New Phytologist Supporting Information

Article title: Comparing Arabidopsis receptor kinase and receptor protein-mediated immune signaling reveals BIK1-dependent differences

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Methods S1 Supplemental Methods
Fig. S1 Time course of flg22 and nlp20-triggered membrane depolarization. Leaves of Arabidopsis Col-0 wild type plants were treated with 100 nM nlp20 or 10 nM flg22, and changes in membrane depolarization ($\Delta V$) were monitored continuously. The response to flg22 was generally higher than nlp20, but the difference was not statistically significant.
Fig. S2 Spatiotemporal analysis of calcium responses to nlp20 and flg22. Kymograph analysis of R-GECO1 signal intensities upon root treatment with 10 μM nlp20 (a) or 10 μM flg22 (b). Signal intensities are normalized for the average signal intensity of the baseline (25 frames). The cartoon above illustrates the different developmental zones of the root along the spatial (horizontal) axes of the kymographs. M, meristem; TZ, transition zone; EZ, elongation zone; DZ, differentiation zone. The calibration bar on the right indicates signal intensities with blue color indicating low and red color indicating high intensity.
**Fig. S3** Time course of flg22 and nlp20-triggered ROS production and MAPK activation.

Arabidopsis leaf discs were treated with flg22 or nlp20 (100 nM or 1 µM), or water as control (mock), and ROS production (a) or MAPK activation (b) was monitored over time as described in Fig. 1. Bars in (a) present means ± SD (n≥6) of relative fluorescence units (RLU). (c) Arabidopsis wild-type seedlings were treated with water or 0.5 µM nlp20 or flg22 for 1 and 6 hours, and isolated RNA was subjected to RNA sequencing as described in Fig. 2. Given are the fold changes (Log2) of MLO12 transcript levels compared the water control. For qRT-PCR, leaves of Arabidopsis wild type plants were infiltrated with water (mock), 0.5 µM flg22 or 5 µM nlp20, and collected 6 hours after treatment. Relative expression of the MLO12 gene was normalized to the levels of EF-1α transcript and calibrated to the levels of mock treatment.
**Fig. S4** Immune responses triggered by RK-ligands flg22 and elf18 compared to RP-ligands nlp20 and PG3.

(a) Arabidopsis leaf discs were treated with 100 nM flg22, elf18, nlp20, or PG3, or water as control (mock), and ROS production was monitored over time. Bars present means ± SD (n=6) of relative luminescence units (RLU). (b) Arabidopsis leaf discs were treated with flg22, elf18, nlp20, or PG3, or water as control (mock), and ethylene production was measured at 3 and 6 hours post incubation. Bars present means ± SD (n=3). For each time point, different letters mean significant differences (P<0.05) by Student’s t-test. (c) PR1 expression by qRT-PCR. Arabidopsis leaves were infiltrated with water (mock) or 500 nM flg22, elf18, nlp20, or PG3 and harvested 6 hours post infiltration. Transcript levels of PR1 were normalized to the levels of EF-1α and calibrated to the levels of mock treatment. Bars present means ± SD (n=3). Different letters mean significant differences (P<0.05) by Student’s t-test. (d) Callose production in Arabidopsis leaves infiltrated with water (mock), each 1 µM flg22, elf18, nlp20, or PG3. Callose deposition was visualized with aniline blue 16 hours after infiltration and evaluated with fluorescence microscopy (left panel). Scale bars, 500 µm. The diagram (right panel) depicts the amount of callose deposits and the bars present means ± SD from 5 pictures. Different letters mean significant differences (P<0.01) by Student’s t-test. (e) CYP71A13A and PAD3 expression by qRT-PCR. Arabidopsis leaves were infiltrated with water (mock), each 0.5 µM flg22, elf18, nlp20, or PG3 and harvested 6 hours post infiltration. Transcript levels were normalized to the levels of EF-1α and calibrated to the levels of mock treatment. Bars present means ± SD (n=3). Different letters mean significant differences (P<0.05) by Student’s t-test. These data were extracted from Fig. 6b and are shown as separate panel here.
Fig. S5 Time course of flg22 and nlp20-triggered ROS production in bak1 and bik1 mutant lines. Arabidopsis leaf discs of the indicated bak1 (a, b) or bik1 (b, c) mutant lines were treated with water (mock), 500 nM flg22 (a, c) or 500 nM nlp20 (b, d) and ROS production was monitored over time as described in Fig. 1. Data present means ± SD (n≥6) of relative fluorescence units (RLU).
Fig. S6 Flg22 and nlp20-triggered ROS production in bik1 and sid2 mutant lines.

Leaf pieces of wild-type, bik1, sid2, or bik1 sid2 plants were treated with water, 500 nM flg22, or 500 nM nlp20, and ROS accumulation was determined as in Fig. 1. The composite 15 independent experiments with each point representing the mean (n=8) total ROS production over 1 h. The mean total ROS production (relative fluorescence units, RLU) for the set of experiments is indicated by a horizontal line. Within each treatment, different letters indicate significant differences (P<0.05) using Student's t-test for all possible individual comparisons.
Fig. S7 Indole glycosinolate levels remain unchanged upon flg22 and nlp20 treatment. 
(a) Levels of the indole glycosinolate indol-3-ylmethyl glucosinolate (I3M) were determined in leaves infiltrated with 1 µM flg22 or nlp20 (also 0.1 µM for 48 hours), or water (mock) and harvested after 12 and 48 hours. Bars (nmol I3M/g fresh weight) present average values ± SD (n = 2). (b) Arabidopsis wild-type seedlings were treated with water or 0.5 µM nlp20 or flg22 for 1 and 6 hours, and isolated RNA was subjected to RNA sequencing. Given are the fold changes (log2) of CYP81F2 transcript levels compared the water control.

![Graph](image)

| Treatment | 12 hr | 48 hr |
|-----------|-------|-------|
| mock      |       |       |
| flg22 1 µM|       |       |
| nlp20 1 µM|       |       |

| Time | CYP81F2 transcript levels |
|------|---------------------------|
|      | flg22 | nlp20 |
| 1h   | 7.78  | 6.99  |
| 6h   | 5.58  | 5.59  |
### Table S1 Arabidopsis thaliana mutant and transgenic lines used in this study.

| Line Name | Locus | Description | Ref. |
|-----------|-------|-------------|------|
| rlp23-1 (SALK_034225) | At2g32680 | insertion | Albert et al. (2015) |
| sobir1-12 (SALK_050715) | At2g31880 | insertion | Gao et al. (2009) |
| bak1-4 (SALK_116202) | At4g33430 | insertion | Heese et al. (2007) |
| bak1-5 | At4g33430 | substitution (C408Y) | Schwessinger et al. (2011) |
| bak1-4/BAK1 | pBAK1:BAK in bak1-4 | | Heese et al. (2007) |
| bak1-4/BAK1_Y403F | pBAK1:BAK_Y403F in bak1-4 | | Perraki et al. (2018) |
| bak1-4/BAK1_S602/3/4AAA | pBAK1:BAK_S602/3/4AAA in bak1-4 | | Perraki et al. (2018) |
| bak1-4/BAK1_S612A | pBAK1:BAK_S612A in bak1-4 | | Perraki et al. (2018) |
| bir2-1 (GK-793F12) | At3g28450 | insertion | Halter et al. (2014) |
| pp2a-a1 (SALK_059903) | At1g25490 | insertion | Segonzac et al. (2014) |
| pp2a-cd (SALK_035009) | At3g58500 | insertion | Segonzac et al. (2014) |
| cpk28-1 (GK_523B08) | At5g66210 | insertion | Monaghan et al. (2014) |
| bik1 (SALK_005291) | At2g39660 | insertion | Lu et al. (2010) |
| pbl1 (SAIL_1236_D07) | At3g55450 | insertion | Zhang et al. (2010) |
| bik1 pbl1 | double mutant | | Zhang et al. (2010) |
| xlg2-1 (SALK_062645) | At4g34390 | insertion | Ding et al. (2008); Liang et al. (2016) |
| agg1 agg2 | At3g63420/At3g22942 | backcrossing agg1-1w with Col-0 to obtain agg1-1c, crossing agg1-1c with agg2-1 to obtain double mutant | Trusov et al. (2007); Liang et al. (2016) |
| bik1/BIK_K105E | pBIK1:BIK1_K105E:HA bik1 | | Lin et al. (2014) |
| bik1/BIK1_Y243F | pBIK1:BIK1_Y243F:HA bik1 | | Lin et al. (2014) |
| bik1/BIK1_Y150F | pBIK1:BIK1_Y150F:HA bik1 | | Lin et al. (2014) |
| bik1/BIK1_Y250F | pBIK1:BIK1_Y250F:HA bik1 | | Lin et al. (2014) |
| bik1 sid2 | At2g39660/At1g74710 | double mutant | Laluk et al. (2011) |
Table S2 GO term list of RNA-seq data from Arabidopsis thaliana treated with flg22 or nlp20. Singular Enrichment Analysis (SEA) was perform by agriGO for transcripts differentially expressed after 0.5 μM flg22 or 0.5 μM nlp20 treatment for 1 h (a-f) and 6 h (g-k). GO terms with FDR ≤ 0.5 are shown. Analysis of 1,638 transcripts up-regulated only by flg22 (a). Analysis of 22 transcripts up-regulated only by nlp20 (b). Analysis of 1,492 transcripts up-regulated by both flg22 and nlp20 (c). Analysis of 1,892 transcripts down-regulated only by flg22 (d). Analysis of 20 transcripts down-regulated only by nlp20 (e). Analysis of 139 transcripts down-regulated by both flg22 and nlp20 (f). Analysis of 1,699 transcripts up-regulated only by flg22 (g). Analysis of 116 transcripts up-regulated only by nlp20 (h). Analysis of 1,184 transcripts up-regulated by both flg22 and nlp20 (i). Analysis of 2,651 transcripts down-regulated only by flg22 (j). Analysis of 17 transcripts down-regulated only by nlp20 (k). Analysis of 140 transcripts down-regulated by both flg22 and nlp20 (l). P: Biological process. F: Molecular function. C: Cellular component. BG/Ref: Background/Reference. FDR: False Discover Rate. Relation and significant levels of GO terms are shown as figures with significant levels and arrow types diagram.

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Table S3  Examples of genes specifically upregulated by flg22 or nlp20 categorized by GO terms.

| 1-hr specifically up-regulated by flg22 | 6-hr specifically up-regulated by flg22 | 6-hr specifically up-regulated by nlp20 |
|----------------------------------------|----------------------------------------|----------------------------------------|
| defense response                       | defense response                       | response to ethylene                    |
| AT3G53260 ATPAL2                       | AT3G52430 PAD4                         | AT2G05520 ATGRP-3                       |
| AT3G45290 ATML03                       | AT1G64280 NPR1                         | AT5G19880                               |
| AT2G17480 ATML08                       | AT3G48090 EDS1                         | AT5G40990 GUP1                          |
| AT5G56030 HSPR1.2                      | AT5G04720 ADR1-L2                      | AT3G23230 ATERF98                       |
| immune response                        | immune response                        | response to reactive oxygen species     |
| AT4G12010 DSC1                         | AT5G51700 PBS2                         | AT4G26070 ATMEK1                        |
| AT5G46520 ACCQ05                       | AT4G26090 RPS2                         | AT3G12580 ATHSF70                       |
| AT5G64900 ATPEP1                       | AT3G07040 RPM1                         | AT2G26150 ATHSFA2                       |
| AT1G66340 ETR1                         | AT1G38950 BHP                          |                                        |
| regulation of signal transduction      | AT5G60410 ATSI21                       |                                        |
| AT2G22430 ATHB6                        | AT3G02140 TMA2                         |                                        |
| AT3G04580 EIN4                         | AT3G45640 MPK3                         |                                        |
| protein modification process           | cellular protein catabolic process     |                                        |
| AT4G05320 UBOQ10                       | AT5G58290 RPT3                         |                                        |
| AT5G07460 ATMSRA2                      | AT4G05050 UBOQ11                       |                                        |
| AT1G10560 PUB18                        | AT4G38630 RPN10                        |                                        |
| AT2G39550 PGTI                         | AT4G31300 PBA1                         |                                        |
|                                        | protein modification process           |                                        |
|                                        | AT5G24240 MOP9.5                       |                                        |
|                                        | AT4G05050 UBOQ11                       |                                        |
|                                        | AT4G35310 CPN5                         |                                        |
|                                        | AT3G62260 PP2C                         |                                        |

Video/Movie S1  Time-lapse recording of cytoplasmic Ca$^{2+}$ elevations in an R-GECO1-expressing root treated with flg22.

Video/Movie S2  Time-lapse recording of cytoplasmic Ca$^{2+}$ elevations in an R-GECO1-expressing root treated with nlp20.
Methods S1: Supplemental Methods

Plant Material

Arabidopsis plants were grown on soil or half-strength Murashige and Skoog (MS) medium as described (Brock et al., 2010). Plants were grown in climate chambers under short-day conditions (8 h : 16 h, light : darkness, 150 μmol/cm²s white fluorescent light, 40-60 % humidity, 22 °C). All mutants used in this study are in Arabidopsis thaliana accession Col-0 background (listed in Table S1).

Elicitors

Flg22, nlp20 and elf18 peptides were synthesized according to the published sequences (Felix et al., 1999; Kunze et al., 2004; Böhm et al., 2014b) by Genscript Inc., prepared as 10 mM stock solutions in ddH₂O, and diluted in ddH₂O prior to use. Botrytis cinerea PG3 was purified from culture filtrates of Pichia pastoris as described previously (Kars et al., 2005).

Ion Flux Measurements

Membrane potential recordings were performed in 5-7-week-old plants. One day before measurements leaves were detached, glued to the base of a chamber (adaxial site), peeled off (abaxial epidermis) and left for recovery overnight in a standard solution containing 0.1 mM KCl, 1 mM CaCl₂ and 5 mM MES adjusted to pH 5.5-5.7 with Tris. During experiments, exposed tissue was constantly perfused with the standard solution (2 ml/min); elicitors were applied for 2 min. For impalements, microelectrodes from borosilicate glass capillaries with filament (Hilgenberg, Malsfeld, Germany) were pulled on a horizontal laser puller (P2000, Sutter Instruments Co, Novato, CA, USA). They were filled with 300 mM KCl and connected via a Ag/AgCl half-cell to a headstage (Axon Inst., Union City, CA, USA) (Scherzer et al., 2015). The tip-resistance was about 20-50 MΩ, while the input resistance of the headstage was 10¹³ Ω. The cells were impaled using an electronic micromanipulator (MM3C, Kleindiek Nanotechnik, Germany). All recordings were amplified with an IPA-2 amplifier (Applicable Electronics, Inc., Forestdale, MA, USA) and stored on a PC (WinEDR software; Windows Electrophysiology Disk Recorder).

Calcium Detection

Polyamide meshes (4 x 1.5 cm, Th. Geyer) with a mesh size of 100 μm were placed on half-strength MS agar plates. Sterile seeds (R-GECO1 (Keinath et al., 2015)) were positioned in a thin row on the upper part of the meshes and grown in an upright position under long day conditions (16 h of light at 21°C, 8 h dark at 18°C). Seedlings were imaged 7-10 days after germination by taking the meshes and placing them in a 1-well chamber on coverglass filled with 2 mL 1/2 MS medium. Treatments were applied by replacing the 1/2 MS medium with 1/2 MS medium containing 10 μM nlp20 or flg22. Imaging of R-GECO1 fluorescence was performed with a time interval of 1 s on a Nikon SMZ18 stereo microscope equipped with an ORCA-Flash 4.0 V2 sCMOS camera (Hamamatsu, Japan), using a filter-set for red fluorescence excitation and detection (excitation: ETS 545/25x; emission: ET605/70m).
**Callose Deposition**

To visualize callose apposition, leaves of 5-week-old Arabidopsis plants were infiltrated with water or the indicated peptides and stained with aniline blue after 24 hours as described (Wang et al., 2009). Pictures were taken with an inverted microscope (Nikon ECLIPSE 80i), then adjusted and analyzed using Photoshop CS5. Quantification of callose was performed by counting dark pixels selected by Magic tool and calculated in % relative to the total pixels of the image.

**Ion Leakage Assay**

Ion leakage assays were performed as described (Lenarčič et al., 2017). Leaves of 5 to 6-week-old Arabidopsis plants were infiltrated with ddH2O or 500 nM PG3. After 10 min of infiltration, leaf discs (Ø 7 mm) were punched out and transferred into a 24-well plate. Two leaf pieces per well were floated on 1 mL ddH2O and shaken at 50 rpm. After 30 min of incubation, leaf pieces were transferred to fresh ddH2O and conductivity was measured at the times indicated using a conductivity meter (QCond2200).

**Indole glucosinolate glucobrassicin determination**

Leaves of 5-week-old Arabidopsis plants were infiltrated with peptide solution or ddH2O. For analysis of I3M (glucobrassicin) 200 mg of fresh plant leaves were harvested and homogenized in liquid nitrogen. Extraction of the analytes was carried out with 500 µl 80 % methanol containing 0.1 % formic acid, followed by a second extraction with 500 µl 20 % methanol containing 0.1 % formic acid. Both supernatants were combined and dried in a speed vac. For analysis with a Waters Acquity UPLC – SynaptG2 LC/MS system the samples were redissolved in 100 µl 20 % methanol containing 0.1 % formic acid. 5 µl were injected onto a Water Acquity HSS T3 reverse phase column. Separation was carried out with a linear 10 min 99 % water to 99% methanol (both solvents containing 0.1 % formic acid) gradient. For detection, the mass spectrometer was operated in negative ESI mode. For quantification of I3M, integrated extracted ion chromatograms were calculated into pmol with a calibration function between 1 nM and 1 mM. The obtained results were then normalized to the exact amount of fresh weight material used.

**Western Blot Analyses**

For MAPK activity assays, Arabidopsis leaves were infiltrated with ddH2O or peptide solution and harvested at the indicated time points. Protein extraction and immunoblot analyses using the anti-phospho p44/42 MAP kinase antibody (Cell Signaling Technology) were performed as described (Brock et al., 2010). Protoplasts were isolated using the protocol as described (Lu et al., 2011). For BIK1 phosphorylation assays, 0.1 mL protoplasts at a density of 2 × 10⁵/ml were transfected with 20 µg of plasmid DNAs carrying BIK1-HA as described (Lu et al., 2010), then treated with flg22 or nlp20. Anti-HA antibody was used for immunoblot analyses.

**Statistical analysis**

Data sets were analyzed using Microsoft Office Excel or JMP® 12.2.0. Comparisons between two groups were made using Student’s t-test. Multiple groups were compared using ANOVA followed by Student’s t-test for all possible individual comparisons.
Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AGB1, At4g34460; AGG1, At3G63420; AGG2, At3G2942; BAK1, At4g34430; BIK1, At2g39660; BIR2, At3g28450; CPK28, At5g66210; CYP71A13, At2g30770; CYP81F2, At5g57220; EF-1α, At1g07920/30/40; EFR, At5g20480; FLS2, At5g46330; MLO12, At3g3200; PAD3, At3g26830; PBL1, At3g55450; PPA2-A1, At1g25490; PPA2-C4, At3g58500; PR1, At2g14610; RLP23, At2g32680; RLP42, At3g25020; SOBIR1, At2g31880; XLG2, At4g34390.

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