Supporting Online Material for

Nuclear Activity of MLA Immune Receptors Links Isolate-Specific and Basal Disease-Resistance Responses

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Published 21 December 2006 on Science Express
DOI: 10.1126/science.1136372

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Materials and Methods

Plant and fungal materials

1. Barley cultivars

Barley cultivar Golden Promise (GP) lacking *MLA* was employed in subcellular localization or functional experiments using MLA10 or *HvWRKY2* chromophore tagged variants. Barley cultivar Morex lacking functional *MLA* was employed for the BSMV VIGS experiments. Barley transgenic line 6E or 9E, harboring a functional single copy of *MLA1-HA* or *MLA6-HA* fusion constructs under control of their native 5’ upstream regulatory sequences respectively (1), were employed in the single-cell transient expression of *HvWRKY2* in various barley genetic backgrounds. *MLA1, MLA10, MLA12* are nearly isogenic barley lines in the genetic background of Pallas (P01, P09, P10 respectively), differing by one or more unique *MLA* specificities (Fig. 3B, 3C) (2). *MLG* is a backcross line in the genetic background of Pallas (3) was generated by seven backcrosses with cv Ingrid.

All barley seedlings were grown at 20°C with 16 h light/8 h darkness, 70% relative humidity in a protected environment.

2. Identification of Arabidopsis wrky insertion mutants and generation of double mutants

The *wrky18* line was derived from T-DNA insertion mutants identified in the GABI-Kat (line 328G03 in Col-0 background; *wrky18-1*) collection. The *wrky60* mutant was derived from the SALK collection (line 120706 in Col-0 background; *wrky60-1*). Individual homozygote lines were obtained by back-crossing and the positions of the insertions (intron 2 of *WRKY18* and exon 1 of *WRKY60*) were confirmed by PCR and sequencing. The *wrky40* mutant was derived from the SLAT collection of dSpm insertion lines in Col-0 background (4). The *wrky40*
mutant was identified within the seed pool SINS:01_02_60 by a PCR-based screen using 4 gene-specific primers as described at NASC (http://arabidopsis.info/info/slat_info1.html) and confirmed by sequencing. The individual wrky40 mutant was back-crossed several times and the position of the dSpm element within intron 1 was verified using gene-specific primers flanking the insertion site. No WRKY40 transcript was detected in the mutant upon 3 h treatment with salicylic acid, a strong inducer of WRKY40 expression in wild-type plants. The double mutant line wrky18/40 was generated by crossing backcrossed homozygote mutant lines wrky18-1 and wrky40 and by determining the genotype of the plants from the progeny by PCR analysis. Double mutants wrky18/60, wrky40/60 and wrky18/40/60 triple mutant are described in (5).

3. *Blumeria graminis* f sp *hordei* (*B. graminis*) fungal strains

Two *B. graminis* isolates used for barley inoculations contain the following relevant avirulence/virulence genes (*Avr*-Avirulence, *vir*-virulence):

- *B. graminis* isolate A6: *AvrMla6, AvrMla10, AvrMla12, AvrMlg; virMla1*
- *B. graminis* isolate K1: *AvrMla1; virMla6, virMla10, virMla12, virMlg*

*B. graminis* strains were maintained on barley plants kept at 20°C/18°C, 70% relative humidity, and 16 h light/8 h darkness after inoculation with *B. graminis* conidiospores.

4. *Golovinomyces orontii* fungus

The powdery mildew fungus *Golovinomyces orontii*, a virulent pathogen on Arabidopsis, was propagated on Col-0 wild-type plants at 20°C and 16 h light/8 h darkness, 80% relative humidity. Conidiospore inoculation of plants and disease phenotype scoring were done according to (6).

**Plasmids constructions**

Unless specified, the plasmids in this study were constructed by Gateway® technology (GW, Invitrogen) following the instructions of the manufacturer (http://www.invitrogen.com/content/sfs/manuals/gatewayman.pdf). Briefly, PCR
products containing inserts flanked by \textit{attB} sites were recombined into the vector pDONR201 (Invitrogen) by a BP reaction to create entry clones with \textit{attL} sites, and subsequently the inserts in the entry clones were recombined into the destination vector by a LR reaction to create the expression constructs.

\textbf{pUbi-MLA10-YFP}
Primer pairs: Sh068xSh069; destination clone: pUbi-GW-YFP. Sense primer (Sh068) 5'-'attB1-TCATGGATATTGTCACC GG C AT, antisense primer (Sh069) 5'-' attB2-GCATTAAATCGTCATCTTTGAGC A-3'.

\textbf{pUbi-MLA10-YFP-NES, pUbi-MLA10-YFP-nes}
PCR amplification of Mla10-YFP with primer pairs: MlaP12sxSh180 or MlaP12sxSh181 added ‘NES’ (NELALKLAGLDINK) or ‘nes’ sequences (NELALKAAAGADANK) to the products. Fragments were digested with SbfI and SacI and inserted into the vector pUbi-MLA10-YFP digested with the same endonucleases. Sense primer (MlaP12s) 5'-'CCACGCAGATATTGT TTGA-3', antisense primer (Sh180) 5'-GGACTGAGCTCTAC TTTGTTAATATCAAGTCCA GCCA CTTAAGAGCAAGCTCGTTTGTACAGCTCGTCCATGC-3', antisense primer (Sh181) 5'-GGACTGAGCTCTACTTTGTTAGCATCTGTGCTCCAGCTGCCT TAAGAGCAAGCTCGTTTGTACAGCTCGTCCATGC-3'.

To facilitate the cloning of multiple baits and preys for yeast two-hybrid analysis we converted the original \textit{pLexA} bait (7) and the \textit{pB42AD} prey vectors (CLONTECH, laboratories, Inc) into the appropriate Gateway® compatible destination \textit{pLexA-GW} and \textit{pB42AD-GW} vectors following the instructions of the manufacturer (detailed procedures or vectors are available upon request). The following constructs were made using GW technology and PCR amplification of appropriate templates:

\textbf{Bait and prey constructs:}
\textbf{pLexA-MLA (1-46)}
Primer pairs: Sh007xSh043. Sense primer (Sh007) 5’- attB1-TCATGGATATTGTCACCGGTGCCATTT-3’, antisense primer (Sh043) 5’- attB2-TCAGGCAGCGTTTCATGCTCTCAAG-3’.

pLexA-MLA1 CC-NB
Primer pairs: Sh007xSh015., antisense primer (Sh015) 5’- attB2-TCACCTTGAAAGAGATGGCATGA-3’.

pLexA-MLA6 CC-NB
Primer pairs: Sh007xSh015, same sequence as above.

pLexA-MLA6 full-length
Primer pairs: Sh007xSh012. Sense primer (Sh007) same sequence as above, antisense primer (Sh012) 5’- attB2-GTCAGTTCTCCTCTCGGCCCTA-3’.

pLexA-WRKY1
Primer pairs: Sh103xSh104. Sense primer (Sh103) 5’- attB1-ACATGGATCCATGGATGGGCAGCCAG-3’, antisense primer (Sh104) 5’- attB2-TTAATTGATGTCCCTGGT.

pLexA-WRKY2
Primer pairs: Sh100xSh101. Sense primer (Sh100) 5’- attB1-ACATGGAGGAGCAGTGATGATC-3’, antisense primer (Sh101) 5’- attB2-TCAAGCAACAGGGATCCGAC-3’.

pLexA-WRKY2 (107-319)
Primer pairs: Sh137xSh101. Sense primer (Sh137) 5’- attB1-ACCTCAGCCTCGTGTAAGGATG-3’, antisense primer (Sh101) same sequence as above.

pB42AD-WRKY1
Primer pairs: Sh103xSh104, same sequence as above.

pB42AD-WRKY2
Primer pairs: Sh100xSh101, same sequence as above.

pB42AD-WRKY2 (107-319)
Primer pairs: Sh137xSh101, same sequence as above.

pB42AD-WRKY2 (178-319)
Primer pairs: Sh111xSh101. Sense primer (Sh111) 5'- attB1-ACCTCAGCCTCGTGGTGAAGGATG-3', antisense primer (Sh101) same sequence as above.

pB42AD-MLA1 CC(1-166)
Primer pairs: Sh007xSh013. Sense primer (Sh007) same sequence as above, antisense primer (Sh013) 5'- attB2-TCACTCTGTCGCTTCAGCATA-3'.

pB42AD-MLA6 CC(1-166)
Primer pairs: Sh007xSh013, same sequence as above.

Expression plasmids in destination vector pUbi-GATE:

pUbi-WRKY1
Primer pair: Sh103x104, Sense primer (Sh103) 5'- attB1 ACATGGATCCATGGATGGGCAGCCAG-3', antisense primer (Sh101) 5'- attB2 TTAATTGATGTCCCTGGTC-3'.

pUbi-WRKY2
Primer pair: Sh100x101, sense primer (Sh100) 5'- attB1 ACATGGAGGAGCAGTGATGATGAC-3', antisense primer (Sh101) 5'- attB2 TCAAGCAACAGGGATCCGAC-3'.
pUbi-SUSIBA2
Primer pair: Sh123x124, sense primer (Sh123) 5’- attB1 ACATGTCCCCCGCGGCTGC-3’, antisense primer (Sh124) 5’- attB2 TCATGGACCCATGACCAAGT-3’; The cDNA sequence of SUSIBA2 was amplified from the pET-15b vector-based construct described elsewhere (8).

pUbi-YFP-WRKY2
Primer pair: Sh100xSh101, same sequence as above, destination vector: pUbi-YFP-GW.

pUbi-CFP-WRKY2
Primer pair: Sh100xSh101, same sequence as above, destination vector: pUbi-CFP-GW

pUbi-CFP-SUSIBA2
Primer pair: Sh123xSh124, same sequence as above, destination vector: pUbi-CFP-GW.

The two HvWRKY1- or HvWRKY2-BSMV silencing vector constructs were generated by PCR amplification of short antisense fragments of both genes (primer pairs Sh131xSh132 or Sh129xSh132), followed by sequential digestion with PacI and NotI and subsequent cloning into the vector BSMV.PDSas180 (9) predigested with the same endonucleases.

pBSMV-WRKY1as
Primer pairs Sh131xSh132. Sense primer (Sh131) 5’- CGCATGCGGCCGCGCTTGAGATGACGGGCTTG-3’, antisense primer (Sh132) 5’- CTGCATTAATACAGGTCACCGACGTGGTCA-3’.

pBSMV-WRKY2as
Primer pairs Sh129xSh132. Sense primer (Sh129) 5'-CGCATGCGGCCGACCTTGGAGACCCTGCTG-3', antisense primer (Sh130) 5'-CTGCATTTAATTAAGTCAGCGCATGATGGCG-3'.

pTNT-MLA1 CC(1-166)-HA
Used for expressing MLA1 CC(1-166)-HA in the TNT Coupled Wheat Germ Extract System (Promega, Mannheim). Prey vector pB42AD-MLA1 CC(1-166) was digested with EcoRI and XhoI, and the short fragment encoding the HA tagged fusion inserted into the EcoRI/XhoI digested pTNT vector.

pGEX-6p-1-WRKY2 (107-319)
Used for expression of GST-HvWRKY2 (107-319) fusion in E. coli. Primer pair SM27xSh119 was used to amplify the HvWRKY2(107-319) fragment and to generate the start codon as well as EcoRI and NotI flanking sites. PCR products were digested with EcoRI and NotI and inserted into EcoRI/NotI predigested vector pGEX-6p-1. Sense primer (SM27) 5'-GGAATTCCGGATGCTCAGCTCGTGGTGAAGGATGGG-3', antisense primer (Sh119) 5'-CGCATGCGGCCGCTCAAGCAACAGGGATCC-3'.

BSMV induced gene silencing
Barley Stripe Mosaic Virus (BSMV) mediated gene silencing was performed essentially as described (10, 11). pBluescript vectors harbored the individual BSMV subgenomes (ND18 strain) and derivatives (obtained from Large Scale Biology Corp. Vacaville, California, USA). Plants were grown in a growth-cabinet under constant conditions (16 h light /8 h dark, 18°C/25°C, 60% moisture, fluorescent light, 400 microEinstein light intensity). The first true leaves of 5 – 7 d old plants were infected with BSMV. Plants were further grown for 14 d to allow emergence of new leaves revealing viral symptoms. Segments of leaves #3 and #4 of the main shoot were collected, transferred to Benzimidazole agar plates and inoculated with powdery mildew spores (B. graminis strain A6) at low density. The fungus was allowed to develop for 48 h, and leaf segments subsequently
fixed in lactoglycerol, stained with coomassie brilliant blue, and microscopically analyzed (12). The plasmids harboring the BSMV-gamma derivatives, containing a ~300 bp insert of the target gene in antisense orientation following the stop codon of ORF gamma-b, were linearized with BssHII (HvWRKY2 with PciI) and used for silencing. These antisense fragments correspond to positions 296-557 of AJ536667 (HvWRKY1), positions 257-517 of AJ853838 (HvWRKY2) (Fig.S2) and positions 3400-3749 of AY270157 (TaLr10) (13).

**Single-cell transient gene expression assay**

Single-cell transient gene expression assays using biolistic delivery of plasmid DNA into plant epidermal cells was essentially done according to Shen et al., 2003 (12). Reporter plasmids containing the ß-glucuronidase (GUS) reporter gene and the respective effector plasmids were mixed prior to coating of the particles. In HvWRKY2 overexpression assays, we adopted a molar ratio of 1:1 (reporter : WRKY) and 3 µg of total DNA; or a molar ratio of 1:1:1 (reporter : HvWRKY : MLA) and 4.5 µg of total DNA. Bombarded leaves were transferred to 1% agar plates supplemented with 85 µM Benzimidazole and incubated at 18°C for 15 h before adequate inoculation with *B. graminis* spores. Leaves were stained for GUS activity 48 h post inoculation and kept in destaining solution. Single leaf epidermal cells challenged by *B. graminis* germlings were evaluated microscopically after the leaves were destained for at least 48 h. In the MLA-YFP fusion functional assay experiments, 1.5 µg of DNA was used for coating of the particles. For FRET experiments, we adopted a molar ratio of 1:3:1 (WRKY : MLA : AVR) and 3 µg of total DNA. Confocal imaging and FRET analyses were performed 36 h after bombardments.
Yeast two-hybrid, immunoblotting analyses, and yeast crude protein extraction

Yeast two-hybrid library screening and targeted analyses were performed essentially as described in (1). Briefly, the bait fusions were constructed by fusing respective *Mla* or *HvWRKY* sequences to the C-terminus of the LexA DNA binding domain in the pLexA-GW vector, and the prey fusions constructed by fusing sequences of *Mla* or *HvWRKY* to the C-terminus of the B42 activation domain in the pB42AD-GW vector. Using the LiAc method (14), both bait and prey plasmid DNA were co-transformed into yeast strain EGY48 that contains an autonomous plasmid (p8op-LacZ) carrying the LacZ reporter gene. Interaction analyses were done according to the user manual (MATCHMAKER LexA Two-Hybrid System User Manual, PT3040-1, CLONTECH, laboratories, Inc). For yeast crude protein extraction and protein immunoblotting, overnight yeast cultures were grown in SD selection media to the same OD and cells pelleted by centrifugation at 3,500 rpm. The yeast cell wall was disrupted and crude protein released by several rounds of freezing and boiling. 200 µl of 2x loading buffer with freshly supplemented DTT was directly added to the samples and stored at –20°C until used. Prior to protein immunoblotting analysis samples were boiled for 5 min and centrifuged for 5 min at 13,000 rpm. 20 µl of supernatant/well was loaded onto the gel and detection achieved with an anti-LexA antiserum (Santa Cruz Biotechnology, CA).

*In vitro* GST pull-down assay

DNA fragment encoding MLA1 (1-166)-HA was cloned into the pTNT Vector and expressed in TNT Coupled Wheat Germ Extract System (Promega, Mannheim) according to manufacturers instruction. DNA fragment encoding *HvWRKY2* (107-319) was cloned into the pGEX-6p-1 vector and expressed as a GST N-terminal fusion in *E.coli* strain Rosetta. The empty vector expressing GST alone was used as control. We purified GST-*HvWRKY2* (107-319) and GST alone on a glutathione matrix (Amersham Pharmacia, Freiburg) using lysis buffer (50mM
Tris pH 7.6; 150mM NaCl; 10% glycerol; 0.1% Tween-20; 1mM DTT, 1x protease inhibitor cocktail complete (Roche, Mannheim)). 100 µg of matrix bound GST or GST-HvWRKY2 (107-319) was incubated with varying amounts of MLA1(1-166)-HA (10µl, 25µl, 50µl, 100µl, 150µl; diluted to 1ml with 50mM HEPES pH 7.5; 150mM NaCl; 10mM EDTA; 1x protease inhibitor cocktail complete) expressed in wheat germ extract for two hours. The matrix was then spun down and washed three times with HEPES buffer. The matrix was loaded onto the gel and subjected to immunoblotting using monoclonal anti-HA antibody (clone 3F10, Roche Mannheim). Protein was detected by Ponceau S red staining.

Note: 50µl of MLA1 (1-166)-HA was used together with 100 µg GST or GST fusions in figure 2B. 10µl of each total incubate was used as input.

**Real-time PCR**

The first true leaves of seven day-old barley plants were detached and kept on Benlimidazole agar plates for 24h, and subsequently inoculated with high density *Bgh* conidiospores. Leaf samples were taken at 0, 3, 6, 9 and 12 hpi, RNA extracted and cDNA prepared as described previously (1). For experiments with flg22, seven day-old barley plants were sprayed with 1µM flg22 solution (+0.04% Silwet) or mock treated. Samples were taken at 0, 0.5, 1, 2 and 3 hours after treatment. Real-time PCR was performed using ABI Prism 7700 Real Time System according to the manufacturer’s instruction. Primers used for actin (1), SM44xSM45 (Chitinase 2a), SM61xSM62 (*HvWRKY1*), SM67xSM68 (*HvWRKY2*); Sense primers: (SM44) 5’- CTACACGTACGACGCCTTCA-3’; (SM61) 5’- ATCCCTGAGCCTCGACCT-3; (SM67) 5’- AACAACCACCACCAGCTCGTT-3’; antisense primers: (SM45) 5’- AGTTGGACCGCCCTGTCGA-3’; (SM62) 5’- GACTCAAGAACCCTCACCTCA-3’; (SM68) 5’-TCACCTTCTGCCCCTACTTC-3’.
Microarrays

Aerial parts of three week-old Arabidopsis Col-0 plants, grown on double-autoclaved soil in a phytochamber under long day conditions at 20°C and 70% humidity, were vacuum-infiltrated for 2 min with a \textit{P. syringae} DC3000 suspension (in 10 mM MgCl$_2$) at a bacterial density of $10^7$ cfu/mL. Control plants were infiltrated with 10 mM MgCl$_2$ solution. Samples were harvested 6 h post infiltration, frozen immediately in liquid nitrogen, and RNA was prepared by first grinding frozen tissue using a mortar and pestle and subsequently using the RNAwiz kit (Ambion Inc.) following the instructions of the manufacturer. RNA was further purified using the RNeasy kit (Qiagen) and included a DNase digestion step as recommended in the instructions. Processing of the RNA, ATH1 GeneChip (Affymetrix) hybridization and raw data collection were performed by the NASC International Affymetrix Service Unit (NASC, Nottingham, UK; http://affymetrix.arabidopsis.info/). Detailed information on the experiment including raw data are available at the NASC website under the NASCArrays reference number 398.

Nuclear fractionation and protein immunoblot analyses

Nuclear fractionation was performed essentially as previously described (15) with the following modifications. The same amounts of fresh weight of different leaf tissue samples were homogenized in Honda buffer (2.5% Ficoll 400, 5% dextran T40, 0.4 M sucrose, 25 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 1 mM DTT, 1 mM PMSF, and 1 x complete protease inhibitor cocktail) using a mortar and pestle, and then filtered through a 62 µm pore-size nylon mesh. After Triton X-100 addition to a final concentration of 5%, the homogenate was incubated on ice for 15 min and centrifuged at 1,500 g for 5 min. The supernatant fraction was saved (soluble fraction) and the pellet was subsequently reprocessed to further purify the nuclear fraction as previously described (15). A 16-fold larger amount of the nuclear fraction compared to the soluble fraction was subjected to immunoblot
analysis as described previously (1). A single lane of the soluble fraction contains protein extract from 10 mg fresh weight leaf tissue.

Anti-histone H3 (Abcam) and anti-HSP90 antibodies (Santa Cruz Biotechnology, CA) were used as nuclear and cytosolic markers, respectively.

Confocal imaging, FLIM-FRET, and APB-FRET analyses

The setup used for the lifetime measurements is depicted in Fig. S5. In all experiments the sample was scanned by a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss GmbH, Jena, Germany). A mode locked Ti:Sapphire laser (Chameleon, Coherent GmbH, Dieburg, Germany) whose emission wavelength was tuned to 860 nm was used for two-photon excitation of the CFP chromophore. The laser beam was attenuated by an accusto-optic modulator (AOM) and directed to the scan head of the laser-scanning microscope. Fluorescence light originating from the sample was directed by a dichroic mirror (KP680) towards the NDD port of the microscope and reflected onto a multichannel-plate photomultiplier (MCP-PMT, 3809U-52, Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany) for time-correlated single photon counting (TCSPC). The MCP-PMT was connected to a TCSPC computer module (SPC730, Becker&Hickl GmbH, Berlin, Germany), which was used to build up a three-dimensional histogram of photon density over spatial (x,y) and temporal (t) coordinates. The actual position (x,y) of the scanning beam was derived from the FrameSync, LineSync, and PixelSync signals of the laser-scanning microscope. TCSPC data were analyzed with a commercial software package (SPCImage V2.8, Becker&Hickl) and our own software. Both approaches use the iterative reconvolution method to recover the fluorescence lifetime from fluorescence decay profiles obtained for each pixel. The best parameters of a given decay are approximated by a modified Levenberg-Marquardt algorithm. The quality of a fit was judged by the value of a reduced $\chi^2$. A model was rejected, when $\chi^2$ exceeded a value of 1.5.
To exclude artifacts that might have been caused by photo-induced reactions, repetitive measurements were carried out. Fluorescence decay curves obtained in two subsequent experiments could be superimposed (data not shown), excluding any photoinduced changes that might have affected the lifetime measurement.

For acceptor photobleaching experiments (APB-FRET) (16) CFP fluorescence was excited with the 458 nm line of the argon laser and recorded in one of the confocal channels using a 480-520 nm bandpass filter. YFP fluorescence was excited with the 514 nm line of the argon laser and detected between 527-570 nm. To minimize cross talk between the channels, images were scanned sequentially switching laser lines and emission filters accordingly. Acceptor photobleaching was performed within a region of interest (ROI, red circle in Fig S7) using the 514 nm laser line at 100% intensity and with 60 scans at 1.6 µs pixel time. CFP and YFP fluorescence images were taken before and after the YFP bleaching procedure to assess changes in donor and acceptor fluorescence intensity. CFP and YFP fluorescence intensities in the ROI were averaged and plotted as a function of time.
Supporting Online Figures

**Fig. S1.** Bait fusions for yeast two-hybrid analyses accumulate in yeast.

Bait fusions of the LexA DNA binding domain with the indicated MLA1/6 domains or full-length MLA6 were expressed in yeast. Overnight cultures were harvested and yeast cell walls were disrupted as described in supporting Methods. Equal amounts of protein extracts were subjected to immunoblot analysis using a LexA-specific antiserum (α-LexA).

**Fig. S2.** *HvWRKY1/2* homologs from monocots and dicots are highly sequence related and share common domains/motifs.

Deduced amino acid sequences of *HvWRKY1/2* and putative wheat *TaWRKY1*, *OsWRKY28/71*, and *AtWRKY18/40/60* were aligned using the Clustal W software. Highly conserved residues are shaded in black, similar residues in gray. The dashed red line marks a polymorphic region between *HvWRKY1* and *HvWRKY2* whose corresponding DNA sequences were engineered into BSMV vector in antisense orientation for VIGS silencing experiments. (LxLxL(I), putative repressor motif; LZ, Leucine zipper motif; NLS, nuclear localization signal; WRKY, WRKY DNA-binding domain)

**Fig. S3.** *HvWRKY1/2* are pathogen and flg22 responsive.

Time course analysis of *HvWRKY1* and *HvWRKY2* transcripts in barley leaves upon inoculation with *B. graminis* or treatment with the flg22 peptide. Transcript levels were determined by real-time PCR using *HvWRKY1* or *HvWRKY2*-specific PCR primers. RNA was extracted from detached leaves of seven day-old barley seedlings and cDNA was prepared as described (1). Representative data of one of three independent real-time PCR experiments are shown. Transcript levels obtained from PCR primers specific for barley actin (1) at time point zero were used as control for normalizing *HvWRKY1* and *HvWRKY2* transcripts.
A. *HvWRKY1* and *HvWRKY2* leaf expression profiles upon challenge with *B. graminis* isolate K1. Leaf samples were taken at the indicated time points from cultivar Golden Promise (GP, compatible interaction) or an *MLA1-HA* transgenic line in GP background (*MLA1-HA* GP, incompatible interaction; (1)).

B. *HvWRKY1* and *HvWRKY2* leaf expression profiles upon treatment with flg22. Leaf samples were taken at the indicated time points from mock-treated or flg22-treated cultivar Golden Promise (GP).

**Fig. S4.** MLA10-YFP co-localizes with CFP-*HvWRKY2* in epidermal nuclei.

A. Confocal image of a barley epidermal cell co-expressing MLA10-YFP and CFP-*HvWRKY2*. The arrowhead points to the nucleus of the cell. The scale bar represents 10 µm.

B. Haustorium index in leaf epidermal cells expressing empty DNA vector (EV) or the indicated plasmid constructs in an *MLA1*-containing barley cultivar subsequent to inoculation with the *AVR A1*-expressing *B. graminis* isolate K1. The haustorium index was microscopically scored at 48 h post inoculation.

**Fig. S5.** FLIM-FRET setup

Scheme of FLIM-FRET setup used in this study. See Supporting Online Material for a detailed description.

**Fig. S6.** *HvWRKY2* and MLA10 association is *AVR A10*-dependent.

A to E. Fluorescence lifetime measurements in barley epidermal nuclei expressing the indicated protein(s). See Fig. 4 legend for a description of the respective panels.
F. Bar graph of CFP fluorescence lifetimes observed in all measured nuclei expressing the indicated protein(s). Mean values are indicated by the yellow bars (mean ± S.E.M.). Grey bars represent the minimal (front) and maximal values (black) observed in all experiments. Only cells subjected to co-delivery of CFP-\textit{HvWRKY2}, MLA10-YFP, and the \textit{B. graminis} AVR\textsubscript{A10} effector that is recognized by MLA10, produced a broad CFP lifetime distribution not seen in any other tested combinations, ranging from 1.32 ns up to 2.17 ns. The marked reduction of CFP lifetime is indicative of close, AVR\textsubscript{A10}-stimulated associations between MLA10 and \textit{HvWRKY2}.

\textbf{Fig. S7}. FRET analysis by acceptor photo bleaching.

\textbf{A}. Confocal images of the nucleus before and after bleaching of the acceptor fluorophore. The indicated donor (CFP-\textit{HvWRKY2} fusions) and potential acceptor proteins (free YFP or MLA10-YFP fusion) were co-expressed in epidermal cells upon biolistic delivery of the corresponding plasmids into barley epidermis. An increase in donor fluorescence could be observed after photobleaching of the acceptor (second row) in cells co-expressing the MLA10-YFP acceptor and the cognate \textit{B. graminis} effector AVR\textsubscript{A10}. Such an increase of CFP fluorescence was not observed in the absence of the MLA10-YFP acceptor (first row) or in nuclei co-expressing the effector AVR\textsubscript{K1} (last row).

\textbf{B}. Time course of donor and acceptor fluorescence intensities in the region of interest (marked by the red line around the nucleus) throughout the bleaching process. Acceptor bleaching was performed between time point 2 and 3 (corresponding to 12 to 34 seconds).
**Fig. S8.** Nuclear activity of MLA immune receptors link race-specific and basal immune responses.

A. Schematic representation of PAMP-triggered basal defense responses during a compatible interaction with *B. graminis*. One or several PAMP receptors are thought to initiate PAMP signalling via intracellular MAPK cascades, which in turn stimulate the induction of unknown WRK complex transcriptional activators (green color) and *HvWRKY1/2* repressors (red color). The WRK complex repressors are thought to restrict the output of PAMP-triggered basal defense gene expression below a detrimental threshold and might prevent a ‘chronic inflammatory state’ of attacked host cells. Inactive MLA immune receptors are assumed to continuously cycle between nucleus and cytoplasm. The dashed oval denotes the nuclear envelope. PM denotes host plasma membrane.

B. Integrated PAMP- and MLA-triggered immune response during an incompatible interaction with *B. graminis*. Perception of a *B. graminis* AVRα isolate-specific effector by MLA stimulates nuclear association between receptor and *HvWRKY1/2* repressors, thereby de-repressing PAMP-triggered immune responses. De-repression of basal defense responses are thought to potentiate expression of defense-related genes (bold arrow) and might drive attacked host cells into cellular suicide. The activated MLA immune receptor is denoted by red color. Whether AVRα is directly or indirectly recognized by the cytoplasmic and/or nuclear MLA pool remains unknown. The dashed oval denotes the nuclear envelope. PM denotes host plasma membrane.
Fig S1

LexA fusions

α-LexA

MLA1-CC(1-166)
LexA
MLA6-CC(1-166)
MLA7 CC-NB
MLA6 CC-NB
MLA6 full-length

96 kD
52 kD
36 kD
29 kD
Fig S2

LxLxL(I) putative repressor motif
LZ
NLS
WRKY

sequences for VIGS
Fig S3

A

B
**Fig S4**

**A**

MLA10-YFP + CFP-HvWRKY2

**B**

<table>
<thead>
<tr>
<th>Transgene</th>
<th>MLA1</th>
<th>AVR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MLA1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CFP-WRKY2</td>
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**Fig. S6**

<table>
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<tr>
<th>Lifetime image</th>
<th>Fluorescence decay</th>
<th>Lifetime distribution</th>
<th>Lifetime histogram</th>
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<tr>
<td><strong>A</strong> CFP-WRKY2 YFP-WRKY2</td>
<td><img src="image" alt="Fluorescence decay" /> ( \tau = 1.07 \text{ ns} )</td>
<td><img src="image" alt="Lifetime distribution" /></td>
<td><img src="image" alt="Lifetime histogram" /></td>
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<tr>
<td><strong>B</strong> CFP-WRKY2 YFP</td>
<td><img src="image" alt="Fluorescence decay" /> ( \tau = 2.08 \text{ ns} )</td>
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<td><img src="image" alt="Lifetime histogram" /></td>
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<td><strong>C</strong> CFP-SUSIBA2 MLA10-YFP AV</td>
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<td><img src="image" alt="Lifetime distribution" /></td>
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<td><strong>D</strong> CFP-SUSIBA2 MLA10-YFP AVRK1</td>
<td><img src="image" alt="Fluorescence decay" /> ( \tau = 2.56 \text{ ns} )</td>
<td><img src="image" alt="Lifetime distribution" /></td>
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<td><strong>E</strong> CFP-SUSIBA2 MLA10-YFP AV</td>
<td><img src="image" alt="Fluorescence decay" /> ( \tau = 2.40 \text{ ns} )</td>
<td><img src="image" alt="Lifetime distribution" /></td>
<td><img src="image" alt="Lifetime histogram" /></td>
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**F**

![Fluorescence lifetime (\( \tau \))](image)
Fig S7

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<td><img src="image9" alt="Image" /></td>
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<td><img src="image11" alt="Image" /></td>
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- **ROI**
- **MLA10-YFP**
- **CFP-WRKY2**
Fig. S8

A

PAMP receptor

PAMP

PM

MAPKKK

MAPKK

MAPK

WRKY

WRKY 1/2

defense genes

MLA

MLA

B

PAMP receptor

PAMP

PM

MAPKKK

MAPKK

MAPK

AVR

MLA

MLA

defense genes

MLA

MLA

25
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<th>AGI number</th>
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<th>10&lt;sup&gt;7&lt;/sup&gt; cfu/ml &lt;i&gt;P. syringae&lt;/i&gt; DC3000&lt;sup&gt;1&lt;/sup&gt;</th>
<th>fold-change</th>
<th>flg22-induced&lt;sup&gt;2&lt;/sup&gt;</th>
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<sup>1</sup>Col-0 leaf material was harvested 6h post infiltration
Filter: raw value with a signal >100 in at least one experiment
Detailed information of the experiment and raw data are available at the NASC website under the NASCArrays reference number-398.

<sup>2</sup>AtGenExpress (http://www.arabidopsis.org/info/expression/atgenexpress.jsp )1h + 4 h flg22 (1µM) infiltrated leaves, and Cell 2006 125: 749-760.
Supporting Online References