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COMMENTARY

Cautionary Notes on the Use of C-Terminal BAK1 Fusion Proteins for Functional Studies

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Detailed phenotypic characterization reveals that several BAK1 fusion proteins with C-terminal tags strongly impair complementation of *bak1* null mutants with respect to responsiveness to the bacterial pathogen-associated molecular patterns flagellin and EF-Tu. This raises concerns about the widespread use of such protein variants of this important regulatory Leu-rich repeat receptor-like kinase (RLK) for functional analyses of RLK-based signaling.

Plants employ numerous surface-localized receptor kinases to control different aspects of their life cycle and to respond appropriately to external stimuli (De Smet et al., 2009; Lehti-Shiu et al., 2009). Recently, it has become evident that the Leu-rich repeat receptor-like kinase (LRR-RLK) BRI1-ASSOCIATED KINASE1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE3 (BAK1/SERK3) and paralogous SERK proteins are major regulators of several RLK-based signaling pathways (Chinchilla et al., 2009; Li, 2010). SERK proteins are a subclass of the LRR-II family of plant RLKs and possess, as characteristic features, five LRRs and a Pro-rich domain in their ectodomain, a transmembrane domain, and an intracellular RD kinase domain (Hecht et al., 2001; Albrecht et al., 2008).

BAK1 was originally identified as a positive regulator of the LRR-RLK BRASSINOSTEROID-INSENSITIVE1 (BRI1) that perceives brassinosteroids (BRs) to control key aspects of growth and development (Li et al., 2002; Nam and Li, 2002). BRI1 forms a ligand-induced complex with BAK1, as well as with SERK1 and BAK1-LIKE KINASE1 (BKK1/SERK4), two additional LRR-RLKs that play functionally redundant roles with BAK1 in modulating diverse aspects of BRI1-mediated responses (Li et al., 2002; Nam and Li, 2002; Karlova et al., 2006; He et al., 2007; Albrecht et al., 2008; Wang et al., 2008; Jeong et al., 2010). BAK1 is also a key positive regulator of innate immune signaling triggered by several LRR-RLKs that act as pattern recognition receptors (PRRs) for pathogen-associated molecular patterns (PAMPs) leading to PAMP-triggered immunity (PTI). The LRR-RLK FLAGELLIN SENSING2 (FLS2) perceives bacterial flagellin (or the conserved peptide flg22) and forms, almost instantaneously, a ligand-induced complex with BAK1 (Chinchilla et al., 2007; Heese et al., 2007; Schulze et al., 2010). Similarly, the LRR-RLK EF-TU RECEPTOR (EFR) that perceives bacterial elongation factor Tu (or the conserved peptide elf18) forms a ligand-induced complex with BAK1 (Roux et al., 2011; Schwessinger et al., 2011). FLS2 and EFR heterooligomerize with other SERKs in addition to BAK1, including BKK1, which appears to act redundantly with BAK1 in FLS2- and EFR-mediated PTI signaling (Roux et al., 2011). Notably, the functional role of BAK1 seems more critical for PTI signaling than for BRI1-mediated responses, as *bak1* mutants are strongly impaired in most flg22- and elf18-induced responses (Chinchilla et al., 2007; Heese et al., 2007; Shan et al., 2008; Ranf et al., 2011; Roux et al., 2011; Schwessinger et al., 2011), while the single mutants display only mild defects in BR-induced responses (Li et al., 2002; Nam and Li, 2002; Karlova et al., 2006; He et al., 2007; Albrecht et al., 2008).

BAK1 is not required for binding of either BR to BRI1 or flg22 to FLS2 (Kinoshita et al., 2005; Chinchilla et al., 2007). The recent elucidation of the crystal structure of the extracellular domain of BRI1 in complex with its ligand suggests that it may act as a platform for binding other proteins, such as the extracellular domain of BAK1 (Hothorn et al., 2011; Jaillais et al., 2011; She et al., 2011). BR binds to the extracellular domain of BRI1, which activates the BRI1 intracellular kinase domain leading to autophosphorylation and transphosphorylation of BAK1 on residues in its kinase domain. This enhances BAK1 autophosphorylation and induces transphosphorylation of BRI1 on residues in the intracellular juxtamembrane and C-terminal regions (Wang et al., 2008). In this sequential phosphorylation model, BAK1 is an amplifier of BRI1 kinase activity and subsequent signaling and thus acts as a regulatory LRR-RLK. We recently demonstrated that BAK1 is not a mere amplifier but can also confer signaling specificity between BR and PTI signaling pathways in a phosphorylation-dependent manner (Schwessinger et al., 2011). In addition, we recently showed that BAK1 that has been activated in response to BR or flg22 perception does not cross-activate PTI or BR signaling, respectively (Albrecht et al., 2011). Here, we demonstrate that the presence of C-terminal tags on BAK1 strongly impacts PTI signaling, while it has a minor effect on a limited number of BR responses. These findings further highlight major differences in the regulation mechanisms imposed by BAK1 on the BR and FLS2/EFR signaling pathways and raise concerns about the use of BAK1 and other SERK fusion proteins for functional studies.

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C-TERMINALLY TAGGED BAK1 VARIANTS ARE MOSTLY FUNCTIONAL IN BR SIGNALING

Transgenic expression of BAK1 fusion proteins in the null mutant *bak1-4* is often used to characterize residues important for BAK1 function or to perform biochemical studies on BAK1 phosphorylation and/or complex formation with BRI1 and/or FLS2. While performing recent detailed studies on BAK1, we realized that such fusion proteins do not always behave as the wild-type protein. To study the potential impact of C-terminal tags on BAK1 function in the BR and PTI signaling pathways, we generated or obtained previously published lines expressing BAK1 proteins fused at their C termini with a triple hemagglutinin (HA_3) tag, an enhanced green fluorescent protein (eGFP), a Myc tag, or a FLAG tag under the control of the native *BAK1* promoter (*pBAK1*) in the null *bak1-4* or *bak1-4 bkk1-1* mutant backgrounds. In all assays, we compared the ability of these BAK1 variants to complement the phenotypes of *bak1-4* or *bak1-4 bkk1-1* in comparison to wild-type Columbia-0 (Col-0) and to *bak1-4* complemented with untagged BAK1. *Arabidopsis thaliana* mutants impaired in BR perception or signaling, including *bak1-4*, display an altered rosette morphology and size under short-day conditions and are hyposensitive to exogenous BR treatments (Li et al., 2002; Nam and Li, 2002; Wang et al., 2008; Schwessinger et al., 2011).

To test quantitatively if the *BAK1* transgenes complement the hyposensitivity phenotype of *bak1-4* (or *bak1-4 bkk1-1*) plants, we analyzed the expression of the BR-responsive genes *CPD* (that is downregulated in response to BR; Figure 1A) and *SAUR-AC1* (that is upregulated in response to BR; Figure 1B), as well as measured root length of light-grown seedlings in response to BR (Figure 1C). All *BAK1* transgenes were able to complement the BR hyposensitivity in all assays (Figures 1A to 1C), with the exception of *BAK1-HA_3*, which was unable to complement the BR insensitivity of *bak1-4* in the root inhibition assay (Figure 1C).

Similarly, we observed that expression of all *BAK1* transgenes complement the rosette phenotype of *bak1-4* (and *bak1-4 bkk1-1*) plants (Figure 1D). Interestingly, we noticed that expression of untagged BAK1 or BAK1-eGFP not only reverted the semidwarf morphology of *bak1-4* plants but also displayed signs of BR hypersensitivity in this assay, as reflected by elongated petioles and leaves (Figure 1D). This hypersensitivity phenotype may be explained by higher expression levels of the untagged *BAK1* and *BAK1-eGFP* transgenes (Figure 1E).

C-TERMINALLY TAGGED BAK1 VARIANTS STILL FORM LIGAND-INDUCED COMPLEXES WITH FLS2

C-terminal BAK1 fusion proteins are often used in *Arabidopsis* and *Nicotiana benthamiana* to study the ability of BAK1 or derived variants to form a ligand-induced complex with FLS2 (Chinchilla et al., 2007; Heese et al., 2007; Shan et al., 2008; Lu et al., 2010, 2011; Zhang et al., 2010; Roux et al., 2011; Schwessinger et al., 2011; Xiang et al., 2011). Using the transgenic lines described in Figure 1, we tested the ability of the different BAK1-tagged proteins to associate with FLS2 in response to flg22 treatment in coimmunoprecipitation experiments. We used specific antibodies recognizing native BAK1 for immunoprecipitation to avoid issues associated with different affinities of different antibodies or binding matrixes. All transgenic lines expressed the FLS2 protein at levels similar to Col-0 (Figure 2, see input). However, the amount of BAK1 protein detected by anti-BAK1 immunoblotting appeared lower in the transgenic lines expressing C-terminally tagged BAK1 proteins compared with Col-0 or *bak1-4* lines expressing untagged BAK1 (Figure 2). The apparent discrepancy between transcript and protein levels of the transgenes (Figures 1E and 2) suggests that the presence of the C-terminal tags may affect recognition of the epitope by the anti-BAK1 antibody. Nevertheless, regardless of the amount of BAK1 being expressed and immunoprecipitated in this assay, the amount of FLS2 in complex with BAK1 after flg22 treatment is comparable between untagged and tagged BAK1. Therefore, the observed impairment of PTI responses is not due to lower protein abundance. This observation is consistent with our recent findings that the amount of BAK1 is not limiting and that only a subset of the total cellular amount of BAK1 proteins associates with FLS2 after elicitation (Albrecht et al., 2011). As expected, no FLS2 protein could be coimmunoprecipitated with any BAK1 variant if plants were not treated with flg22 (Figure 2), showing that the presence of a C-terminal tag did not lead to constitutive association with FLS2.

C-TERMINALLY TAGGED BAK1 VARIANTS ARE NOT FULLY FUNCTIONAL IN PTI RESPONSES TRIGGERED BY FLG22 AND ELF18

We tested the ability of the different BAK1 variants to complement the hyporesponsive phenotype of *bak1-4* during FLS2- and EFR-mediated innate immune responses (Chinchilla et al., 2007; Heese et al., 2007; Shan et al., 2008; Ranf et al., 2011; Roux et al., 2011; Schwessinger et al., 2011). An early response triggered by flg22 and elf18 perception is the rapid production of reactive oxygen species (ROS) in an oxidative burst. The amount of ROS produced during this burst can be plotted as the total amount of relative light units emitted in a luminol-based assay during 40 min following elicitation. In this assay, leaf discs of *bak1-4* plants displayed a greatly reduced flg22- and elf18-triggered ROS burst when compared with Col-0 (Figures 3A and 3B), as previously described (Chinchilla et al., 2007; Heese et al., 2007). Expression of untagged BAK1 fully complemented the reduced ROS burst of *bak1-4* leaf discs in response to flg22 and elf18 (Figures 3A and 3B). However, none of the tagged BAK1 fusion proteins could complement the *bak1-4* phenotype, revealing that the presence of the C-terminal tags blocked the ability of BAK1 to initiate the oxidative burst. In all cases, the kinetics of the ROS burst was similar over the recorded 40-min period.

A late response triggered by flg22 and elf18 is the inhibition of the growth of *Arabidopsis* seedlings. This can be depicted as the relative growth (as measured by fresh weight) of treated seedlings in comparison to untreated seedlings 1 week after treatment (Figures 3C and 3D). Notably, the null *bak1-4*

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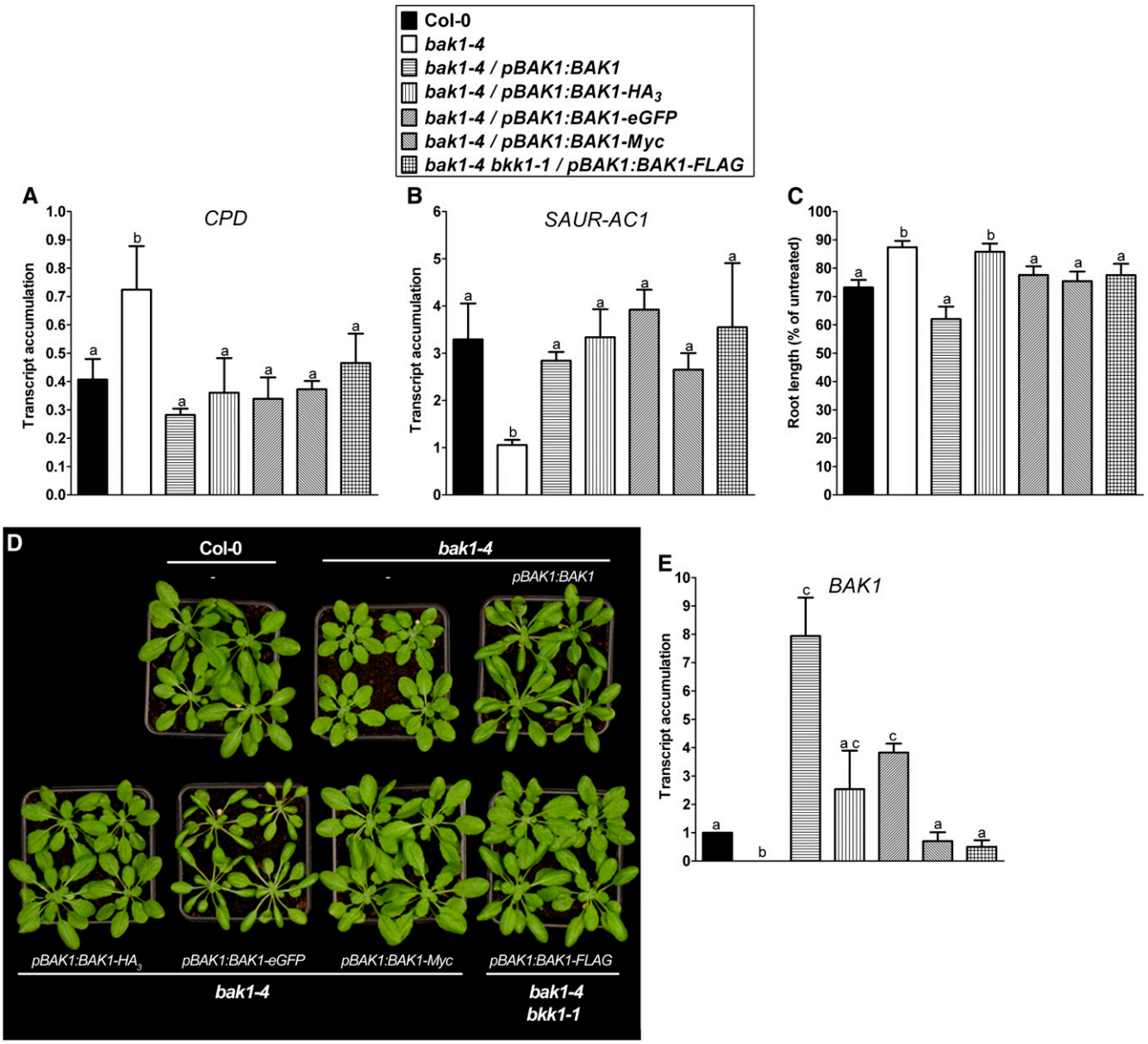

Figure 1. C-Terminally Tagged BAK1 Proteins Complement the BR-Insensitive Phenotype of *bak1-4* and *bak1-4 bkk1-1* Mutant Plants.

(A) and **(B)** BR marker genes expression. Seedlings of all genotypes were pretreated for 16 h with brassinazole before 3-h treatment with water or 200 nM BL. Gene expression of *CPD* and *SAUR-AC1* was measured by quantitative RT-PCR. Data averaged from two biological repeats are presented as transcript accumulation relative to water-treated Col-0. Bars represent SD ($n = 6$).

(C) BR-induced root length inhibition. Relative root growth of 7-d-old seedlings grown on medium supplemented or not with 1 nM BL is shown. Root length is presented relative to untreated control for each genotype. Bars represent SE ($n = 30$). This experiment was repeated twice with similar results.

(D) Representative pictures of short-day-grown *Arabidopsis* plants (4.5 weeks old) of the indicated genotypes.

(E) Accumulation of *BAK1* transcript in BAK1-tagged lines was measured by quantitative RT-PCR on 14-d-old seedlings and is presented as relative to Col-0. Data averaged from three biological repeats are shown. Bars represent SD ($n = 9$).

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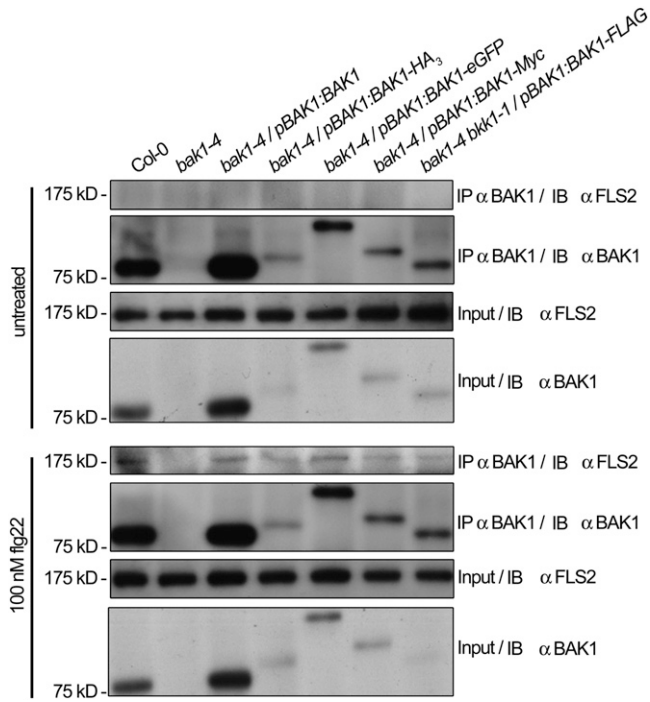

Figure 2. All BAK1 Fusion Proteins Form a Ligand-Induced Complex with FLS2.

Fourteen-day-old seedlings were left untreated or treated with 100 nM flg22 for 2 min as indicated. Extracted proteins were subjected to immunoprecipitation (IP) with anti-BAK1 antibodies and IgG beads followed by immunoblot (IB) analysis using either anti-BAK1 (α BAK1) or anti-FLS2 (α FLS2) antibodies. Seedling genotype is indicated at the top of each lane. These experiments were repeated twice with similar results.

mutation strongly affects flg22-induced inhibition of seedling growth, rendering *bak1-4* seedlings almost insensitive to 100 nM flg22 in this assay, but has only a very minor effect on elf18-induced inhibition of seedling growth (Figures 3C and 3D; Chinchilla et al., 2007; Roux et al., 2011; Schwessinger et al., 2011). Expression of untagged BAK1 in *bak1-4* seedlings fully restored wild-type responsiveness to flg22 (Figure 3C). In contrast with what was observed in the ROS burst assay (Figure 3A), expression of BAK1-HA_3, BAK1-eGFP, and BAK1-Myc partially restored flg22 responsiveness of *bak1-4* seedlings (Figure 3C). More strikingly, expression of the same variants rendered *bak1-4* seedlings even less sensitive to elf18 in the seedling growth inhibition assay than untransformed *bak1-4* seedlings (Figure 3D), revealing that the presence of the HA_3, eGFP, and Myc tags has a dominant-negative effect on EFR-mediated signaling leading to seedling growth inhibition. Thereby, the potentially reduced BAK1 protein level in these lines cannot account for the observed impairment in PTI signaling (Figure 3). In response to both flg22 and elf18, BAK1-FLAG complemented the *bak1-4* phenotype to the same extent as untagged BAK1 (Figures 3C and 3D) in contrast with its inability to restore a ROS burst (Figures 3A and 3B). This could suggest that the presence of this tag impairs BAK1 function in early responses (e.g., ROS burst) but still enable the establishment of late responses (e.g., inhibition of seedling growth).

CONCLUSIONS AND IMPLICATIONS

Taken together, our results revealed that the presence of C-terminal tags on the regulatory LRR-RLK BAK1 results in the inability of these fusion proteins to fully complement the hyporesponsiveness of the null *bak1-4* mutant to the PAMPs flg22 and elf18 during innate immunity. Strikingly, all BAK1 fusion proteins were fully functional for BR-mediated responses, as previously reported for BAK1-GFP, BAK1-YFP, BAK1-FLAG, and BAK1-CITRINE (Albrecht et al., 2008; Wang et al., 2008; Jaillais et al., 2011). In only one case did we find that BAK1-HA_3 was not fully complementing the hyporesponsiveness of the null *bak1-4* mutant to BR in the root inhibition assay. This highlights that the regulatory molecular mechanisms underlying BAK1-dependent BR- or PAMP-induced responses are mechanistically distinct; something that became evident recently with the characterization of the *bak1-5* allele that specifically impairs PAMP-induced responses but not BR-triggered responses (Schwessinger et al., 2011). In addition, all BAK1 fusion proteins were still able to form a ligand-induced complex with FLS2, consistent with our

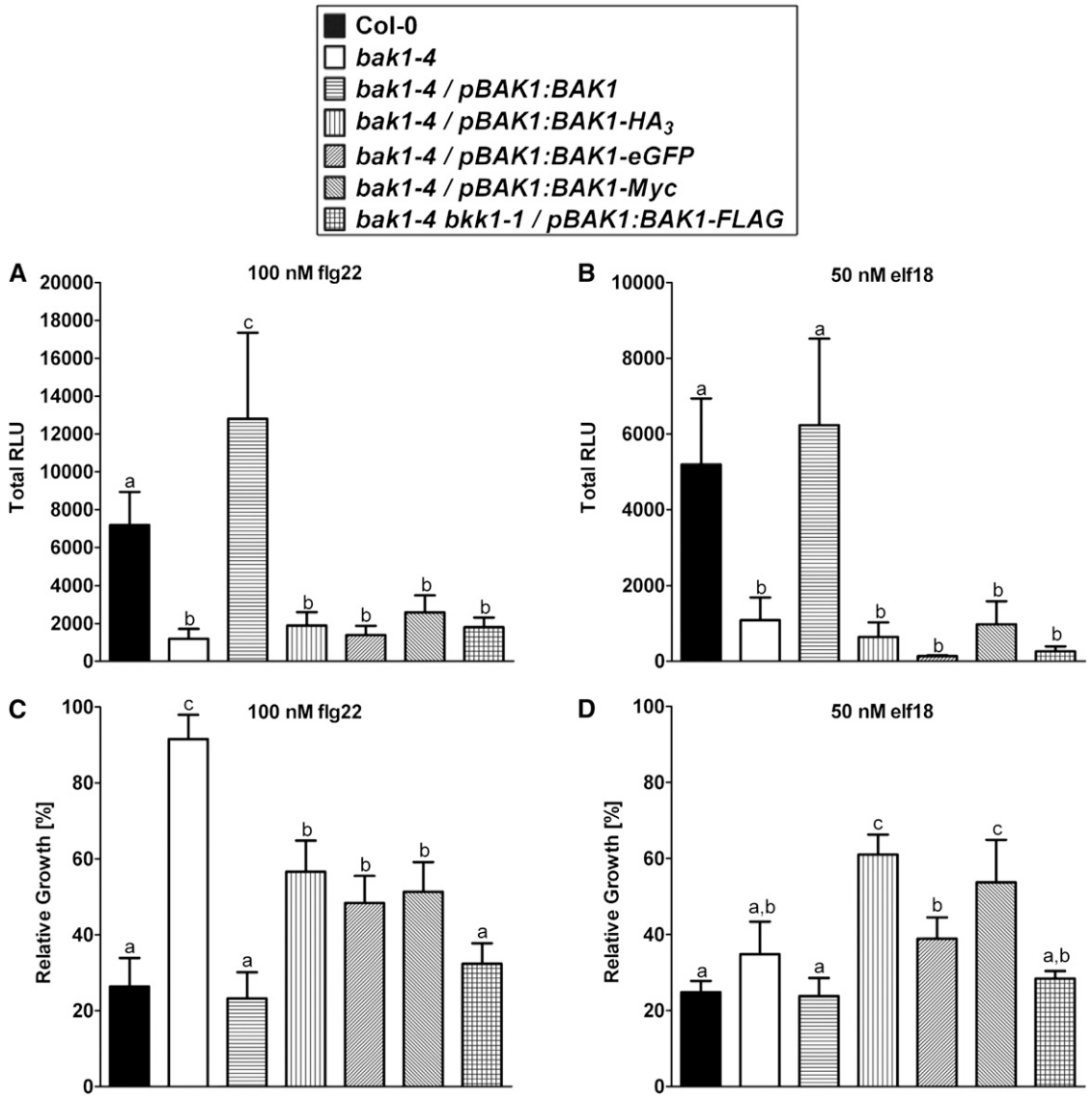

Figure 3. C-Terminally Tagged BAK1 Proteins Are Unable to Complement Fully the *bak1-4* PTI Phenotypes.

(A) and **(B)** PAMP-triggered ROS burst was measured as relative light units (RLUs) emitted in a luminol-based assay within 40 min after treatment with flg22 **(A)** or elf18 **(B)**.

(C) and **(D)** PAMP-triggered seedling growth inhibition. Relative growth measured as fresh weight of seedlings 1 week after treatment with flg22 **(C)** or elf18 **(D)**. Results are average ± SE ($n = 8$); different letters indicates statistically significant differences ($P < 0.05$; analysis of variance test). These experiments were repeated at least twice with similar results.

previous observation that native and tagged BAK1 proteins in both wild-type and transgenics plants are both able to form ligand-induced complexes with FLS2 or EFR (Roux et al., 2011). This suggests that C-terminally tagged BAK1 fusion proteins can be used to study ligand-induced heterooligomerization with PRRs, although the use of native antibodies is clearly preferred. Importantly, our unexpected findings that the presence of a C-terminal tag on BAK1 affects FLS2- and EFR-mediated signaling demonstrates that caution must be exercised when using BAK1 fusion proteins to analyze PAMP-triggered immune signaling downstream of the PRR-BAK1 complex. It is conceivable that the presence of the C-terminal tags could

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affect (in a nonmutually exclusive manner) phosphorylation events within the PRR-BAK1 complex, association and/or dissociation with downstream components, and phosphorylation events with direct substrates. It is becoming increasingly clear that, in addition to their role in BR responses, BAK1 and other SERKs in *Arabidopsis* and other plant species are master regulators of plant innate immunity (Heese et al., 2007; Shan et al., 2008; Fradin et al., 2009, 2011; Santos et al., 2009; Bar et al., 2010; Krol et al., 2010; Chaparro-Garcia et al., 2011; Mantelin et al., 2011; Roux et al., 2011; Yang et al., 2011) as well as of cell death control (He et al., 2007; Kemmerling et al., 2007; Gao et al., 2009; Wang et al., 2011), pollen development (Albrecht et al., 2005; Colcombet et al., 2005), and organ abscission (Lewis et al., 2010). One must not assume that the model based on analyses between BRI1 and BAK1 is universal, and, in light of our results, it is essential that researchers analyze in detail and quantify the impact of modifying the SERK proteins in transgenic approaches when studying SERK-dependent signaling pathways.

METHODS

Plant Materials, Generation of Transgenic Plants, and Growth Conditions

Arabidopsis thaliana ecotype Col-0 was the background for all mutants and transgenic lines used in this study. Plants were grown at 20 to 21°C with a 10-h photoperiod on soil or on plates containing Murashige and Skoog (MS) salt medium (Duchefa), 1% Suc, and 1% agar with a 16-h photoperiod. The mutant *bak1-4* and the double mutant *bak1-4 bkk1-1* were previously described (Chinchilla et al., 2007; He et al., 2007; Schwessinger et al., 2011). The *bak1-4*/*pBAK1*:*BAK1-Myc* construct, containing the whole genomic region of *BAK1* including a 1-kb promoter fragment upstream of the start codon, and the *bak1-4 bkk1-1*/*pBAK1*:*BAK1-FLAG* construct, containing the coding sequence of *BAK1* ligated to a 1.7-kb promoter fragment, were previously described (Chinchilla et al., 2007; Wang et al., 2008; Oh et al., 2010). The *pBAK1*:*BAK1-HA*$_3$ construct was generated as described previously for *pBAK1*:*BAK1* and *pBAK1*:*BAK1-eGFP*, containing the whole genomic region of *BAK1*, including a 1.5-kb promoter fragment (Schwessinger et al., 2011), with the exception of using *epiGreenB*(HA_3) as final binary vector. The *pBAK1*:*BAK1*, *pBAK1*:*BAK1-HA*$_3$, and *pBAK1*:*BAK1-eGFP* constructs were transformed into *bak1-4* by floral dipping. Homozygous lines carrying a single transgene insertion were selected, and T3 plants were used for detailed phenotypic characterization.

Quantitative RT-PCR

Sterilized seeds were sown on MS medium supplemented with 1% Suc and 0.8% agar. Plates were kept at 4°C for 2 d and then placed in the light chamber for 5 d. Seedlings were transferred in 800 μL liquid MS medium supplemented with 1% Suc and kept in the light chamber for an additional 7 d. For the brassinolide (BL)–regulated gene assay, 16 h prior to the treatment, the medium was replaced and supplemented with 2.5 μM brassinazole. Seedlings were treated with water or 200 nM BL for 3 h. The relative expression values were determined using *U-box* (At5g15400) as reference gene and the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$). Primers used for quantitative PCR are as follows: BAK1, 5′-GACAACCGCAGTGCGTGGGA-3′ and 5′-TCGCGAGGCGAGCAAGATCA-3′; CPD, 5′-CCCAAACCACTTCAAAGATGCT-3′ and 5′-GGGCCTGTCGTTACCGAGTT-3′; SAURAC1, 5′-TTGGGTGCTAAGCAAATTATTCG-3′ and 5′-TCTCCTACATAGACCGCCATGA-3′; U-box, 5′-TGCGCTGCCAGATAATACACTATT-3′ and 5′-TGCTGCCCAACATCAGGTT-3′.

BL Root Length Inhibition

Sterilized seeds were sown on half-strength MS medium supplemented with 1 nM BL and 0.8% agar. Plates were kept at 4°C for 4 d and then placed in the light chamber for 7 d. Root length was measure for 30 seedlings per genotype and plotted as inhibition percentage compared with untreated roots.

Protein Extraction, Immunoprecipitation, and Immunoblotting

Leaves were ground in liquid nitrogen, and extraction buffer (150 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM DTT, 5 mM EDTA, 10 mM NaF, 1 mM $Na_2MoO_4.2H_2O$, 1 mM PMSF [Sigma-Aldrich], 10 mM Na_3VO_4, 1% [v/v] P9599 protease inhibitor cocktail [Sigma-Aldrich], and 1% [v/v] IGEPAL CA-630 [Sigma-Aldrich]) was added. Samples were cleared by centrifugation at 25,000*g* for 30 min at 4°C and adjusted to 2 mg/mL total protein concentration. Immunoprecipitations were performed on 1.5 mL volume by adding 25 μL true-blot anti-rabbit Ig beads (Ebioscience) and 20 μL anti-BAK1 antibody (Schulze et al., 2010) and incubation at 4°C for 3 to 4 h. Beads were washed five times with extraction buffer and boiled for 10 min in 25 μL SDS loading buffer. Immunoblotting was performed as previously described (Schwessinger et al., 2011).

ROS Burst Assay

Eight leaf discs (4-mm diameter) of at least four 3- to 4-week-old plants were sampled using a cork borer and floated overnight on sterile water. The following day, the water was replaced with a solution of 17 μg/mL (w/v) luminol (Sigma-Aldrich) and 10 μg/mL horseradish peroxidase (Sigma-Aldrich) containing 50 nM elf18 or 100 nM flg22. Luminescence was captured using a Photek camera.

Seedling Growth Inhibition

Seeds were surface sterilized, sown on MS medium, stratified for 2 d at 4°C in the dark, and transferred to light. Five-day-old seedlings were transferred into liquid MS with or without the indicated concentration of peptide and incubated for an additional 8 d. Fresh weight of six replicates per

treatment was measured using a precision scale (Sartorius) and plotted relative to untreated control.

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AUTHOR CONTRIBUTIONS

V.N., B.S., C.S., and C.Z. designed the research and analyzed data. V.N., B.S., and C.S. performed the research. V.N., B.S., C.S., and C.Z. wrote the article.

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