

# Conservation of NLR-triggered immunity across plant lineages

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The nucleotide-binding domain and leucine-rich repeat (NLR) family of plant receptors detects pathogen-derived molecules, designated effectors, inside host cells and mediates innate immune responses to pathogenic invaders. Genetic evidence revealed species-specific co-evolution of many NLRs with effectors from host-adapted pathogens, suggesting that the specificity of these NLRs is restricted to the host or closely related plant species. However, we report that an NLR immune receptor (MLA1) from monocotyledonous barley is fully functional in partially immunocompromised dicotyledonous *Arabidopsis thaliana* against the barley powdery mildew fungus, *Blumeria graminis* f. sp. *hordei*. This implies ~200 million years of evolutionary conservation of the underlying immune mechanism. A time-course RNA-seq analysis in transgenic *Arabidopsis* lines detected sustained expression of a large MLA1-dependent gene cluster. This cluster is greatly enriched in genes known to respond to the fungal cell wall-derived microbe-associated molecular pattern chitin. The MLA1-dependent sustained transcript accumulation could define a conserved function of the nuclear pool of MLA1 detected in barley and *Arabidopsis*. We also found that MLA1-triggered immunity was fully retained in mutant plants that are simultaneously depleted of ethylene, jasmonic acid, and salicylic acid signaling. This points to the existence of an evolutionarily conserved and phytohormone-independent MLA1-mediated resistance mechanism. This also suggests a conserved mechanism for internalization of *B. graminis* f. sp. *hordei* effectors into host cells of flowering plants. Furthermore, the deduced connectivity of the NLR to multiple branches of immune signaling pathways likely confers increased robustness against pathogen effector-mediated interception of host immune signaling and could have contributed to the evolutionary preservation of the immune mechanism.

innate immunity | defense phytohormone

The nucleotide-binding domain and leucine-rich repeat (NLR)-containing proteins in plants and animals mediate pathogen-sensing. Unlike animal NLRs that detect mostly conserved microbial molecules, plant-encoded NLRs typically recognize the presence of isolate-specific pathogen effectors (1). Most plant NLRs are modular, containing coiled-coil (CC) or TOLL/IL-1 receptor domains at the N terminus, a central nucleotide-binding domain, and C-terminal leucine-rich repeats. Plant NLRs are often polymorphic between individual plants of a host population, and the sum of genes encoding NLRs in a host population defines the repertoire available for the detection of structure or action of polymorphic pathogen effectors (1).

Interfamily transfer of NLR function was shown by coexpression of an NLR, its cognate effector and the effector target (e.g., *Arabidopsis* RPS5, *Pseudomonas syringae* AvrPphB, and *Arabidopsis* PBS1; reviewed in ref. 2). However, these data are based on transient gene expression and use host cell death as proxy for NLR activity even though cell death has been uncoupled from NLR-mediated pathogen growth restriction in several cases (1). Those functional recapitulations of the NLR-mediated host cell death response were demonstrated only between *Brassicaceae* plants (e.g., *Arabidopsis thaliana*) and *Solanaceae* plants (e.g., *Nicotiana benthamiana*) (2). To date, interfamily transfer of NLRs has failed to produce stable transgenic plants with expected disease resistance

ability (2, 3). These attempts, however, are not conclusive evidence that the disease resistance activity of a given NLR is necessarily restricted to the host or closely related plant species.

To address this longstanding question of plant NLR biology, we transferred the mildew A (MLA) gene from the monocotyledonous plant barley (*Hordeum vulgare*) to the dicotyledonous plant *A. thaliana*, which were separated by evolution ~200 Mya (4). The polymorphic barley *MLA* locus encodes functionally diversified CC-type NLRs, each recognizing a cognate isolate-specific effector of the powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*) (5). Although WT *Arabidopsis* plants are fully resistant to *Bgh*, this fungus is able to reproduce on leaves of partially immunocompromised *Arabidopsis* plants (*pen2 pad4 sag101* background). On this triple mutant, early invasive growth (i.e., haustorium formation) is followed by epiphytic growth with fungal microcolonies containing conidia maturing at ~7 d after spore inoculation (6). PEN2 functions as a myrosinase in the metabolism of indole glucosinolates and is required for effective preinvasive resistance to nonadapted fungi including *Bgh* (7), whereas PAD4 and SAG101 are indispensable and redundantly acting cofactors for functions of lipase-like EDS1 (8) and confer postinvasive defense responses against the nonadapted *Bgh* fungus (6). As the combined activities of PAD4 and SAG101 are essential for TOLL/IL-1 receptor-type NLR but not CC-type NLR functions (8, 9), and the indole glucosinolate substrates and products of PEN2 are lineage-specific innovations of the Capparales [which includes the *Brassicaceae* (7, 10)], we reasoned that MLA activities in *Arabidopsis* should become detectable in the absence of PEN2 PAD4 SAG101. We selected *MLA1* among different *MLA* specificities in view of higher steady-state levels *in planta* of the encoded receptor protein (11).

Here we report that barley *MLA1* confers *Bgh* isolate-specific disease resistance in *A. thaliana*. We then investigate the evolutionarily conserved molecular mechanism of MLA1-mediated disease resistance in the model plant *Arabidopsis* by exploiting genomic and genetic tools that are presently unavailable in barley. We also discuss models for MLA1 signaling that could permit increased robustness against pathogen effector-mediated interception of host immune signaling, despite the receptor's connectivity to conserved immune signaling targets.

Author contributions: T.M. and P.S.-L. designed research; T.M. and S.V. performed research; T.M., B.K., S.V., E.V.L.v.T., and P.S.-L. analyzed data; and T.M. and P.S.-L. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo) (accession no. GSE39463).

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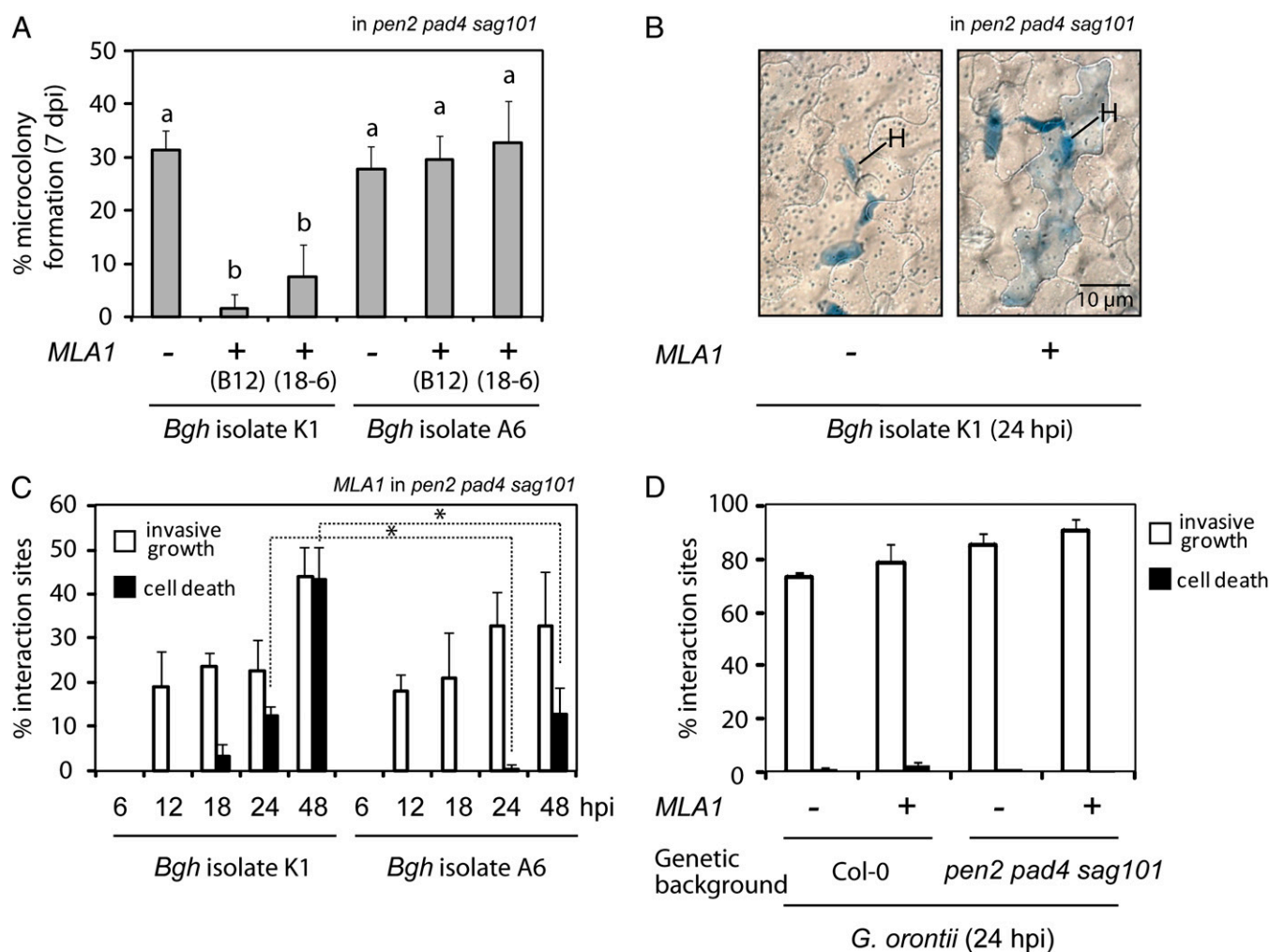
This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1218059109/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1218059109/-DCSupplemental).

## Results

**Barley *MLA1* Functions in *Arabidopsis*.** We introduced an epitope-tagged *MLA1* construct into partially immunocompromised *Arabidopsis* plants (*pen2 pad4 sag101* background) because the absence of these gene products enables the nonadapted *Bgh* fungus to form invasive infection structures (i.e., haustoria) on *Arabidopsis* leaves and permits completion of its asexual life cycle by conidiation (6). Despite the use of the strong *cauliflower mosaic virus 35S* promoter, the steady-state levels of *MLA1*-HA in *Arabidopsis* leaves were comparable or lower than those in transgenic barley driven by the native *MLA1* promoter (Fig. S1). Remarkably, microcolony formation of the avirulent *Bgh* isolate K1 expressing the cognate AVR<sub>A1</sub> effector was significantly reduced on leaves of two independent transgenic *Arabidopsis* lines expressing *MLA1*-HA (line B12 and line 18-6) in comparison with *pen2 pad4 sag101* triple mutant plants lacking *MLA1* (Fig. 1A). In contrast, the same transgenic lines permitted similar levels of microcolony formation compared with the triple mutant upon inoculation with the *Bgh* isolate A6

expressing other AVR<sub>A</sub> effectors (Fig. 1A). This *MLA1*-mediated growth restriction of the fungal pathogen included a localized host cell death response at attempted infection sites (Fig. 1B and C), which accompanies the activation of most tested plant NLRs (1).

Many filamentous pathogens including powdery mildew fungi form dedicated invasive infection structures (i.e., haustoria) for nutrient uptake and likely for effector delivery (12). It has been shown that *Bgh* genes encoding candidates for secreted effector proteins are predominantly expressed in haustoria (13). To correlate haustoria development (i.e., invasive growth) and *MLA1* activation, we measured host cell death as a proxy for receptor activation in the established heterologous system. Time-course experiments revealed that cell death became detectable at 18 h post inoculation (hpi) only in host cells containing haustoria of the avirulent AVR<sub>A1</sub>-expressing isolate, and, at 48 hpi, almost all haustorium-containing cells were dead (Fig. 1C). In contrast, cell death was undetectable in host cells containing haustoria of the virulent A6 isolate at 18 and 24 hpi and remained low (12%) at 48

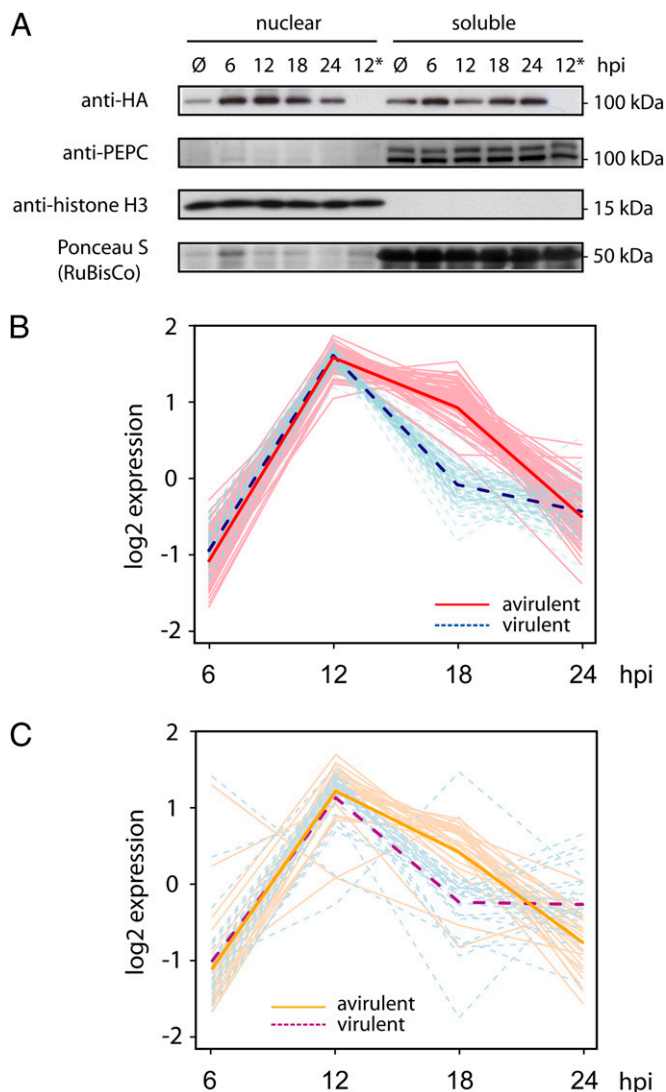


**Fig. 1.** Barley *MLA1* confers isolate-specific immunity to the barley powdery mildew (*Bgh*) in *Arabidopsis*. (A) Frequency of fungal microcolony formation at *Bgh* interaction sites on leaves of *Arabidopsis* plants. Two independent transgenic lines were used (line B12 and line 18-6). dpi, days post inoculation. (B) *MLA1*-dependent single-cell death upon *Bgh* entry into *Arabidopsis* leaf epidermal cells. Retention of trypan blue in epidermal cells. Apart from staining of the fungus, staining of an infected epidermal cell, indicative of cell death, was only observed in the *MLA1* line. (H, haustorium.) (C) Frequency of invasive growth and cell death at *Bgh* interaction sites on leaves of *Arabidopsis* plants. (D) Frequency of invasive growth and epidermal single-cell death of the host-adapted virulent powdery mildew *G. orontii* on WT (Col-0), *pen2 pad4 sag101* (*pps*) triple mutant, and transgenic *Arabidopsis* expressing *MLA1*-HA in either Col-0 or *pps* mutant background. The plant genotypes used in pathogenicity assays are indicated for each panel as with (+) or without (–) the *MLA1*-HA construct. *Bgh* isolate K1 expresses AVR<sub>A1</sub> and isolate A6 lacks AVR<sub>A1</sub>. Different letters and asterisks indicate significant differences ( $P < 0.01$ ). hpi, hours post inoculation. Data in A, C, and D show average and SD from two independent experiments ( $n \geq 4$ ), three independent experiments ( $n \geq 5$ ), and two independent experiments ( $n \geq 3$ ), respectively.

hpi (Fig. 1C). As a result of the absence of host cell death before fungal invasion, the AVR<sub>AI</sub> effector is likely delivered into host cells coincident with or after the differentiation of invasive infection structures. The transgenic *Arabidopsis* lines retained susceptibility to a host-adapted powdery mildew, *Golovinomyces orontii*, and host cell death was hardly detectable upon haustoria formation (Fig. 1D). This excludes the possibility that *MLA1* expression constitutively activated immune signaling to the host-adapted powdery mildew in these plants. Taken together, an authentic *MLA1*-mediated isolate-specific disease resistance response was recapitulated in transgenic *Arabidopsis* lines against the barley powdery mildew *Bgh*.

**MLA1-Mediated Transcriptional Reprogramming in *Arabidopsis*.** Nucleocytoplasmic partitioning of *MLA* is pivotal for receptor function in barley (14, 15). The nuclear receptor pool is indispensable for disease resistance in barley (14), whereas cell death signaling depends on the cytoplasmic pool (15). To examine the subcellular distribution of *MLA1* in *Arabidopsis*, we purified nuclei from leaves before and after challenge with the avirulent *Bgh*. Immunoblot analyses detected *MLA1*-HA in nuclear extracts and nuclei-depleted soluble fractions in *Arabidopsis* leaves, as in barley (Fig. 2A). It has been postulated that, in barley, the nuclear receptor pool amplifies or sustains microbe-associated molecular pattern-triggered defense responses to the *Bgh* fungal pathogen (14). RNA-seq experiments in *Arabidopsis* detected a sustained expression of a large *MLA1*-dependent gene cluster (Fig. 2B and Fig. S2) at 18 hpi. This cluster is greatly enriched in genes known to respond to the fungal cell wall-derived microbe-associated molecular pattern chitin (44 of 87 genes; Fig. S2). An evolutionarily conserved regulon was previously identified in barley and *Arabidopsis* upon comparative coexpression analysis of genetically defined immune components that restrict the growth of fungal pathogens, including powdery mildews (16). Strikingly, the functionally validated antifungal regulon also showed sustained transcript accumulation at 18 hpi in our data set (Fig. 2C), implying a functional link between *MLA1*-dependent transcriptional outputs and disease resistance.

**MLA1-Triggered Immunity Is Retained in Ethylene, Jasmonic Acid, and Salicylic Acid Signaling Depleted Background.** A model of the plant innate immune system suggests that its robustness is achieved through the formation of an interconnected signaling network with synergistic and compensatory links between several overlapping signaling pathways, as exemplified by the well-characterized defense phytohormone sectors [i.e., ethylene (ET), jasmonic acid (JA), and salicylic acid (SA)] (17). To examine if this defense phytohormone network contributes to *MLA1*-triggered immunity, we simultaneously impaired the ET, JA, and SA sectors by introducing *ein2*, *dde2*, and *sid2* mutations in the aforementioned *MLA1*-expressing *Arabidopsis* lines lacking *PEN2 PAD4 SAG101*. The resulting *MLA1*-expressing sextuple mutant fully retained isolate-specific disease resistance and cell death activation to *Bgh* (Fig. 3), suggesting that the defense phytohormone-dependent signaling sectors are dispensable for *MLA1*-dependent transcriptional reprogramming (i.e., sustained gene expression in Fig. 2B). This also demonstrates the existence of additional immune mechanisms, including processes underlying *MLA1*-triggered cell death, which act independently of all defense phytohormone sectors. As *PAD4* and *SAG101* are indispensable for the function of *EDS1*, an immune regulator (8, 9), the resultant sextuple mutant is expected to lack also the *EDS1* signaling sector. Thus, our data are consistent with the previous observation that CC-type NLRs function independently from *EDS1* (9). Furthermore, our results demonstrate that the defense phytohormone and *EDS1* sectors do not act redundantly in CC-type NLR-triggered immunity, which contrasts with a previous report (18).

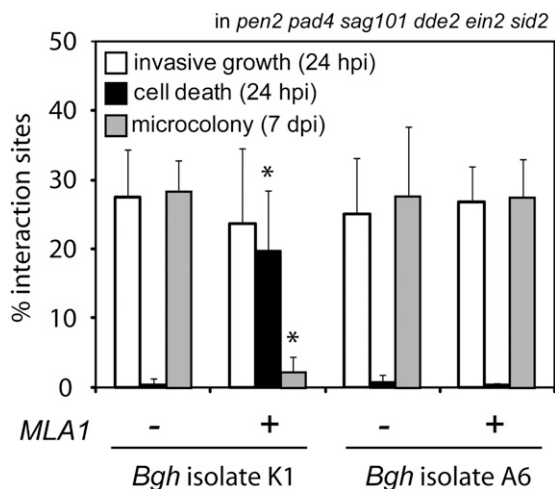


**Fig. 2.** *MLA1*-mediated transcriptional reprogramming in *Arabidopsis*. (A) Immunoblotting of *MLA1*-HA in nuclear and soluble fractions of healthy or *Bgh*-challenged *Arabidopsis* leaves. Purified nuclear and nuclei-depleted soluble fractions were prepared from leaves of a transgenic line expressing *MLA1*-HA in the *pen2 pad4 sag101* (*pps*) background after inoculation with the avirulent *Bgh* isolate K1 at the indicated time points. (0, noninoculated controls.) This loading represents an ~20-fold overrepresentation of nuclear proteins on a per-tissue amount basis. Histone H3, phosphoenolpyruvate carboxylase (PEPC), and Ponceau S staining of ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCo) were used as fraction markers. The *pps* mutants were used as a negative control for the specificity of *MLA1*-HA detection (asterisks). (B) Gene expression pattern of the major *MLA1*-dependent cluster of genes in Fig. S2 that were significantly differentially regulated ( $FDR < 0.05$ ) in response to the avirulent *Bgh* isolate. (C) Gene expression pattern of 27 of 28 genes in the antifungal regulon (16) that were significantly differentially regulated ( $FDR < 0.05$ ) in response to the avirulent *Bgh* isolate in our study. A total of 24 of these differentially regulated genes showed a similar pattern of sustained gene expression at 18 hpi as detected for the cluster in B, with a Pearson correlation coefficient  $r > 0.8$ . Gene expression in B and C is given as genewise standardized log<sub>2</sub>-transformed counts per million. hpi, hours post inoculation.

## Discussion

Our data revealed conservation of *MLA1*-mediated immunity to *Bgh* in both major classes of flowering plants. This has implications for the evolutionary conservation of components required for *MLA1*-mediated pathogen perception and downstream sig-





**Fig. 3.** Retained MLA1-HA-triggered immunity in ET, JA, and SA signaling-depleted background. Frequency of invasive growth, cell death, and fungal microcolony formation at *Bgh* interaction sites on leaves of *Arabidopsis* plants. The plant genotypes used in the pathogenicity assays are indicated as with (+) or without (–) the *MLA1-HA* construct. *Bgh* isolate K1 expresses *AVR<sub>A1</sub>* and isolate A6 lacks *AVR<sub>A1</sub>*. dpi, days post inoculation; hpi, hours post inoculation. Asterisks indicate significant differences ( $P < 0.01$ ). Data show average and SD from three independent experiments ( $n \geq 4$ ).

naling. Our findings necessarily imply that the CC-type MLA1 receptor connects to at least one conserved downstream signaling pathway that acts independently of all three phytohormone-mediated defense mechanisms and EDS1. Previous work has shown a differential requirement for these phytohormone-dependent mechanisms in disease resistance mediated by the CC-type *Arabidopsis* NLRs RPM1 and RPS2 to the bacterial pathogen *Pseudomonas syringae* DC3000 (17). In the phytohormone-depleted plants, RPM1- and RPS2-mediated pathogen growth restriction was impaired by 20% and 80%, respectively, demonstrating quantitatively different dependencies on defense phytohormone sectors in CC-type NLR-conditioned immunity. Together with our findings, this implies the existence of at least one evolutionarily conserved and phytohormone-independent signaling mechanism mediated by all CC-type NLRs. Accordingly, this mechanism might contribute 20% of pathogen growth restriction in the case of RPS2—but most or all in the case of RPM1—and MLA1-mediated pathogen growth restriction.

It has recently been shown that independently evolved pathogen effectors can target the same highly connected host proteins, designated cellular hubs, which are likely conserved in flowering plants (19). Fifteen of 17 tested hubs were shown to have immune functions. A potential link between such hubs and NLR functions may be given by the *Arabidopsis* NLRs RRS1 and RPS4 that act together and appear to detect three independently evolved effectors from different pathogen species (20). As NLRs have been shown to detect nonself or modified-self molecules inside host cells, these hubs are potential NLR guarders for indirect effector recognition (21, 22). It is possible that MLA1 “guards” a conserved hub, enabling indirect detection of the cognate *Bgh* effector in *Arabidopsis*. Alternatively, MLA1 binds directly to this effector and initiates downstream signaling through evolutionarily conserved pathway(s). Both molecular models are compatible with extensive diversification of MLA recognition specificities at the *MLA* locus in barley each conferring isolate-specific immunity to *Bgh* strains expressing cognate *AVR<sub>A</sub>* effectors (5) and presumed iterative cycles of effector and receptor adaptations (referred to as a co-evolutionary arms race).

It is still unclear how fungal effectors enter host plant cells (23). Fungal effectors are typically small proteins with aminoterminal

secretion signals, and several of those were shown to interfere with host immunity (23). These effectors are secreted from haustoria and accumulate at the host–pathogen interspace, designated extrahaustorial matrix. As the extrahaustorial matrix is separated by an extrahaustorial membrane from the host cytoplasm, effectors released into the extrahaustorial matrix subsequently need to pass through the extrahaustorial membrane to enter the host cytoplasm (12). Although binding of effectors to host-specific phosphatidylinositol-3-phosphate has been proposed for effector internalization into host cells (24), the general applicability of this model remains controversial (23). Our data strongly suggest that the *AVR<sub>A1</sub>* effector of the barley powdery mildew *Bgh* is delivered into live *Arabidopsis* cells, thereby activating intracellular MLA1 as in barley. Thus, if a host-encoded machinery contributes to effector internalization into host cells, the underlying mechanism must be conserved in flowering plants.

The existence of an evolutionarily conserved immediate target(s) of MLA1 is counterintuitive because pathogens are likely to have evolved effectors that sabotage the activity of such conserved immune components. One way to rationalize this paradox is that a single plant NLR initiates immune signaling via multiple downstream signaling pathways. Recent studies revealed cell compartment-specific activities in the cytoplasm and nucleus for MLA10 and RPS4 (15, 25), suggesting that receptors interact with different host proteins to initiate functionally redundant, but mechanistically distinct, resistance responses. Thus, the existence of multiple NLR signal outputs would confer increased robustness against pathogen effector-mediated interception of host immune signaling, despite the receptor’s connectivity to conserved immune signaling targets. Finally, our findings suggest that NLRs can, in principle, be exploited for disease resistance breeding in a much wider range of plant species than previously thought.

## Materials and Methods

**Plant and Fungal Materials and Pathogenicity Assay.** Transformation of *A. thaliana* was described previously (6). The binary transformation vector, pAMPAT-MCS (GenBank accession no. AY436765) containing *H. vulgare* *MLA1* cDNA with a single HA epitope-sequence at the C terminus was used to generate the *Arabidopsis* stable transgenic lines expressing MLA1-HA under the *cauliflower mosaic virus 35S* promoter (35Sp). Two phosphinothricin-resistant lines, 1-1 and 47-1 (35Sp:MLA1-HA in Col-0 background) were independently selected by immunoblotting of MLA1-HA. Because *pen2-1 pad4-1 sag101-2* triple mutant (*pps*) enables the barley powdery mildew (as detailed later) to reproduce on *Arabidopsis* leaves (6), these lines were crossed with *pps* to generate transgenic lines expressing MLA1-HA in *pps* mutant background. Line 18-6 and line B12 (35Sp:MLA1-HA in *pps* background) were established from line 1-1 and line 47-1, respectively. The transferred DNA (T-DNA) copy number of *MLA1-HA* constructs was examined by scoring the segregation of *MLA1-HA* in an  $F_2$  population generated by crossing with WT plant. Line B12 appears to harbor a single integration of *MLA1-HA*. Line B12 was crossed with *pad4-1 ein2-1 dde2-2 sid2-2* quadruple mutant (17) to generate transgenic lines expressing MLA1-HA in *pen2-1 pad4-1 sag101-2 ein2-1 dde2-2 sid2-2* mutant background. Siblings of *MLA1-HA<sup>+/−</sup> pen2-1 pad4-1 sag101-2 ein2-1 DDE2/dde2-2 sid2-1* plants were genotyped and used for the pathogenicity assay shown in Fig. 3. Unless otherwise indicated, line B12 or its derivative lines were used in this study.

Plant growth and pathogenicity assays with *Bgh* isolate K1 expressing *AVR<sub>A1</sub>*, *Bgh* isolate A6 lacking *AVR<sub>A1</sub>*, and *G. orontii* (anonymous isolate) were performed as described previously (6, 14), except for the siblings of *MLA1-HA<sup>+/−</sup> pen2-1 pad4-1 sag101-2 ein2-1 DDE2/dde2-2 sid2-1* plants. The siblings were initially grown on MS-agar plates for 2 wk under sterile conditions and subsequently transferred to Jiffy pots supplemented with a fertilizer, Wuxal TopN (Aglukon Spezialdünger), and grown for an additional 2 wk. Four week-old plants were used for pathogenicity assay. Histochemical analysis of plant cell death and fungal structure with lactophenol-trypan blue was described previously (15). Haustoria formation in epidermal cells and retention of trypan blue in epidermal cells were scored as invasive growth and cell death, respectively. Barley (*H. vulgare*) transgenic line 6E expressing a functional single copy of *MLA1-HA* under the 5′ native regulatory sequences was described previously (11).

**RNA-seq Analysis.** A  $2 \times 2 \times 4$  factorial design was used with plant-genotypes (MLA1-HA+/-), pathogen-isolates (A6 and K1 isolates) and time (6, 12, 18, and 24 hpi) as factors. Each combination contained three biological replicates totaling 48 samples. RNA-purification with the RNeasy Plant Mini Kit (Qiagen) was performed according to the manufacturer's protocol. RNA-seq libraries were prepared from an input of 4  $\mu$ g total RNA according to recommendations of the supplier (TruSeq RNA sample preparation v2 guide; Illumina). Libraries were quantified by fluorometry, immobilized and processed onto a flow cell with a cBot (Illumina), followed by sequencing-by-synthesis with TruSeq v3 chemistry on a HiSeq2000 system. Library construction and RNA sequencing were performed by the Max Planck Genome Centre Cologne and resulted in 20 to 60 million 100-bp-long reads per sample (total, 2 billion). Reads were mapped to the *Arabidopsis* genome (tair10) using TopHat and transformed into a count per gene per sample by using the BEDTools suite (function coverageBed). The RNA-seq data used in this study are deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE39463).

**Nuclear Fractionation and Protein Immunoblot Analyses.** Nuclear fractionation was performed as previously described (14). A 20-fold larger amount of the nuclear fraction compared with the soluble fraction on a per-tissue amount basis was subjected to immunoblot analysis as previously described (26). A single lane of the soluble fraction contains protein extract from 3 mg fresh weight leaf tissue. Anti-histone H3 (Abcam) and anti-phosphoenolpyruvate carboxylase antibodies (Rockland) were used as nuclear and cytosolic markers, respectively.

**Data Analysis.** Statistical analysis of epidermal single-cell death, invasive growth, and microcolony formation was performed by using R version 2.11.0 (<http://www.r-project.org/>). Data were evaluated by using ANOVA followed by Tukey–Kramer honestly significant difference test. A significance level of 0.01 was used for statistical analysis.

**Statistical Analysis of RNA-seq.** For the statistical analysis of the RNA-seq data, genes with fewer than 100 reads in all samples were discarded, and, subsequently, the count data of the remaining genes was log-transformed and normalized by the function voom from the R package limma to yield  $\log_2$  counts per million. To each gene, a linear model was fit by using function lmFit with the following terms: “virulence\_time + plant Genotype + pathogen isolate”. To find genes with patterns of differential expression in the virulence–time interaction, we used a moderated *t* test over seven contrasts. Six contrasts define the interaction between two time points and virulence, and the remaining contrast defines the pairwise comparison between avirulent and virulent samples at time point six (27). Resulting *P* values were adjusted for false discoveries caused by multiple hypothesis testing via the Benjamini–Hochberg procedure. In total, 4,694 genes have a significant virulence–time interaction as defined by the *t* test [false discovery rate (FDR) < 0.05]. The top 100 most significant genes were clustered by using complete linkage hierarchical clustering with the Pearson correlation as distance measure (Fig. S2). To identify the prevalent expression pattern among these genes, the obtained dendrogram was cut at height 0.6 to yield five clusters, and the largest of these clusters (87 genes) was chosen for visualization. To find Gene Ontology (GO) terms enriched among the genes in the largest cluster, we used the Generic GO Term Finder online tool (<http://go.princeton.edu/cgi-bin/GOTermFinder>). The largest cluster in Fig. S2 is most significantly enriched with genes categorized by GO as “response to chitin” (corrected  $P = 1.78 \times 10^{-55}$ , 44 of 87 genes). Gene expression is given as genewise standardized  $\log_2$ -transformed counts per million.

**ACKNOWLEDGMENTS.** We thank the Max Planck Genome Centre Cologne for RNA-seq and plant genotyping, Fumiaki Katagiri for seeds of the quadruple mutant, and Stefan Mauch and Petra Köchner for technical assistance. This work was supported by German Research Foundation in the Collaborative Research Centre SFB670 (to T.M., B.K., S.V., and P.S.-L.) and by the Max Planck Society (E.V.L.v.T. and P.S.-L.).

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