Rapid Heteromerization and Phosphorylation of Ligand-activated Plant Transmembrane Receptors and Their Associated Kinase BAK1

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In plants leucine-rich repeat receptor kinases (LRR-RKs) located at the plasma membrane play a pivotal role in the perception of extracellular signals. For two of these LRR-RKs, the brassinosteroid receptor BRI1 and the flagellin receptor FLS2, interaction with the LRR receptor-like kinase BAK1 (BRI1-associated receptor kinase 1) was shown to be required for signal transduction. Here we report that FLS2-BAK1 heteromerization occurs almost instantaneously after perception of the ligand, the flagellin-derived peptide flg22. Flg22 can induce formation of a stable FLS2-BAK1 complex in microsomal membrane preparations in vitro, and the kinase inhibitor K-252a does not prevent complex formation. A kinase dead version of BAK1 associates with FLS2 in a flg22-dependent manner but does not restore responsiveness to flg22 in cells of bak1 plants, demonstrating that kinase activity of BAK1 is essential for FLS2 signaling. Furthermore, using in vivo phospholabeling, we are able to detect de novo phosphorylation of both FLS2 and BAK1 within 15 s of stimulation with flg22. Similarly, brassinolide induces BAK1 phosphorylation within seconds. Other triggers of plant defense, such as bacterial EF-Tu and the endogenous AtPep1 likewise induce rapid formation of heterocomplexes consisting of de novo phosphorylated BAK1 and proteins representing the ligand-specific binding receptors EF-Tu receptor and Pep1 receptor 1, respectively. Thus, we propose that several LRR-RKs form tight complexes with BAK1 almost instantaneously after ligand binding and that the subsequent phosphorylation events are key initial steps in signal transduction.

One of the central themes in cell biology is the sensing of extracellular chemical signals through cell surface receptors: How does the event of receptor-ligand interaction on the outside of the cell activate a signal transduction chain in the inside of the cell? In higher plants, the most prominent class of membrane receptors is formed by proteins with intracellular serine/threonine-type protein kinases. These receptors account for ~2.5–4% of all proteins encoded by the genome of a plant. Despite their importance, there is still little experimental evidence on the molecular activation mechanisms of plant transmembrane receptor kinases. Current models are based on the precedent of animal receptor tyrosine kinases where ligand binding causes receptor tyrosine kinases to form homo- or hetero-oligomers, followed by transphosphorylation (2). In the case of the epidermal growth factor receptor, these phosphorylation events occur within 60 s of receptor activation (3).

The best studied plant transmembrane receptor kinase is BRI1, the receptor for the brassinosteroid growth hormones (4). BRI1 is one of the 224 members of LRR-RKs in Arabidopsis (5). Upon ligand binding BRI1 interacts with a second LRR receptor-like kinase named BAK1 (6, 7). Two further well-characterized plant LRR-RKs are the flagellin receptor FLS2 (flagellin sensing 2) (8) and the EF-Tu receptor (EFR) (9). FLS2 perceives a generally conserved 22-amino acid epitope (flg22) of bacterial flagellin (8), and EFR senses the N-terminal amino acids (elf18/elf26) of bacterial elongation factor EF-Tu (10). FLS2 and EFR are typical pattern recognition receptors involved in plant immunity and render plants more resistant to bacterial attack (10, 11). However, pattern recognition receptors are also involved in the recognition of endogenous peptide signals, such as the wound-induced AtPep1, which is recognized by the LRR-RK PEPR1 and is suggested to be part of a positive feedback loop to amplify the plant immune response (12–14). Surprisingly, in recent work we and others found that stimulation with flg22 leads to heteromerization of FLS2 with BAK1, the same associated kinase employed by the hormone receptor BRI1 (15, 16). Consistently, bak1 mutants show strongly reduced responses to flg22, indicating that complex formation of FLS2 with BAK1 is essential for flg22 signaling. Despite these recent advances, the biochemical function of BAK1 and the precise mechanism underlying BAK1-FLS2 interaction and activation of the flagellin receptor remain unclear. In the case of brassinosteroid perception, it was shown by means of mobility shift and mass spectrometry that BRI1 and BAK1 are transphosphorylating in vivo, resulting in amplification of brassinosteroid signaling (17–19). However, these phos-
phorylation events have been studied only after a considerable lag time of 20–90 min following ligand perception. In contrast, there is currently no direct evidence for phosphorylation of BAK1 and FLS2 in response to flagellin treatment. This is of particular interest because FLS2 does not exhibit a conserved arginine-aspartic acid (RD) motif in the catalytic site. This special class of non-RD kinases generally shows weak autophosphorylation activity (20).

In previous work we reported that FLS2 associates with BAK1 within ~2 min of treatment with the flg22 ligand, concomitantly with the early physiological responses observed in Arabidopsis cells (15). Here we performed a more detailed kinetic analysis, showing that heteromerization of FLS2 and BAK1 occurs almost instantaneously and is independent of kinase activity. Using an in vivo labeling approach with radioactive orthophosphate, we studied the dynamics of phosphorylation events occurring within the receptor complex. Thereby, we observed that rapid heteromerization with BAK1 and phosphorylation induced by ligand perception are features common to various LRR-RKs, including BRI1 and the pattern recognition receptors EFR and PEPRI. Thus, the in vivo phospholabeling approach described in this study represents a new tool to detect plant receptors that biochemically interact and rely on BAK1 as a signaling partner.

**EXPERIMENTAL PROCEDURES**

**Materials, Plants, and Bioassays**—The flagellin peptides flg22, flg22-Δ2, and the EF-Tu peptides elf18 and elf26, as well as AtpPep1 used in this study were described previously (10, 12, 21, 22). The peptides were used at a concentration of 1 μM if not otherwise stated. Chitin fragments were used at a final concentration of 50 μg/ml (23) and the Penicillium-derived elicitor Pen at 1 μg/ml (24). Brassinazole (TCI America), brassinolide, calyculin A (Alexis), K-252a (Alexis), and estradiol (Sigma) were prepared as stock solutions of 1 mM in Me2SO or ethanol and used at the final concentrations indicated. Arabidopsis thaliana ecotype Columbia, bak1-4 and bak1-4-expressing BAK1-Myc (15) and other transgenic plants used in this study were grown as one plant/pot at 18/21 °C with an 8-h photoperiod or in liquid Murashige and Skoog medium (Duchefa) with 1% sucrose under continuous light. Cell cultures of A. thaliana, originally derived from plant tissue of accession Landsberg erecta were grown as described (22). Assays for extracellular pH alkalization and generation of reactive oxygen species were performed as previously described (9, 22).

**Western Blot and Immunoprecipitation**—Seedlings (~200 mg fresh weight) grown for 2 weeks in liquid Murashige and Skoog medium were frozen in liquid nitrogen and homogenized in 100 μl of cold extraction buffer (50 mM Tris-HCl, pH 8, 50 mM NaCl, and protease inhibitor mixture; Sigma). Equal amounts of proteins were separated by 7% SDS-PAGE and analyzed by Western blot with polyclonal antibodies directed against the C-terminal peptide (DSTSQIENEPSPGPR) of BAK1 (EZ Biolab). For immunoprecipitation, Arabidopsis cells (~20–200 mg fresh weight) were frozen in liquid nitrogen and homogenized in 0.05–0.5 ml of cold IP buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% (v/v) Nonidet P-40, and protease inhibitor mixture). After incubation for 1 h at 4 °C with gentle shaking, this preparation was centrifuged three times at 10,000 × g for 10 min. The supernatant was incubated 1 h at 4 °C with protein A-Sepharose beads (Amersham Biosciences) and polyclonal anti-FLS2 (8) or anti-BAK1 antibodies. The beads were collected and washed three times with ice-cold IP buffer and once with 50 mM Tris-HCl, pH 7.5. The proteins retained on the beads were separated by 7% SDS-PAGE and analyzed by Western blot. For analysis of transgenic plants, Western blot analysis and co-IP experiments were conducted as described above on respectively 20 and 200 mg of tissue from adult plants.

**In Vitro Complex Formation**—Arabidopsis cells (~200 mg fresh weight) were frozen in liquid nitrogen and homogenized in 0.5 ml of cold extraction buffer (50 mM Tris-HCl, pH 8, 0.5 mM sucrose, and protease inhibitor mixture). After a first centrifugation at 3,000 × g for 10 min at 4 °C, the supernatants were centrifuged at 150,000 × g for 30 min to isolate microsomes. The microsomes were further treated or not with 10 μM of peptides (flg22, flg22-Δ2, or elf18 as indicated) for 10 min at room temperature (unless specified) and solubilized for further IP with anti-BAK1 antibodies as indicated above.

**Chemical Cross-linking of FLS2 and BAK1**—Aliquots of cells were treated with flg22, flg22-Δ2, or brassinolide in the presence or absence of 2 mM bis(sulfosuccinimidy) sulfate. The reactions were stopped after 3 or 5 min by the addition of Tris buffer (50 mM, pH 8.5) and the freezing of cells. Solubilized membrane proteins were adsorbed to concanavalin A-Sepharose, resolubilized in SDS sample buffer, and analyzed on separate Western blots by immunodetection with anti-FLS2 and anti-BAK1 antibodies.

**In Vitro Phosphorylation Assay**—IPs from Arabidopsis cells were washed in 50 mM Hepes, pH 7.5, 3 mM MnCl2, and incubated in the same buffer in presence of [γ-32P]ATP (1 μCi, 0.66 nm; Hartmann Analytic) and unlabelled ATP (1 μM) for 5 min at 25 °C. The reaction was stopped by the addition of SDS loading buffer and denaturation of proteins at 95 °C. The proteins were subjected to SDS-PAGE and blotted on membrane, and the membrane was exposed to a Phospho Storage Screen (Bio-Rad) to record radioactive signals. Subsequently, the membranes were analyzed by Western blot with anti-FLS2 and anti-BAK1 antibodies.

**Pulse Labeling of Arabidopsis Cell Cultures with [32P]Orthophosphate**—Aliquots (~0.6 ml, 50 mg) of cultured cells (7 days after subculturing) were treated with 1 μM elicitor. For pulse labeling 20 μCi of carrier-free [32P]orthophosphoric acid (Hartmann Analytic) was added to the cells at the time point specified for each experiment. Labeling was stopped by freezing the cells in liquid nitrogen 1–5 min after [32P]phosphate addition. The samples were thawed on ice and homogenized in 200 μl of cold IP buffer, and IP was performed as described above. K-252a (25 mM) and calyculin A (2 mM) were added during protein solubilization to suppress in vitro kinase and phosphatase activities. The proteins were subjected to SDS-PAGE and blotted on membrane, and the membrane was exposed to a Phospho Storage Screen (Bio-Rad) to record radioactive signals. Subsequently, the membranes were analyzed by Western blot with anti-FLS2 and anti-BAK1 antibodies. The uptake of [32P]phosphate to the cell was monitored by separating 20 μl of the protein solubilize on SDS-PAGE, drying the gel, and exposing it...
to a Phospho Storage Screen. If not otherwise shown, the various treatments had no effect on the phosphate uptake and thus on overall protein phosphorylation. Quantification of signals was performed using Quantity One Software (Bio-Rad).

**Generation of Transgenic Plants and Transformation of Protoplasts**—The full coding sequence of the BAK1 gene was amplified by PCR from genomic DNA and cloned into pDONR207 (Gateway system; Invitrogen). Using site-directed mutagenesis, BAK1 kinase dead mutant (BAK1-KD) was generated containing two mutations: one at the ATP-binding site (substitution of the conserved lysine residue 317 by an aspartate) (7) and a second in the kinase subdomain XI (substitution of the glutamine 537 with an arginine). Both the wild type BAK1 and BAK1-KD constructs were verified by sequencing. For generation of transgenic plants, we used pMDC7 as an acceptor plasmid to express the BAK1 or BAK1-KD genes under the control of an estradiol-inducible promoter (25). The final construct was used to generate stable lines in the bak1-4 genotype (15). Plants of the T2 generation were chosen for functional analysis and were treated overnight with 1 μM estradiol to induce transgene expression.

For protoplast transformation the BAK1 and BAK1-KD genes were transferred into the acceptor plasmid pMDC32 under the control of the double 35 S promoter (26). Transient expression in leaf mesophyll protoplasts was performed as described (27) using bak1-4 mutant plants. Aliquots of 80,000 protoplasts were co-transformed with 0.5 μg of plasmid DNA encoding firefly luciferase under the FRK1 promoter (28) and with 0.5 μg of plasmid DNA encoding either wild type BAK1 or BAK1-KD. The protoplasts were resuspended in 400 μl of W5 solution (27) and split in four samples (100 μl, 20,000 protoplasts). The cells were incubated in a 96-well plate and 16 h after transformation were supplied with 50 μM luciferin (D-Luciferin, firefly; PJK). Then protoplasts were treated with 100 nM flg22, and luminescence was quantified in vivo.

**RESULTS**

**Generation of Antibodies against BAK1**—We raised polyclonal antibodies against the C terminus of BAK1. On immunoblots these antibodies specifically detect a band of ~75 kDa, corresponding to the glycosylated BAK1 in the extracts of leaves from wild type Arabidopsis but not in those from the bak1-4 mutant (supplemental Fig. S1A). BAK1 is also termed SERK3 because it belongs to the five-member family of somatic embryogenesis receptor kinases (SERKs) (29). Competitive IP assays show that the antibodies recognize the C termini of BAK1 and its homologues SERK4 and SERK5, but not SERK1 or SERK2 (supplemental Fig. S1B). However, because the ~75-kDa signal was absent in extracts of bak1-4 mutant plants, either cross-reactivity with SERK4 and SERK5 is weak, or BAK1 is strongly predominating in these plant extracts. Using Arabidopsis cultured cells and the anti-BAK1 antibodies for co-IP experiments, we were able to detect and identify by mass spectrometry the FLS2 polypeptide in an anti-BAK1 IP performed on extracts of flg22-induced cells, but not on those of non-stimulated cells (supplemental Fig. S1C).

**Characterization of the FLS2-BAK1 Interaction**—With similar co-IP assays we proceeded to examine the association of BAK1 and FLS2 in the first seconds after stimulation with flg22 (Fig. 1A). Complex formation of FLS2 with BAK1 was almost instantaneous and fully completed even in cells that were immediately frozen after treatment with flg22 (<1 s time point; Fig. 1A). In contrast, the addition of flg22 to the extracts of cells did not lead to detectable complex formation. This extremely rapid association might reflect the presence of BAK1 and FLS2 in close vicinity in the plasma membrane, possibly as preformed heteromers. To test for the presence of preformed complexes, we exposed cells to the cross-linker bis(sulfosuccinimidyl)suberate, before or after a 3- or 5-min treatment with flg22 (supplemental Fig. S2). Although a band of high molecular weight corresponding to covalently linked FLS2-BAK1 was clearly detectable in the flg22-treated cells, there was no such band in the untreated cells or in cells treated with the C-terminal truncated peptide flg22-Δ2, which binds to FLS2 but does not activate signaling (22). This result demonstrates that FLS2 and BAK1 form a tight cross-linkable heteromeric complex, only after productive binding of the flg22 ligand to FLS2.

In view of this rapid formation of a stable complex between FLS2 and BAK1, we attempted to reconstitute this process in vitro from microsomal membrane preparations of untreated cells. Indeed, formation of the FLS2-BAK1 complex was triggered also in microsomes (Fig. 1B). As in intact cells, the FLS2-BAK1 complex was induced by flg22 but neither by the inactive flg22-Δ2 nor by the EFR ligand elf18. Complex formation in vitro reached ~20% of the complex found in an equivalent amount of microsomes from cells stimulated with flg22 in vivo. However, efficiency of heteromerization was strongly reduced when microsomes were kept at 4 °C, and no complex formation was observed after pretreatment with the detergent Nonidet P-40 (Fig. 1, C and D). Thus, FLS2-BAK1 interaction can be induced by flg22 in vitro, however only in the presence of functional membranes.
In Vivo Phospholabeling of the FLS2-BAK1 Complex—Characteristically for plant transmembrane receptors, both FLS2 and BAK1 present a cytoplasmic kinase domain, which exhibits protein kinase activity when purified from bacteria (6, 30). To examine the potential of the FLS2-BAK1 complex to become phosphorylated, we incubated IPs with [γ-32P]ATP in vitro (supplemental Fig. S3). FLS2 and BAK1 showed increased phosphorylation when co-precipitated from flg22-induced cells but not when both proteins were concomitantly immunoadsorbed from untreated cells and when flg22 was added in vitro. Thus, phosphorylation seems to be dependent on the ligand-induced tight interaction between FLS2 and BAK1.

The above results indicate that flg22-dependent phosphorylation of the FLS2-BAK1 complex might occur as early events of receptor activation in vivo. Extensive previous surveys using quantitative phosphoproteomics have revealed a number of plasma membrane proteins that are specifically phosphorylated in response to flg22 (31, 32). Surprisingly, however, neither FLS2 nor BAK1 were among them. Thus, we reinvestigated this question using a method of in vivo labeling of cultured plant cells with short, 1–5-min pulses of [32P]orthophosphate described previously (33). As reported in this earlier publication, plant cells in culture tend to have a very strong phosphate uptake, and >90% of radioactive tracer phosphate applied accumulates within the cells in less than a minute. Because of its intrinsically rapid turnover, the phosphate group at the γ-position of ATP shows maximal specific labeling within the first minute of tracer application. Thus, labeling with these short [32P]phosphate pulses not only allowed us to identify de novo phosphorylation events but also to resolve the kinetics of these changes after treatment with flg22. FLS2 and BAK1 phosphorylation was monitored by IP of the receptor proteins from Arabidopsis cells labeled with 1-min pulses of [32P]phosphate at various time points relative to flg22 stimulation (Fig. 2A). In fact, FLS2 and BAK1 exhibited a strong increase in de novo phosphorylation, which was clearly detectable in cells that were analyzed only 15 s after the addition of flg22 (Fig. 2B). When IPs were performed in the presence of excess antigenic peptide, neither the proteins nor the radioactive signal were precipitated (Fig. 3A). Similarly, phospholabeling was not detectable in untreated cells or cells treated with the inactive peptide flg22-Δ2 (Fig. 3A).

Quantification of the signals observed for BAK1 and FLS2 revealed that the radioactive labeling for both proteins was maximal between 30 and 60 s and was slowly declining at later time points (Fig. 2, B and C). Phosphorylation kinetics for FLS2 were the same as for BAK1, and importantly, the kinetics were independent whether proteins were pulled down directly (IP) or indirectly (co-IP). Within the limits of this experimental setup, phosphorylation of FLS2 and BAK1 occurred simultaneously with formation of the FLS2-BAK1 complex but clearly before physiological responses such as medium alkalinization, which is measurable only after a lag phase of ~50 s (Fig. 2C). Phosphate pulses applied during the ongoing alkalinization process resulted in progressively weaker [32P]phosphate incor-

![In Vivo Phosphorylation Dynamics of the FLS2-BAK1 Complex](https://example.com/in vivo-fig2.png)
The Importance of Kinase Activity in FLS2 Signaling—In the experiments above, formation of the FLS2-BAK1 complex and its de novo phosphorylation appeared as a concomitant process. To investigate how these phosphorylation events themselves influence complex formation, we used the potent protein kinase inhibitor K-252a, which has been shown to inhibit elicitor responses within 30 s of application in tomato and Arabidopsis cells (21, 33). In previous studies with BAK1-Myc constructs, we found a slight reduction of the FLS2-BAK1 complex formation (15). In contrast, in cell cultures pretreatment with K-252a completely blocked phosphorylation of FLS2 and BAK1, whereas association of FLS2 with BAK1 was not affected (Fig. 3B). Strikingly, when K-252a was given 15 s after flg22, it had no effect on [33P]phosphate labeling of BAK1 and FLS2, again indicating that the phosphorylation process was completed rapidly after stimulation (Fig. 3B). The action of K-252a in inhibiting cellular responses to flg22 treatment thus seems to lie downstream of FLS2-BAK1 complex formation. To study the functional role of the kinase domain of BAK1 in the regulation of flg22 signaling in more detail, we constructed a kinase-deficient version of BAK1 (BAK1-KD) mutated on critical residues in the kinase domain. For an initial analysis of this construct, leaf mesophyll protoplasts of bak1-4 mutants were co-transformed with a construct containing BAK1-KD or wild type BAK1 and a second construct carrying a luciferase gene under the FRK1 promoter, used as a reporter of flg22 signaling (28). Both BAK1 and BAK1-KD were similarly well expressed in the protoplasts (Fig. 4B); however, only wild type BAK1 was able to restore responsiveness to flg22, whereas the kinase-deficient version BAK1-KD was not (Fig. 4A). Subsequently, bak1-4 plants were transformed with wild type BAK1 or BAK1-KD; an estradiol-inducible promoter (25) was used to avoid possible BRI1-related interferences on plant growth (6, 7). Among the transformants, two T2 lines were selected for each construct that showed strong estradiol-inducible transgene expression (Fig. 4D). In the absence of estradiol, all of these four transgenic lines were only marginally responsive to flg22 in a bioassay for production of reactive oxygen species, just like the parent bak1-4 line (Fig. 4C). However, after preincubation with estradiol, the plants expressing wild type BAK1 responded as strongly as Col-0 wild type plants, demonstrating functional complementation, whereas BAK1-KD was unable to complement the bak1-4 phenotype (Fig. 4C). Importantly, based on an immunoprecipitation experiment with antibodies against BAK1, both the BAK1 and the BAK1-KD proteins were similarly able to form a stable complex with FLS2 upon stimulation with flg22 (Fig. 4E), confirming that kinase activity itself is not required for the formation of the FLS2-BAK1 complex (Fig. 3B). In some cases, we observed a reduction of reactive oxygen species production in response to flg22 in BAK1-KD-expressing plants (Fig. 4C, line A) compared with the residual response of bak1-4 plants. This suggests that while interacting with FLS2, BAK1-KD may compete with proteins acting redundantly to BAK1.

De Novo Phosphorylation of BAK1 in Response to the Phytohormone Brassinolide—Originally described as associated kinase of the brassinosteroid receptor BRI1, BAK1 was reported to be phosphorylated within the BRI1 complex in

In Vivo Phospholabeling of the FLS2-BAK1 Complex

The EC_{50} values for the DEP1-A complex formation of FLS2 and BAK1 is only induced in the presence of the active ligand flg22. A, cells were treated simultaneously with [33P]phosphate and flg22 or flg22-Δ2 for 1 min. Solubilized membrane proteins were immunoprecipitated with antibodies against FLS2 and BAK1 (top panels) or anti-BAK1 (bottom panels). Phosphorylation and Western blot (WB) signals were absent in IPs competed with their respective antigenic peptides (+flg22 + antigen). B, formation of the FLS2-BAK1 complex is not dependent on kinase activity. Preincubation with the kinase inhibitor K-252a (1 μM) 2 min before (−120 s) the 1-min pulse with [33P]phosphate and flg22 prevents phosphorylation but does not complex formation of FLS2 and BAK1. In contrast, K-252a given 15 s after (−15 s) flg22 treatment had no effect on phosphorylation. Controls without K-252a were treated with an equivalent amount of solvent (dimethyl sulfoxide, shown as + DMSO). autorad., autoradiography.
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response to brassinolide (BL) treatment in Arabidopsis tissue (17). We therefore investigated whether we could detect rapid phosphorylation of BRI1 and BAK1 in a time course experiment with BL treatment of Arabidopsis cells. For depletion of endogenous BL levels, the cells were preincubated in the presence of brassinazole, an inhibitor of BL synthesis (36). Indeed, we found a clear increase of de novo phosphorylation of BAK1 after the addition of BL in these cells (Fig. 5A). In analogy to labeling after elicitation with flg22 (Fig. 2B), the signal for BAK1 showed comparable kinetics with a maximum at 60–120 s. However, although reproducible phospholabeling was observed for BAK1, there was no co-IP of a clear band that would indicate the presence of de novo phosphorylated BRI1. Nevertheless, because BAK1 itself is not the ligand receptor site for BL (37), we infer that the rapid phosphorylation of BAK1 observed must involve interaction with BL-activated BRI1.

De Novo Phosphorylation of BAK1-RK Complexes in Response to Other Elicitors than flg22—Since the finding of FLS2-BAK1 interaction, it is emerging that BAK1 is also required for the function of several other pattern recognition receptors (15, 16, 38, 39). Thus, we set out to fish for further receptor kinases involving BAK1 as a signaling partner using the IP of BAK1 in combination with in vivo phospholabeling. Two peptide elicitors, the bacterially derived elf26 and the Arabidopsis endogenous danger signal AtPEP1 (12), were used, because for these two elicitors the receptor-binding sites have already been characterized (10, 13). Additionally, we investigated BAK1 phosphorylation after treatment with two carbohydrate-based elicitors: the fungal peptidoglycan Pen (24) and chitin. Pulldown of BAK1 from [32P]phosphate-labeled Arabidopsis cells revealed increased phosphorylation of BAK1 after treatment with not only elf26 but also AtPEP1 (Fig. 5B). In contrast, no clear increase in labeling of BAK1 was observed after treatment with the fungal elicitor preparations, although these stimulated medium alkalization similar to flg22, elf26, or AtPEP1 (supplemental Fig. S7). Importantly, treatment with elf26 and AtPEP1 led to co-IP of phospholabeled higher molecular mass proteins clearly different from the FLS2 polypeptide: a polypeptide of ~150 kDa in cells treated with elf26 and one of ~160 kDa in cells treated with AtPEP1, respectively. These bands in all probability represent the phosphorylated EFR for elf26 and PEPR1 for AtPEP1, because they match the sizes reported for the corresponding LRR-RK-binding sites (10, 13).

**DISCUSSION**

The aim of this study was to investigate how early signaling events such as phosphorylation of plant LRR-RKs, exemplified by FLS2 and BAK1, are temporally connected with the activation of these receptors. We used in vivo labeling with short pulses of [32P]orthophosphate, which allowed us to study de novo phosphorylation

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**FIGURE 4.** A functional kinase domain of BAK1 is required for flg22-dependent response activation. **A,** leaf mesophyll protoplasts of bak1-4 mutant plants were co-transfected with luciferase under the flg22-responsive FRK1 promoter and either the wild type BAK1 or the kinase-deficient BAK1-KD. The samples were mock treated or treated with 100 nM flg22 (time 0), and luminescence was quantified as relative light units (RLU). The values show the means of two replicates with standard deviations bigger than ±60 relative light units (RLU) shown as error bars. **B,** expression of BAK1 and BAK1-KD in bak1-4 protoplasts under similar conditions as shown in **A.** Although the full response to flg22 was already gained after transformation of bak1-4 protoplasts with low amounts of plasmid (0.5 μg), protein accumulation was more obvious in samples transfected with higher amounts of DNA (5 μg). **C,** responsiveness of bak1-4 plants to flg22 was restored with wild type BAK1, but not BAK1-KD. Two T2-lines each of bak1-4 plants, transformed with either BAK1 (line-2 and line-4) or BAK1-KD (line-A and line-M) under the control of an estradiol-inducible promoter, were selected and compared with the parent bak1-4 line and with wild type Col-0 plants. Expression was induced or not in leaf pieces by treatment with 1 μM estradiol, and production of reactive oxygen species was quantified as RLU after treatment or not with 100 nM flg22. The data represent the averages ± S.D. (n = 3). **D,** expression of BAK1 and BAK1-KD in plants induced or not with estradiol under the conditions shown in **C.** E, analysis of FLS2 co-immunoprecipitation with BAK1 after 5 min of treatment with 1 μM flg22 both in BAK1 line-4 and in the BAK1-KD line A. Col-0 seedlings were used as positive control of co-IP between BAK1 and FLS2. WB, Western blot.

**FIGURE 5.** Phosphorylation of BAK1 and co-immunoprecipitation of specific LRR-RKs in response to various signals. **A,** phosphorylation of BAK1 in response to stimulation with BL. Arabidopsis cell cultures were preincubated with brassinazole (2 μM for 6 days) and treated with BL (1 μM) or ethanol as control and were pulse-labeled with the same sampling scheme as in Fig. 2A. Solubilized membrane proteins were immunoprecipitated (IP) with anti-BAK1, separated by SDS-PAGE, blotted, and analyzed by autoradiography (autorad., upper panel) and Western analysis (WB, lower panel). B, BAK1 serves as interaction partner of several transmembrane receptors. Arabidopsis cells were treated with the peptide elicitors flg22, elf26, and AtPEP1, and the fungal elicitors Pen and chitin and were simultaneously incubated with [32P]phosphate for 5 min.
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events occurring during the first seconds after ligand stimulation. This approach complements and differs from other methods like mass spectrometry or Western blot analysis detecting phospho-residues that have been successfully used for the characterization of the phosphorylation status of the BRI1-BAK1 system (17–19). In this study we show that phosphorylation of the FLS2-BAK1 complex is concomitant to the heteromerization process and is an extremely quick and specific event because it appears within 15 s of stimulation with flg22. In accordance with a role in receptor-mediated signaling, these phosphorylations clearly precede all of the responses and intracellular signaling events known so far (40).

Phosphorylation of BAK1 and phosphorylation of FLS2 seem to be tightly connected because both events exhibit a dose dependence for the flg22 ligand that correlates closely with the saturation curve for binding of flg22 to FLS2 (22), supporting the model that each flg22 molecule that binds to a FLS2 receptor site triggers formation and phosphorylation of a FLS2-BAK1 complex. Whether these complexes represent heterodimers or higher oligomerization states with a different stoichiometry remains to be studied.

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Importantly, we confirm that BAK1 gets phosphorylated in a delayed time course in response to BL perception (28). However, in this case the FLS2-BAK1 complex formation was not investigated. Alternatively, because the association and phosphorylation between BRI1 with BAK1 was determined not until 90 min after BL application, the BRI1 kinase activity may be important for long term stability rather than formation of the complex.

One intriguing question remains: how do these LRR-RKs complexes share the same signaling partner, BAK1? It is tempting to hypothesize that ligand-binding receptors are present in preformed, inactive complexes with BAK1 in the absence of ligands. The existence of preformed dimers has been reported in case of the epidermal growth factor receptor in animal cells (41). Supporting this, complex formation between BAK1 and FLS2 was found to be too quick to resolve the kinetic of interaction in a time course. However, using cross-linking agents, we were not able to confirm preassociation of the FLS2 and BAK1 proteins, although cross-linking was successful in samples from stimulated cells. As a complement to the signalosome or to the BRI1 signaling partner, BAK1 may also be due to dissociation of the BRI1-BAK1 complex during IP or to the low abundance of this protein in Arabidopsis cultured cells. Considering the small amount of cell tissue used in our experiments, the signal for labeled BRI1 may be below the detection threshold.

Moreover, the function of BAK1 seems not restricted to the regulation of the FLS2 and BRI1 signaling pathways. Plants affected in BAK1 have been reported previously to exhibit reduced responses to various microbial elicitors such as the bacterial EF-Tu, cold shock protein, or the oomycete elicitor INF1 (15, 16, 38). Additionally, the BAK1 cytosolic domain was reported to interact with the PEPR1 protein in a yeast two-hybrid assay (39). Here, we give biochemical evidence that BAK1 is phosphorylated quickly in Arabidopsis cells after the perception of various peptidic elicitors like flg22, elf26, and AtPep1. Moreover, distinct phosphorylated polypeptides corresponding in size to the expected ligand binding LRR-RKs were coinmunoprecipitated with BAK1 in response to the peptidic elicitors. The fact that BAK1 phosphorylation was not detectable after elicitation of Arabidopsis cells with carbohydrate-based elicitors like chitin is in good agreement with genetic analyses indicating that BAK1 is not required for chitin signaling (38). Interestingly, all of the ligand-binding proteins reported here to interact with BAK1 (BRI1, FLS2, EFR, and PEPR1) belong to the LRR receptor kinase family. Thus, the present study supports that recruitment and rapid de novo phosphorylation of BAK1 are general activation mechanisms to many LRR receptor kinases. Further analysis with other elicitors will serve to investigate the functional repertoire of BAK1 and identify new unknown ligand-binding sites, relying on BAK1 as a common signaling partner.

Based on the findings, we may draw a model where ligand binding provokes interaction of the extracellular LRRs of FLS2...
and BAK1 and thus brings the intracellular kinase domains into contact. Initiation of intracellular signaling can thus be induced by transphosphorylation events between the BAK1 and FLS2 cytosolic domains. In analogy, the crystal structures of the ectodomains of animal innate immune receptors revealed ligand-induced dimerization, which was proposed to stabilize a receptor complex and thus initiate signaling (45, 46). With respect to the specificity of signaling output, it will be of importance to investigate whether bacterial elicitors and BL treatment lead to phosphorylations of the same or different sites in BAK1, especially at the early time points defined here.

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