al. 2003). *NHO1* encodes a glycerol kinase but its precise function during resistance is still unknown. *PEN1* encodes a syntaxin and is supposed to have a role in mediating fusion of vesicles containing toxic cargo at the plasma membrane during attempted fungal invasion into leaf epidermal cells. A mutation in the *PEN1* homolog in barley, *Ror2*, originally was identified as suppressor of another form of pathogen resistance termed non-race-specific resistance or broad spectrum resistance against virulent isolates of *B. graminis* f. sp. *hordei* (Freialdenhoven et al. 1996). Broad spectrum *B. graminis* f. sp. *hordei* resistance in barley is conferred by loss-of-function alleles (*mlo*) of *Mlo* (Büschges et al. 1997). The finding that *A. thaliana* PEN1 and barley ROR2 are functionally homologous syntaxins points to the existence of shared molecular components in broad-spectrum and nonhost resistance responses in mono- and dicotyledonous plant species.

Plants have the capacity to recognize pathogen-derived molecules or structures that are either conserved in multiple microbial species or occur in one or few pathogen isolates (Nürnberger et al. 2004). Recognition of the former is mediated by membrane-resident receptors such as *Arabidopsis* FLS2 (Gómez-Gómez and Boller 2000) and mutations in the corresponding gene were shown to result in enhanced disease susceptibility (super-susceptibility) to virulent *P. syringae* (Zipfel et al. 2004). Recognition of isolate-specific pathogen effectors is mediated either by membrane-resident or intracellular race-specific resistance (R) proteins (Bonas and Lahaye 2002). Most isolated *R* genes encode intracellular proteins containing a nucleotide-binding (NB) domain and C-terminal leucine-rich repeats (LRRs). Isolate-specific effectors, recognized by R proteins, are encoded by so-called avirulence (*Avr*) genes (Flor 1971). Currently, much research is being undertaken to unravel the molecular mechanics of effector recognition (direct or indirect) and to understand downstream events triggered by *R* genes. Mutational analyses uncovered genes required for *R* gene function in a number of plant–microbe interactions (Freialdenhoven et al. 1994; Hammond-Kosack et al. 1994; Salmeron et al. 1994). Among these was the *Rar1* gene in barley, which originally was shown to be required for race-specific resistance triggered by the NB-LRR type R protein MLA12 toward *B. graminis* f. sp. *hordei* isolates containing *AvrMla12* (Freialdenhoven et al. 1994; Shen et al. 2003; Torp and Jørgenson 1986).

Rar1 encodes an intracellular Zn^{2+}-binding protein (Shirasu et al. 1999) and subsequent analyses has shown that RAR1 is required for several *R*-gene-mediated responses against different pathogen classes in monocotyledonous and dicotyledonous plant species (Shirasu and Schulze-Lefert 2003). RAR1 is re-

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quired for the function of two subtypes of NB-LRR proteins, which differ from each other by the presence of a coiled-coil (CC) or a Toll and Interleukin-1 receptor (TIR)-like domain at the N terminus. This is in contrast to 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 subclasses (Aarts et al. 1998; Century et al. 1997, Falk et al. 1999). Barley RAR1 interacts in yeast and in vivo with SGT1, an intracellular protein that, similar to RAR1, is also required for a number of resistance responses triggered by both NB-LLR R protein subclasses in diverse plant species (Azevedo et al. 2002; Shirasu and Schulze-Lefert 2003). The biochemical roles of RAR1 and SGT1 in NB-LRR-mediated resistance are poorly understood. However, physical association of RAR1 with cytosolic HSP90 and structural features in plant SGT1 and animal RAR1 homologs that resemble co-chaperones have led to the idea that these proteins might either alone or together exert cochaperone-like activities in the folding of R protein containing recognition complexes or downstream signaling complexes (Hubert et al. 2003; Liu et al. 2004; Takahashi et al. 2003). Consistent with this is the observation that the abundance of the *Arabidopsis* NB-LRR RPM1 protein is drastically reduced in non-challenged *rar1* mutant plants (Tornero et al. 2002). Interestingly, gene silencing experiments in *Nicotiana benthamiana* suggest that SGT1 and cytosolic HSP90 contribute to nonhost resistance responses, indicating a potential link between race-specific resistance and nonhost resistance (Kanzaki et al. 2003; Peart et al. 2002).

The barley *Mla R* locus is highly polymorphic and encodes more than 28 characterized race-specific resistance specificities to *B. graminis* f. sp. *hordei* 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 *Mla* (Halterman and Wise 2004; Halterman et al. 2001; Shen et al. 2003; Wei et al. 2002; Zhou et al. 2001). Despite the high sequence identity of the molecularly isolated *Mla R*-gene specificities (>90%), only a subset of MLA proteins engage RAR1 for efficient resistance (Jørgenson 1996). MLA chimeras have been generated in which *AvrMla6* recognition specificity was uncoupled from RAR1 dependence (Shen et al. 2003) and a single amino acid substitution in the LRR domain alleviates RAR1 dependence of *Mla6* and *Mla13* resistance reactions (Halterman and Wise 2004). These data demonstrate that subtle changes in MLA proteins can affect RAR1 engagement in race-specific resistance and might indicate a physical activity close to MLA. The resistance response triggered by *Mla R*-gene specificities invariably is linked to a cell death reaction of the attacked epidermal host cell and few subtending mesophyll cells (hypersensitive response; HR) (Boyd et al. 1995). In susceptible *Mla12 rar1* mutant plants, both *Mla12*-triggered host cell death and a preceding whole-cell oxidative burst, that may serve as a signal for race-specific resistance responses (Hückelhoven and Kogel 2003), is severely impaired (Shirasu et al. 1999).

The objective of this work was to uncover presumptive negative control elements of race-specific resistance triggered by *Mla12*. We made a suppressor screen based on the assumption that the impaired resistance response in susceptible *Mla12 rar1* mutant plants can be restored by the removal of negative regulators and identified a mutant, *rom1*, that shows restoration of *Mla12*-mediated resistance responses. Altered nonhost defense responses to two tested inappropriate powdery mildew species in *rar1* and *rom1* mutant plants led us to propose that the corresponding wild-type genes might have a dual role in both race-specific and nonhost resistance.

RESULTS

Seed of both the *rar1*-1 mutant M82 and the *rar1*-2 mutant M100 (Freialdenhoven et al. 1994) were mutagenized with NaN_3, and approximately 4,000 independent M_2 families comprising approximately 50,000 M_2 seedlings were screened for a modified infection phenotype upon inoculation with the *AvrMla12*-containing powdery mildew isolate A6. In all, 152 putative mutants were selected and classified into three classes with respect to a modified infection phenotype compared with the corresponding susceptible *rar1* mutant. The first class consisted of four candidates with a fully resistant infection phenotype, likely to be putative *mlo* mutants. Into the second class we grouped 85 candidates displaying enhanced disease resistance to the powdery mildew infection, and the last class contained 63 candidates with a lesion mimic phenotype prior to inoculation. Eighty-nine candidates survived to produce M_3 seed. We focused on mutants of the second class and reduced the total number of candidates to seven by further inoculation tests. Here we will give a detailed phenotypic and genetic description of the first mutant of this class. The remaining mutants will be described elsewhere.

Enhanced disease resistance to *B. graminis* f. sp. *hordei.*

In order to verify that mutant 348/1a originated from the *rar1*-2 mutagenized M_2 population, we performed a fingerprint analysis with cleaved amplified polymorphic sequence (CAPS) markers that previously were used for high-resolution mapping of the *Rar1* locus (Lahaye et al. 1998). In addition, mutant 348/1a displayed a characteristic anthocyanin deficiency in the leaf sheath, a phenotype that is diagnostic for Sultan5, the barley cultivar in which the mutant population was generated (Freialdenhoven et al. 1994). Thus, our analyses ensured that the identified mutant line was not due to seed contamination. The infection phenotypes of the *Mla12*-resistant wild type, the *rar1*-2 mutant, and mutant 348/1a upon inoculation with the *AvrMla12*-containing isolate A6 is shown in Figure 1. In *Mla12 Rar1*-resistant wild-type plants, only small necrotic lesions but no sporulating colonies were visible (Fig. 1A). In the *rar1*-2 mutant, resistance was impaired and the inoculated primary leaves were covered by fungal mycelium (Fig. 1B). In mutant 348/1a, the number of sporulating colonies was drastically reduced, and this was accompanied by a necrotic response (Fig. 1C). Notably, the necrotic response was much stronger than in resistant wild-type Sultan5. At a later stage of the infection, the necrosis in mutant 348/1a often led to the collapse of the inoculated primary leaf, a phenotype that is not observed in Sultan5. However, progeny of the new mutant grown under pathogen-free conditions (not shown) and leaf tissue protected with tape prior to inoculation (Fig. 1D) did not show a spontaneous or spreading necrosis, indicating that lesions result only upon powdery mildew attack.

In order to test whether the new infection phenotype was due to a mutation in host factors that are required for powdery mildew fungi to fulfill their life cycle, we inoculated mutant 348/1a with the virulent powdery mildew isolate K1 that lacks *AvrMla12*. Mutant 348/1a was fully susceptible (Fig. 1E). The mutant also was fully susceptible to another biotrophic fungus, a virulent yellow rust isolate that colonizes mesophyll tissue (*Puccinia graminis* f. sp. *hordei*) (Fig. 1F). Thus, mutant 348/1a supports unrestricted growth of two tested virulent biotrophic fungi but is affected in the *Mla12*-triggered race-specific resistance response. In accordance to the observed infection phenotype in mutant 348/1a, we designated the corresponding wild-type locus "restoration of *Mla12*-specified resistance" (*Rom1*) and refer to mutant 348/1a as the *rom1* mutant plant.

Restored pathogen triggered whole-cell H_2O_2 accumulation and cell death in the *rom1* mutant.

Barley *rar1* mutant plants are impaired in two commonly observed race-specific resistance responses, accumulation of reactive oxygen species and cell death of attacked epidermal cells (Shirasu et al. 1999); therefore, we compared wild-type (WT) (*Mla12 Rar1 Rom1*), *rar1*-2 mutant (*Mla12 rar1-2 Rom1*), and *rom1* mutant plants (*Mla12 rar1-2 rom1*) in a time course experiment for the above-described cytological reactions upon infection with the fungal isolate A6 (*AvrMla12*). The results are summarized in Figure 2.

Staining with 3,3′-diaminobenzidine (DAB), which allows detection of H_2O_2, did not uncover genotype-specific differences at an early stage of the interaction (24 h postinoculation [hpi]) where DAB staining is restricted mainly to cell wall appositions (CWAs) underneath sites of attempted fungal penetration (not shown). At 48 hpi, the fungus had penetrated epidermal host cells in up to 60% of interaction sites in all three genotypes (Fig. 3). Detection of DAB polymerization showed similar whole-cell accumulation of H_2O_2 for wild-type and *rom1* mutant plants (Fig. 2A and C). In contrast, the *rar1*-2 mutant essentially lacked DAB whole-cell staining despite the presence of fully developed fungal haustoria (Fig. 2B). No DAB whole-cell staining was detected in the *Rar1* wild-type, *rar1*-2, and *rom1* mutant plants when being inoculated with the virulent fungal isolate K1, which lacks *AvrMla12* (not shown).

The *Rar1* wild-type, *rar1*-2, and *rom1* mutant plants also were studied by trypan blue, a stain that marks cells that are committed to die (Koegh et al. 1980). In *Rar1* wild-type plants, clusters of stained mesophyll cells were visible at 72 hpi and hyphal growth of the fungus ceased (Fig. 2D). In the *rar1-2* mutant, trypan blue was rarely detectable in attacked host cells indicative of an impaired cell death reaction (Fig. 2E). In contrast, the *rom1* mutant plants showed trypan blue staining in attacked epidermal host cells (Fig. 2F). At 96 hpi, trypan blue also became visible in mesophyll cells. This reaction might lead to the severe necrotic infection phenotype 7 days postinoculation. Taken together, our data demonstrate that, in contrast to *rar1* mutant plants, in *rom1* mutant plants both typical cytological events of race-specific resistance are reinstated: generation of reactive oxygen species and cell death in host cells that are attacked by *B. graminis* f. sp. *hordei*.

***Rom1* dependent resistance kinetics.**

The kinetics of the *Mla12*-triggered resistance response can be changed by *Mla12* over-expression from a slow post-penetration to a rapid pre-penetration resistance in a transient single-cell gene expression system. Despite its altered kinetics, this altered *Mla12* resistance response retains its requirement for *Rar1* (Shen et al. 2003). We have investigated *rom1* mutant plants using this transient test system (Fig. 3).

Penetration frequencies were measured as the formation of a fully developed haustorium in attacked epidermal host cells, which were marked by β-glucuronidase staining to monitor transformation efficiency. Delivery of an empty vector resulted in approximately 60% fungal penetration in all three genotypes after inoculation with isolate A6 (*AvrMla6* and *AvrMla12*). This demonstrates that neither *rar1* nor *rom1* mutant plants interfere with the ability of *B. graminis* f. sp. *hordei* to switch from surface to invasive growth. Bombardment with the *Mla12* over-expression construct reduced the amount of successful penetration in wild-type plants to approximately 5%. This shift of the resistance kinetics is *Rar1* dependent, as shown by an unaltered high-penetration frequency (approximately 60%) in the *rar1*-2 mutant. However, in the *rom1* mutant, an intermediate penetration frequency of approximately 37% was obtained (χ^2 test, $P < 0.05$ compared with the *rar1*-2 mutant). Therefore, we conclude that, in a *rar1*-2 mutant background, *rom1* plants (genotype *Mla12 rar1*-2 *rom1*) partially restore the ability to shift the resistance kinetics upon *Mla12* over-expression.

In order to test whether, in *rom1* mutant plants, the *Rar1*-dependent *Mla6*-triggered defense response (Halterman et al. 2001) is affected, we conducted bombardment experiments using an *Mla6* over-expression construct. In the wild type, no fungal penetration into bombarded cells was detected, a typical feature of the rapid pre-penetration resistance response triggered by *Mla6*. Dependence of the response on *Rar1* was detected by 10% haustorium formation in the *rar1*-2 mutant. Although, in *rom1* plants, the penetration frequency appeared to be reduced compared with *rar1*-2 plants (7.5%), this reduction was statistically insignificant. Control experiments with the *Rar1*-independent *Mla1* gene (Zhou et al. 2001) and the powdery mildew isolate K1 (*AvrMla1*) resulted in early termination of fungal growth prior to haustorium formation in all three tested genotypes (not shown).

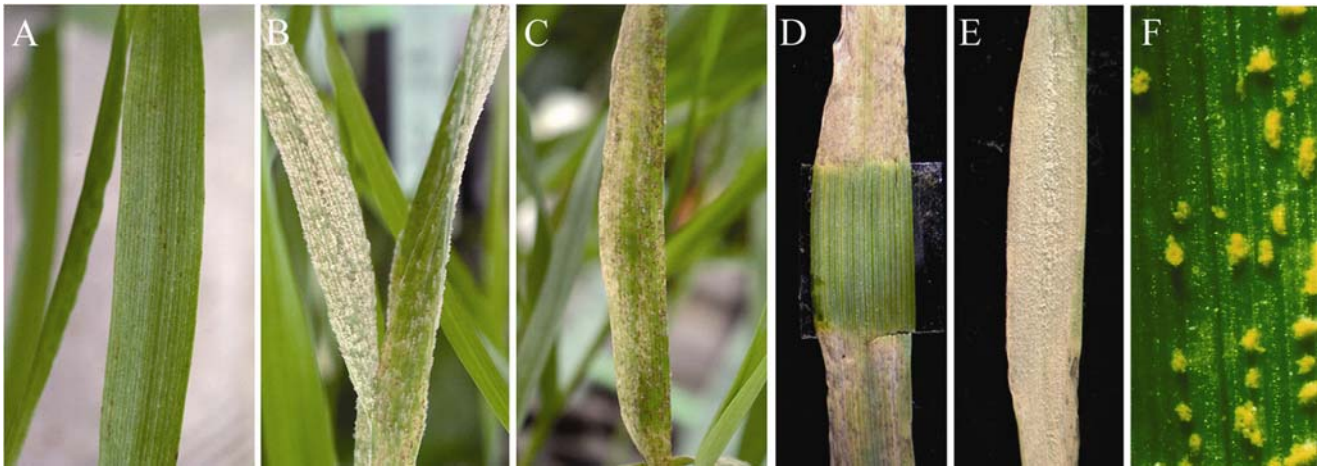

Fig. 1. Infection phenotypes of wild-type and mutant plants. Infection phenotype of **A,** wild-type Sultan5 (*Mla12 Rar1 Rom1*), **B,** *rar1* mutant M100 (*Mla12 rar1*-2 *Rom1*), and **C,** *rom1* mutant 348/1a (*Mla12 rar1*-2 *rom1*) 7 days after inoculation with powdery mildew isolate A6 (*AvrMla12*). **D,** Restriction of necrosis to powdery mildew-attacked tissue in the *rom1* mutant. **E,** Full susceptible infection phenotype of the *rom1* mutant 7 days after inoculation with virulent powdery mildew isolate K1. **F,** Full susceptible infection phenotype of the *rom1* mutant 14 days after inoculation with virulent yellow rust isolate JIW28.

***Rom1* is involved in nonhost defense responses.**

We previously have shown that an HR-like cell death response is triggered in barley by inoculation with the wheat powdery mildew (*B. graminis* f. sp. *tritici*) isolate JIW28 (Peterhänsel et al. 1997). Therefore, we investigated the reaction of the wild type (*Mla12 Rar1 Rom1*), *rar1*-2 (*Mla12 rar1*-2 *Rom1*), and *rom1* (*Mla12 rar1*-2 *rom1*) mutant upon inoculation with isolate JIW28. No colony formation was observed in any of the genotypes tested. Inspections under UV light revealed whole-cell autofluorescence in the wild type indicative of a pathogen-induced cell death response (Fig. 4A). The response was drastically reduced in the *rar1*-2 mutant background (Fig. 4B). In contrast, inoculated *rom1* mutant plants showed a hyper-autofluorescence of attacked tissue, which was significantly stronger compared with the wild type (Fig. 4C). Microscopic evaluation of infection sites revealed that in no case had the fungus penetrated barley epidermal cells (not shown). However, staining of the specimen with aniline blue and inspection under UV showed that, in wild-type Sultan5 and *rom1* plants, whole-cell deposition of callose occurred in response to fungal attack which, in the case of *rom1* plants, was spreading into the mesophyll layer (Fig. 4G and I). In the *rar1*-2 mutant, this reaction was restricted mostly to CWAs underneath fungal appressoria (Fig. 4E and H). Importantly, we obtained a comparable result in inoculation experiments using the inappropriate powdery mildew species *Golovinomy-*

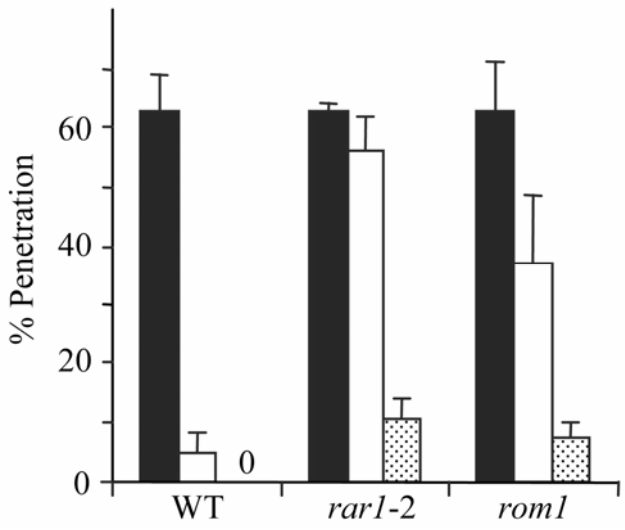

Fig. 3. Haustorium formation of powdery mildew in epidermal cells of barley. Mean values of developed haustoria in single epidermal cells of wild-type Sultan5 (WT; *Mla12 Rar1 Rom1*), *rar1*-2 mutant (*Mla12 rar1*-2 *Rom1*), and *rom1* mutant (*Mla12 rar1*-2 *rom1*). Detached barley leaves were bombarded with a β-glucuronidase reporter construct together with an empty vector (black bars) or a construct over-expressing the resistance gene *Mla12* (white bars) or *Mla6* (dotted bars) under transcriptional control of the maize ubiquitin promoter.

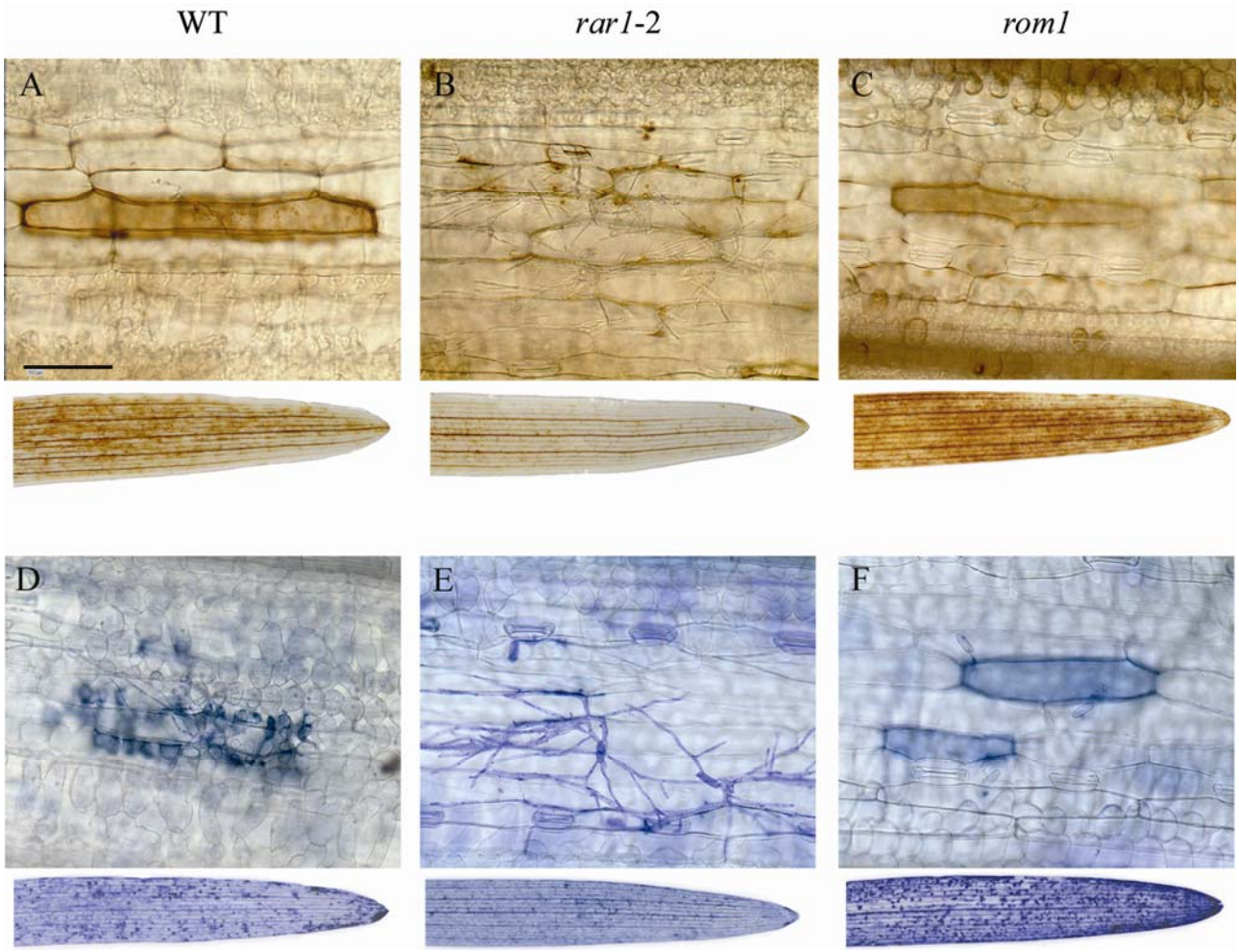

Fig. 2. Oxidative burst and cell death during powdery mildew attack. Accumulation of hydrogen peroxide monitored by 3,3′-diaminobenzidine staining in epidermal cells 48 h postinfection: **A,** wild-type Sultan5 (WT; *Mla12 Rar1 Rom1*), **B,** *rar1*-2 mutant (*Mla12 rar1*-2 *Rom1*), and **C,** *rom1* mutant (*Mla12 rar1*-2 *rom1*). Retention of trypan blue in epidermal cells indicative for a cell death reaction 72 h postinfection: **D,** wild-type Sultan5, **E,** *rar1*-2 mutant, and **F,** *rom1* mutant. Scale bar = 100 μm.

ces orontii, which causes disease only on dicot plants and for which barley is a nonhost (not shown). Collectively, this shows that *Rar1* and *Rom1* are active in both race-specific and nonhost interactions of barley with powdery mildews.

Unaltered RAR1 and SGT1 protein abundance in the *rom1* mutant.

The *rar1*-2 allele is mutated in an invariant nucleotide within the 3′ splice site consensus sequence of intron 2 leading to undetectable RAR1 protein levels in Western blot analyses (Azevedo et al. 2002; Shirasu et al. 1999); therefore, we tested whether the *rom1* infection phenotype is due to restored RAR1 protein accumulation. Protein extracts from the wild type, *rar1*-2, and *rom1* mutant were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and probed with an RAR1-specific antibody (Fig. 5). The approximately 30-kDa RAR1 protein was detected in the wild-type but absent in both the *rar1*-2 and *rom1* mutant genotypes. In addition, we examined the abundance of the RAR1 interacting protein SGT1 (approximately 45 kDa) which was unaltered in both mutant genotypes compared with the wild type.

Genetic analyses of the *rom1* mutant.

In order to determine the inheritance of the novel infection phenotype in the *rom1* mutant 348/1a, *rom1* plants were crossed with several barley lines containing the *Mla12* resistance gene. The F_2 populations were inoculated with isolate A6 and scored for the outcome of the interaction (Table 1).

The segregation in the backcross with the *rar1*-2 mutant (M100) showed that the inheritance of the *rom1* infection phenotype (mutant 348/1a) is consistent with a monogenic recessive trait ($P > 0.95$). This result was confirmed in the cross with the *rar1*-1 mutant M82 ($P > 0.3$). Thus, the *rom1* locus is able to restore features of *Mla12*-triggered race-specific resistance independent from the *rar1* mutant allele. In F_2 populations derived from crosses with barley wild-type genotypes Sultan5 and Nudinka, segregation of the *rom1* infection phenotype did not fit the expected 12:3:1 ratio ($P < 0.05$) for two unlinked loci (*Rar1* and *Rom1*). However, the data were all in accordance with a 3:1 segregation of wild-type to mutant plants (*rar1*-2 and *rom1* mutant infection phenotypes taken together). These results suggested linkage between the *rar1*-2 mutant allele and the *rom1* locus. Therefore, we performed an

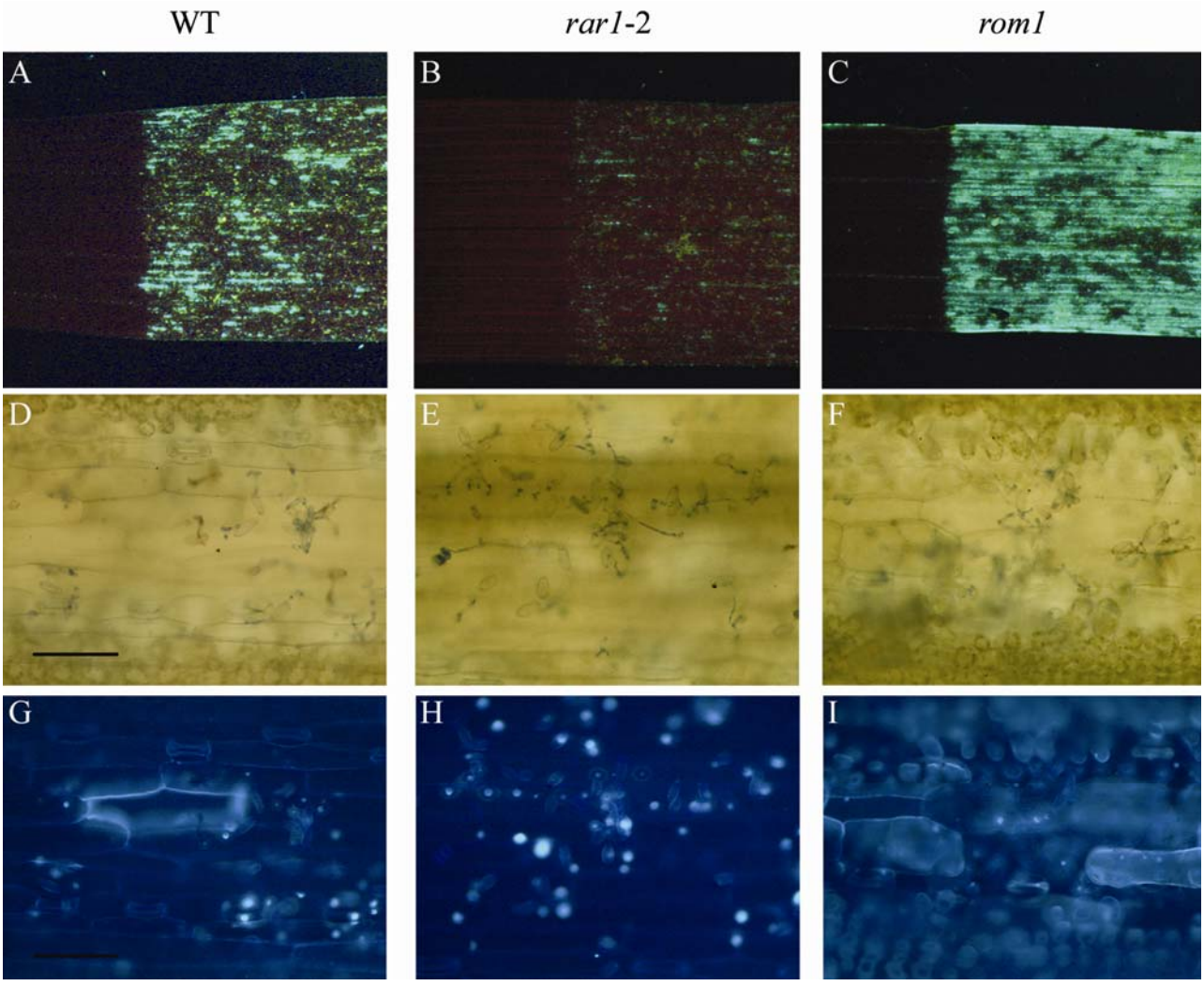

Fig. 4. Resistance response triggered by infection with *Blumeria graminis* f. sp. *tritici*. **A** through **C,** The right half of the barley leaf was inoculated with tritici powdery mildew isolate JIW28 and inspected 5 days later for autofluorescent cells under UV light with Leica filter GFP1 (425/60 nm excitation filter, 480 barrier filter). The left half of the leaf was covered with tape prior to inoculation. Magnification is ×5. **D** tbrough **F,** Infection sites under the light microscope. Fungal structures are stained by Coomassie blue. Note: no penetration into epidermal cells was detected (*not shown*). **G** through **I,** Same infection sites as shown in D through F under UV light. Callose deposition is visible due to aniline blue staining of the specimen. A, D, and G are wild-type Sultan5 (WT, *Mla12 Rar1 Rom1*); B, E, and H are *rar1*-2 mutant (*Mla12 rar1*-2 *Rom1*); and C, F, and I are *rom1* mutant (*Mla12 rar1*-2 *rom1*). Scale bar = 100 µm.

amplified fragment length polymorphism (AFLP) mapping approach (Becker et al. 1995, Castiglioni et al. 1998) on F_2 segregants of the cross with cv. Nudinka by taking advantage of AFLP markers known to map on chromosome 2H (*Rar1* location) and assigned the chromosomal map position of *Rom1* (Fig. 6). The *Rom1* locus co-segregated in all 17 tested *rom1* mutant plants with AFLP marker E4044-5 (34 chromosomes). Tested *rar1* mutants showed at least one recombinant chromosome for marker E4044-5 (23 recombinant chromosomes out of 42; 21 *rar1*-2 mutant plants tested). AFLP marker E3546-13 was positioned distal from *Rom1* (1 recombinant chromosome out of 34).

DISCUSSION

We provide genetic, molecular, and histochemical data that we have identified a locus, *Rom1*, which negatively affects an RAR1-dependent race-specific resistance response in barley. The finding that both *Rar1* and *Rom1* also are required for defense responses triggered by inappropriate powdery mildews of barley extends the number of common components in race-specific and nonhost resistance reactions. RAR1 dependency in race-specific resistance is a widespread phenomenon in different pathogen classes in diverse plant species (tobacco, *Arabidopsis*, barley) (Shirasu and Schulze-Lefert 2003); therefore, the *rom1* mutant broadens our knowledge about the regulation of plant immunity.

Restoration of H_2O_2 accumulation as a trigger of cell death?

The accumulation of hydrogen peroxide in barley upon powdery mildew attack occurs in a timely and spatially regulated manner and, on this basis, can be divided into three phases (Hückelhoven and Kogel 2003). The first and second phases are characterized by spatially restricted hydrogen peroxide accumulation (i) beneath the primary fungal germ tube and (ii) beneath the fungal appressorium. In the third phase, hydrogen peroxide accumulation is no longer restricted to subcellular compartments but occurs throughout the whole attacked cell. Accumulation of hydrogen peroxide beneath the primary germ tube and the fungal appressorium was not altered in *Rar1* or *Rom1* mutants in our studies (first phase, not shown; second phase, Fig. 2). However, whole-cell hydrogen peroxide accumulation of attacked host cells was dramatically decreased in *rar1* mutant plants and restored to wild-type-like frequency in the *rom1* mutant plants. Whole-cell accumulation of reactive oxygen intermediates is known to be closely linked with the onset of a subsequent cell death reaction in *Mla*-triggered race-specific resistance (Shirasu et al. 1999; Thordal-Christensen et al. 1997). Indeed, our dye exclusion assay with trypan blue clearly showed that the restoration of this response coincides with the restoration of pathogen-triggered cell death in *rom1* mutants (Fig. 2E). We hypothesize that the *rom1* mutant identifies a locus which negatively affects the outcome of a *Rar1*-dependent race-specific resistance response.

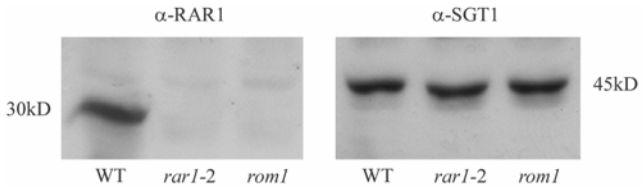

Fig. 5. Protein abundance of RAR1 and SGT1 in wild-type and mutant plants. Western blots were probed with RAR1- (α-RAR1) or SGT1- (α-SGT1) specific antibody. Protein extracts were from uninoculated leaf tissue of wild-type Sultan5 (WT; *Mla12 Rar1 Rom1*), *rar1*-2 mutant (*Mla12 rar1*-2 *Rom1*), and *rom1* mutant (*Mla12 rar1*-2 *rom1*).

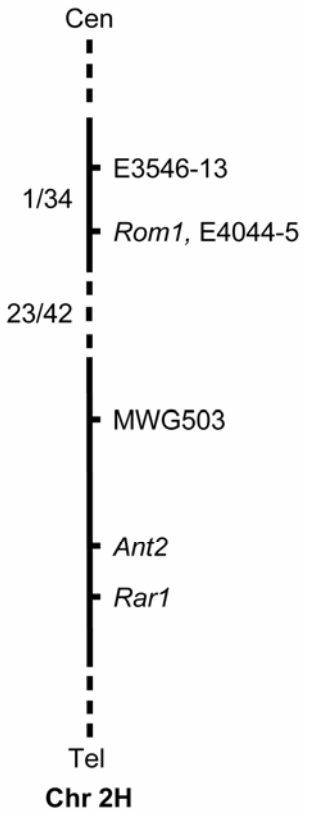

Fig. 6. Map position of the barley *Rom1* locus. Based on the analyses of F_2 plants segregating for the *rar1*-2 and *rom1* mutant infection phenotype, the *Rom1* locus could be positioned on chromosome 2H of barley. Amplified fragment length polymorphism (AFLP) marker E4044-5 co-segregated with all 17 *rom1* mutant plants tested. All 21 plants being scored as *rar1* mutant were recombinant for AFLP marker E4044-5 (23 recombinant chromosomes out of 42). AFLP marker E3546-13 is separated by a single recombination event from the *Rom1* locus (1 recombinant chromosome out of 34). MWG503 is a restricted fragment length polymorphism marker; *Ant2* = anthocyanin locus 2. Note that spaces between markers do not represent defined physical distances.

Table 1. Phenotypes and segregation ratios from crosses of the *rom1* mutant 348/1a with different *Mla12*-containing barley lines

	F_2 infection phenotype					
Cross 348/1a (*rar1*-2 *rom1*)	**Wild type (*Rar1 Rom1*)**	**M100/M82 (*rar1 Rom1*)**	**348/1a (*rar1*-2 *rom1*)**	**Ratio**	**χ^2 for segregation**	***P***
× M100 (*rar1*-2 *Rom1*)	0	82	27	3:1	0.0031	>0.95
× M82 (*rar1*-1 *Rom1*)	0	33	14	3:1	0.5763	>0.3
×	223	35	23	12:3:1	8.333	<0.02
Sultan5 (*Rar1 Rom1*)	223	58		3:1	2.848	>0.05
×	124	19	19	12:3:1	12.09	<0.01
Nudinka (*Rar1 Rom1*)	124	38		3:1	0.206	>0.5

An exaggerated cell death response appears to be the consequence of deregulated resistance in *rom1* plants.

A hallmark of cell death in the context of race-specific resistance is the spatial confinement to a few cells at sites of attempted infection. The strong necrotic phenotype of *rom1* mutant plants upon powdery mildew infection is suggestive of a deregulation of the resistance response, as has been described for the *Arabidopsis lsd1* mutant (Jabs et al. 1996). However, the *rom1* mutant is not a typical lesion mimic mutant because we did not observe necrosis spreading into unchallenged tissue (Fig. 1E). The observed infection phenotype of *rom1* mutants might be due to a delayed defense response which supports fungal escapes and, thus, increases the amount of cell death and necrotic tissue. This might reflect the situation observed in *Arabidopsis rar1* mutant plants impaired in *RPP5*-triggered resistance to *Peronospora parasitica*. The growing mycelium is followed by a trailing necrosis indicative of a delayed *RPP5*-mediated HR response in these plants (Muskett et al. 2002). In our analysis of the *rom1* mutant (Fig. 2), we observed, in most cases, halt of fungal growth prior to ramification of fungal hyphae. Therefore, in the *rom1* mutant, the severity of the resistance response appears higher than in the wild type. Exposure of *rom1* and *Rom1* leaves to different dosages of paraquat, which is known to provoke a light-inducible oxidative damage in plant cells leading to cell death (Dodge 1971), did not reveal significant differences between the genotypes (not shown). Similar results were obtained upon treatment with Rose Bengal, known to induce oxidative stress by the production of singlet oxygen (Affek and Yakir 2002; not shown). This suggests that the *rom1* mutant is not generally impaired in the responsiveness to oxidative stress.

Restoration of the resistance response in *rom1* plants also could be the consequence of constitutive activation of pathogenesis-related (*PR*) genes (Lorrain et al. 2003). However, absence of spontaneous necrosis, absence of constitutive *PR*-1 gene expression in unchallenged *rom1* plants (not shown), and retained susceptibility to virulent *B. graminis* f. sp. *hordei* and *Puccinia hordei* isolates suggests that *rom1* plants are fundamentally different from previously described *Arabidopsis cpr* mutants that show a constitutive expression of defense-related genes (Bowling et al. 1994, 1997; Jirage et al. 2001). Likewise, retained susceptibility to the virulent *B. graminis* f. sp. *hordei* isolate distinguishes the *rom1* mutant from *Arabidopsis pmr* and *edr1* mutants that exhibit enhanced disease resistance to virulent *Erysiphe cichoracearum* (Frye et al. 2001; Vogel and Somerville 2000).

***Rar1* and *Rom1*: A link between nonhost and race-specific resistance?**

Challenge inoculation with conidiospores of a wheat and a dicot-infecting powdery mildew species resulted in whole-cell autofluorescence and callose accumulation, both being indicative of induced defense responses. A similar whole-cell autofluorescence has been described upon inoculation of *B. graminis* f. sp. *hordei* spores on the nonhost *Arabidopsis* in cases where the fungus was able to penetrate into attacked epidermal cells (Collins et al. 2003). Our analysis revealed that whole-cell autofluorescence is strongly diminished in an *rar1* background whereas the *rar1 rom1* genotype showed not only a restored but even an enhanced fluorescence reaction compared with wild-type plants. This indicates that both *Rar1* and *Rom1* participate in nonhost reactions to the inappropriate powdery mildews (Fig. 4). The exaggerated response in the *rom1* mutant is consistent with the proposed negative activity of the *Rom1* wild-type locus in disease resistance. To date, *Rar1* has been shown to be required for a subset of race-specific resistance responses mediated by intracellular NB-LRR type R proteins (Holt et al. 2003; Shirasu and Schulze-Lefert 2003). Our findings suggest that *Rar1* also is required for nonhost interactions of barley. However, the *rar1* mutation on its own was not sufficient to support enhanced growth of the inappropriate fungi. The latter could be explained by unaffected penetration resistance that is under control of barley *Ror* and *Arabidopsis PEN* genes (Collins et al. 2003; Peterhänsel et al. 1997). If NB-LRR R proteins participate in nonhost resistance to powdery mildew fungi, it is conceivable that the diminished autofluorescence response in *rar1* plants reflects impaired recognition of conserved powdery mildew effectors that are present in *B. graminis* f. sp. *hordei*, *B. graminis* f. sp. *tritici*, and *G. orontii*. Interestingly, a function for the RAR1 interactor SGT1 in nonhost resistance has been shown in *N. benthamiana* interactions with inappropriate *Xanthomonas* spp. (Peart et al. 2002).

Plants have the capacity to recognize isolate-specific effectors and conserved pathogen-associated molecular patterns (PAMPs). Recognition of both types of molecules by receptors elicits immune responses that can include MAP kinase signaling and induction of defense-related genes (Asai et al. 2002; Jin et al. 2002). However, resistance in *Arabidopsis* to *Pseudomonas syringae* induced by the PAMP flg22, a flagellin-derived peptide that is recognized by the membrane resident receptor FLS2 (Gómez-Gómez and Boller 2000), does not require tested signaling components known to be involved in *R*-gene-triggered resistance such as EDS1, RAR1, or SGT1 (Zipfel et al. 2004). In addition, flg22, like many other PAMPs, does not provoke a cellular suicide of plant cells, demonstrating that *R*-gene- and PAMP-receptor-triggered immune responses are at least partially dissimilar (Asai et al. 2002; Gómez-Gómez and Boller 2002; Nürnberger et al. 2004). If lack of cell death in PAMP-mediated resistance involves negative regulation, the exaggerated resistance response in *rom1* plants might reflect a potentiated PAMP-triggered response in which cell death suppression is impaired. Recently, virulent but not inappropriate powdery mildews have been shown to suppress plant defense responses in *Arabidopsis* (Zimmerli et al. 2004). This finding could explain retained susceptibility in *rom1* mutants to the virulent *B. graminis* f. sp. *hordei* isolate. Future studies should reveal whether the *Rom1* wild-type locus controls yet unknown cross-talk between PAMP-triggered and race-specific plant immunity.

MATERIALS AND METHODS

Plant material, mutagenesis, and seedling inoculations.

The *rar1* mutant plants M82 and M100 were derived from the double-haploid barley line Sultan5 (Torp and Jørgensen 1986). Cv. Nudinka was used to establish a general AFLP map of barley (Becker et al. 1995) and contained the *Mla12* resistance gene. Crosses were performed by pollination of mutant 348/1a with pollen derived from barley lines indicated in Table 1. Chemical mutagenesis with NaN_3 and tests for resistance using different powdery mildew isolates were described previously (Freialdenhoven et al. 1994, 1996). The frequency of chlorophyll-defective M_2 seedlings (6%) and the parallel isolation of two independent *mlo* mutant plants in the population (*mlo-29*) (Piffanelli et al. 2002) indicated that the mutation rate was in the general range for chemical mutagenesis of barley (Jende-Strid 1978).

Histochemical analysis and single-cell transient expression assay.

Uptake and polymerization of DAB (Sigma, St. Louis) was monitored according to the protocol of Thordal-Christensen and associates (1997). Trypan blue retention was carried out as

described by Peterhänsel and associates (1997). Staining for callose was performed with 0.01% aniline blue (wt/vol) (Sigma-Aldrich) in 150 mM KH_2PO_4 (pH 9.5) for 48 h. Microscopic inspection of specimens was conducted as described previously (Peterhänsel et al. 1997). For the single-cell transient expression assays, the protocol and over-expression constructs described by Shen and associates (2003) were used. Each bar in Figure 3 is the result of at least three independent shooting experiments and a minimum of 150 single-cell interaction sites.

Molecular analysis.

RAR1 and SGT1 protein detection are described in Azevedo and associates (2002). For each genotype, a protein mixture of five independent primary leaves was used for the Western blot shown in Figure 5. DNA from single F_2 plants was isolated for AFLP analysis which was performed as described by Becker and associates (1995). The F_2 infection phenotypes were confirmed by inoculation experiments of derived F_3 segregants. AFLP and restriction fragment length polymorphism marker designations are according to Castiglioni and associates (1998) and Graner and associates (1991), respectively.

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