INTRODUCTION

Plants are equipped with a multilayered recognition system that detects and activates immunity against microorganisms potentially detrimental to the plant health. Plant immune responses are initiated by perception of highly conserved pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by cell-surface-localized pattern recognition receptors (PRRs). Here, we investigated the role in PTI of Arabidopsis thaliana brassinosteroid-signalling kinases 7 and 8 (BSK7 and BSK8), which are members of the receptor-like cytoplasmic kinase subfamily XII. BSK7 and BSK8 localized to the plant cell periphery and interacted in yeast and in planta with FLS2, but not with other PRRs. Consistent with a role in FLS2 signalling, bsk7 and bsk8 single and bsk7,8 double mutant plants were impaired in several immune responses induced by flg22, but not by other PAMP/DAMPs. These included resistance to Pseudomonas syringae and Botrytis cinerea, reactive oxygen species accumulation, callose deposition at the cell wall, and expression of the defence-related gene PR1, but not activation of MAP kinases and expression of the FRK1 and WRKY29 genes. bsk7, bsk8, and bsk7,8 double mutant plants also displayed enhanced susceptibility to P. syringae and B. cinerea. Finally, BSK7 and BSK8 variants mutated in their myristoylation site or in the ATP-binding site failed to complement defective phenotypes of the corresponding mutants, suggesting that localization to the cell periphery and kinase activity are critical for BSK7 and BSK8 functions. Together, these findings demonstrate that BSK7 and BSK8 play a role in PTI initiated by recognition of flg22 by interacting with the FLS2 immune receptor.

KEYWORDS

damage-associated molecular pattern, pathogen-associated molecular pattern, pattern recognition receptor, plant immunity, receptor-like cytoplasmic kinase, signal transduction
actions of BSK7 and BSK8 with FLS2. By loss-of-function analysis, we reveal physical inter-
complexes with PRRs and downstream components, regulation of ROS
subfamily XII have been shown to play roles in PTI by formation of
Arabidopsis
RLCK
Rao et al., 2018; Shi et al., 2013). Members of the
MAPKKK5 to regulate immunity (Yan et al., 2018). Accordingly,
PTI signalling. BSK1 associates with the PRR FLS2 and phosphorylates
et al., 2008). Recent studies revealed that certain BSKs are involved in
signalling and growth (Ren et al., 2019; Sreeramulu et al., 2013; Tang
subfamily XII are named brassinosteroid- signalling kinases (BSKs)
in ROS accumulation and defence gene expression upon PAMPs/
mitogen-activated protein kinases (MAPKs), deposition of callose
responses referred to as pattern-triggered immunity (PTI) that inclu-
des generation of reactive oxygen species (ROS), activation of
mitogen-activated protein kinases (MAPKs), deposition of callose
at the plant cell wall, and accumulation of defence-related proteins
(Tang et al., 2017).
Plant PRRs mainly consist of receptor-like kinases (RLKs) and
receptor-like proteins (RLP) (Tang et al., 2017). RLKs contain an extracellu-
lar domain for PAMP/DAMP perception, a transmembrane domain,
and a cytoplasmic kinase domain. RLPs have a similar domain composi-
tion as RLKs but lack a cytoplasmic kinase domain. The most extensively
studied PRRs in Arabidopsis thaliana are FLS2, which recognizes the flg22 epitope of flagellin (Zipfel et al., 2004);
EFR, which binds the elf18 epitope of elongation factor Tu (Zipfel et al., 2006); and PEPR1 and PEPR2, which recognize the DAMP pep1 (Krol et al., 2010). Plants also encode a large repertoire of receptor-like cytoplasmic kinases (RLCKs) that are evolutionarily related to RLKs but lack an extracellular domain and a transmembrane domain (Liang & Zhou, 2018). RLCKs are typically recruited by PRRs to transduce ligand perception to downstream signalling compo-
nents (Liang & Zhou, 2018).

RLCKs from the 46-member subfamily VII and the 12-member
subfamily XII have been shown to play roles in PTI by formation of complexes with PRRs and downstream components, regulation of ROS production and MAPK activation by phosphorylation, and induction of defence-related genes (e.g., Liang & Zhou, 2018; Majhi et al., 2019; Rao et al., 2018; Shi et al., 2013). Members of the Arabidopsis RLCK subfamily XII are named brassinosteroid-signalling kinases (BSKs) because of the established role of several of them in brassinosteroid signalling and growth (Ren et al., 2019; Sreeramulu et al., 2013; Tang et al., 2008). Recent studies revealed that certain BSKs are involved in PTI signalling. BSK1 associates with the PRR FLS2 and phosphorylates MAPKKK5 to regulate immunity (Yan et al., 2018). Accordingly, bsk1 mutant plants accumulate reduced levels of salicylic acid, are impaired in ROS accumulation and defence gene expression upon PAMPs/DAMPs treatment, and display enhanced susceptibility when chal-
gened with fungal and bacterial pathogens (Shi et al., 2013). BSK3 was shown to interact in vivo with multiple RLKs involved in immunity (Xu et al., 2014), but its function remains to be elucidated. BSK5 associates with multiple PRRs and plays a role in ROS production, callose depo-
sition, and defence gene expression mediated by the flg22, elf18, and pep1 PAMPs/DAMPs (Majhi et al., 2019). Accordingly, bsk5 mutant plants are more susceptible than wild-type plants to fungal and bac-
terial pathogens (Majhi et al., 2019), whereas plants overexpressing
BSK5 are more resistant to pathogens (Majhi & Sessa, 2019).

It is still unknown whether other Arabidopsis BSK family members play a role in plant immunity. In previous studies, BSK8 was found to associate with FLS2 in a protein complex (Qi et al., 2011) and to be phosphorylated upon flg22 treatment (Benschop et al., 2007). In this study, we demonstrate that BSK8 and the closely related BSK7 play a role in PTI initiated by the PRR FLS2. By protein–protein interaction studies in yeast, in planta, and in vitro, we reveal physical inter-
actions of BSK7 and BSK8 with FLS2. By loss-of-function analysis, we show that BSK7 and BSK8 are required for flg22-induced PTI against Pseudomonas syringae and Botrytis cinerea, and for proper ROS production, callose deposition, and expression of the defence gene PR1, but not for MAPK activation. Finally, we provide evidence suggesting that association with the cell plasma membrane and ki-
nase activity are required for BSK7 and BSK8 biological function.

2 | RESULTS

2.1 | BSK7 and BSK8 interact in yeast with the FLS2 PRR

BSK1 and BSK5, which are members of the Arabidopsis BSK fam-
ily of RLCKs, were recently found to play important roles in PTI
signalling by interacting with plant PRRs (Majhi et al., 2019; Shi
et al., 2013). To identify additional BSKs that are involved in plant
immunity, we used a yeast two-hybrid system to test the interac-
tion of BSK family members (BSK1–11) with the cytoplasmic do-
main of the PPR1 (PEPR1-CD) RLK, which recognizes the DAMP
pep1 (Krol et al., 2010), and with that of the EFR (EFR-CD) and FLS2
(FLS2-CD) RLKs, which are well-characterized receptors of the bac-
terial PAMPs EF-Tu and flagellin, respectively (Zipfel et al., 2004,
2006). BSKs were individually used as baits and PEPR1-CD, EFR-CD,
or FLS2-CD were used as prey. The catalytically inactive forms
PEPR1-CDK855E, EFR-CDK849N, and FLS2-CDK997A were also used as
preys to stabilize interactions that otherwise may be transient and
not detectable. Expression in yeast of bait and prey proteins was
confirmed by western blot analysis (Majhi et al., 2019). BSK1 specifi-
cally interacted with FLS2-CDK997A (Figure 1), in agreement with the
BSK1–FLS2 association reported to occur in planta (Shi et al., 2013);
BSK3, which was shown to associate with multiple immunity-related
RLKs (Xu et al., 2014), interacted with PEPR1-CDK855E; BSK5 inter-
acted with the catalytically active PEPR1-CD and with the inactive
PEPR1-CDK855E and EFR-CDK849N as we previously reported
(Majhi et al., 2019); and finally, BSK7 and BSK8, whose amino acid
sequences are closely related (89% identity), interacted with FLS2-
CDK997A in line with the report that BSK8 forms a complex with FLS2
(Qi et al., 2011), and revealing a novel interaction between BSK7 and
FLS2. BSK2, BSK4, BSK6, BSK9, BSK10, and BSK11 did not interact
with any of the tested PRRs (Figure S1). Because of the novelty of
the BSK7–FLS2 interaction and the lack of a functional characteriza-
tion for BSK7 and its closely related homolog BSK8, we focused our
investigation on validating the association of these two proteins with
FLS2, and elucidating their involvement in FLS2-mediated signalling.

2.2 | BSK7 and BSK8 interact in planta with FLS2, but they are not phosphorylated by FLS2 in vitro

To validate the observations detected in yeast, the interactions of
BSK7 and BSK8 with full-length FLS2, EFR, and PEPR1 were exam-
ned in Arabidopsis protoplasts by split luciferase complementation
assays. BSK7 and BSK8 were fused to the C-terminal half of the luciferase protein (C-LUC) and coexpressed in protoplasts along with PEPR1, EFR, or FLS2 fused to the N-terminal half of luciferase (N-LUC). As negative control, C-LUC-BSK7 or C-LUC-BSK8 were coexpressed with N-LUC-GFP. Expression of the examined fusion proteins was confirmed by western blot analysis (Figure S2a; Majhi et al., 2019). The interaction of BSK7 and BSK8 with each PRR was monitored by measuring luminescence at 8 hr after cotransfection of the tested protein pairs. C-LUC-BSK7 and C-LUC-BSK8 interacted with N-LUC-FLS2, but not with N-LUC-PEPR1 or N-LUC-EFR (Figure 2a). Similar interactions were observed when the same protein pairs were expressed via *Agrobacterium tumefaciens* in *Nicotiana benthamiana* leaves (Figures 2b and S2b). To assess whether the interactions of BSK7 and BSK8 with FLS2 are direct or involve additional proteins, the GST-BSK7 and GST-BSK8 fusion proteins were expressed in *Escherichia coli* and immobilized onto glutathione agarose beads. The His-tagged catalytically inactive forms PEPR1-CDK855E, EFR-CD849N, and FLS2-CD997A were used for pull-down assays. FLS2-CD997A, but not PEPR1-CDK855E and EFR-CD849N, was pulled down by GST-BSK7 and GST-BSK8 (Figure 2c,d), indicating a direct interaction between BSK7 and BSK8 with FLS2. Taken together, these results obtained in different experimental systems indicated that BSK7 and BSK8 physically interact with the FLS2 PRR, but not with EFR or PEPR1.

To explore the hypothesis that BSK7 and BSK8 are signalling components acting downstream of FLS2, we tested whether BSK7 and BSK8 are substrates of FLS2 phosphorylation. We used a kinase assay to assess whether FLS2 phosphorylates BSK7 and BSK8 in vitro. BRI1, which was previously shown to phosphorylate BSK8, was used as a positive control (Sreeramulu et al., 2013). BSK7 and BSK8, and the kinase domain of FLS2 (FLS2-KD) were fused to glutathione-S-transferase (GST), while the BRI1 kinase domain was fused to maltose-binding protein (MBP). Fusion proteins were expressed in *E. coli* and purified. GST-FLS2-KD and MBP-BRI1-KD were incubated with each of the GST-BSK fusions in the presence of MgCl₂, MnCl₂, and [γ-³²P]ATP. Reactions were then fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidine difluoride (PVDF) membrane, and exposed to autoradiography. As expected, MBP-BRI1-KD autophosphorylated and phosphorylated the GST-BSK fusions (Figure S3a,b). However, FLS2 autophosphorylation or phosphorylation of BSK7 or BSK8 by FLS2 were not detected (Figure S3a,b). These observations might be due to the fact that FLS2 displays very weak or no kinase activity in vitro, as previously reported (Schwessinger et al., 2011; Zhang et al., 2010).

### 2.3 | BSK7 and BSK8 contribute to immunity against *P. syringae pv. tomato* and *B. cinerea*

To investigate the function of BSK7 and BSK8 in plant immunity, we tested susceptibility of *bsk7* and *bsk8* T-DNA insertion mutants to the biotrophic bacterial pathogen *P. syringae pv. tomato* (Pst).
In bsk7 the T-DNA insertion is in the 5′ untranslated region (UTR) while in bsk8 the insertion is in the fourth exon (Figure 3a). Leaves of 5-week-old Arabidopsis thaliana Col-0 wild-type, bsk7, and bsk8 homozygous mutant plants were inoculated by syringe-infiltration with a bacterial suspension (10^5 cfu/ml). Bacterial populations were determined in leaf tissues sampled at 0 and 4 days postinoculation (dpi). Pst bacteria displayed a significantly higher growth in both bsk7 and bsk8 mutant plants as compared to wild-type plants (Figure 3b). To confirm that the observed phenotype was caused by BSK7 and BSK8 loss of function, transgenic plants expressing the BSK7-HA or BSK8-HA genes driven by their native promoters were generated in the bsk7 and bsk8 mutant backgrounds, respectively (bsk7/BSK7-HA and bsk8/BSK8-HA). Independent transgenic lines (two for each BSK) were selected that expressed either BSK7-HA or BSK8-HA, as detected by western blot analysis using anti-HA antibodies (α:His) and stained with Coomassie blue. Experiments were repeated three times with similar results.

Next, we tested susceptibility of bsk7 and bsk8 mutant plants to the necrotrophic fungal pathogen B. cinerea. Wild-type, bsk7, and bsk8 mutant plants were inoculated by placing a droplet of B. cinerea spore suspension (5 x 10^5 conidia/ml) on their leaves. Infected leaves were monitored for the development of lesions whose diameter was measured at 3 dpi. Symptoms developed more rapidly and lesions were significantly larger in leaves of bsk7 and bsk8 mutants than in wild-type plants or bsk7/BSK7-HA and bsk8/BSK8-HA complemented plants (Figure 3c,d).

Given the high amino acid similarity of BSK7 and BSK8, a bsk7,8 double mutant was generated and included in the pathogenicity assays described above to assess whether the bsk7 and bsk8
mutations have an additive or redundant effect on disease susceptibility. As shown in Figure 3b–d, bsk7,8 plants displayed significantly higher growth of Pst bacteria and larger disease lesions on B. cinerea infection than the bsk7 and bsk8 single mutants. These results indicate that BSK7 and BSK8 are involved with an additive effect in Arabidopsis immunity to both Pst bacteria and the fungus B. cinerea.

**FIGURE 3** Enhanced susceptibility of bsk7, bsk8, and bsk7,8 mutant plants to *Pseudomonas syringae* pv. *tomato* (Pst) and *Botrytis cinerea*. (a) T-DNA insertion sites in bsk7 (locus, At1g63500; mutant, WiscDsLox413-416), bsk8 (locus, At5g41260; mutant, Salk_077982) mutants. Black boxes indicate exons, lines represent introns, and white boxes represent untranslated regions. (b) Leaves of the indicated genotypes were inoculated by infiltration with a suspension of Pst (10^5 cfu/ml). Bacterial growth was measured at 0 and 4 days postinoculation (dpi). (c, d) Leaves of the indicated genotypes were droplet-inoculated with a suspension of B. cinerea spores (5 × 10^5 conidia/ml). Representative leaves were photographed (c) and the size of disease lesions was measured at 3 dpi (d). Data in (b) and (d) are means ± SE of three biological replicates each including five plants. Data were analysed for significant differences between the means based on Duncan’s multiple range test using analysis of variance (p < .05). Columns with different letters are significantly different from each other.

### 2.4 BSK7 and BSK8 are required for flg22-induced immunity

Because BSK7 and BSK8 interacted with the FLS2 receptor, we tested their requirement for PTI triggered by flg22. To this aim, wild-type (Col-0), bsk7, bsk8, and bsk7,8 mutants, and bsk7/BSK7-HA and bsk8/BSK8-HA complemented plants were pretreated with flg22 or water, and 24 hr later were infected either by infiltrating leaves with a Pst bacterial suspension (10^5 cfu/ml) or by placing on the leaves a droplet of *B. cinerea* spore suspension (5 × 10^5 conidia/ml). *fls2* mutant plants, which carry a mutation in the FLS2 receptor and are insensitive to flg22, were used as control (Zipfel et al., 2004). Pst bacterial populations were determined in leaf tissues sampled at 0 and 4 dpi. In wild-type plants pretreated with flg22, bacterial growth at 4 dpi was lower compared to plants pretreated with water (Figure 4a). However, in bsk7 and bsk8 mutant plants, the effect of pretreatment with flg22 on bacterial growth was significantly reduced than in wild-type plants and severely reduced in bsk7,8 double mutant plants (Figure 4a). In bsk7/BSK7-HA and bsk8/BSK8-HA complemented plants, the growth pattern of Pst was very similar to that observed in wild-type plants (Figure 4a). In plants inoculated with *B. cinerea* lesion size was measured at 3 dpi. In wild-type, bsk7/BSK7-HA, and bsk8/BSK8-HA complemented plants pretreated with flg22 lesion size was significantly smaller as compared to plants pretreated with water (Figure 4b). However, in bsk7 and bsk8 mutant plants the effect of flg22 pretreatment on lesion size was significantly reduced compared to wild-type plants and severely reduced in bsk7,8 double mutant plants (Figure 4b). As expected flg22-induced immunity to Pst and *B. cinerea* was completely abolished in *fls2* mutant plants, which showed similar bacterial growth and lesion size when treated with flg22 or water (Figure 4a,b). Together, these results demonstrate that BSK7 and BSK8 play an important role in PTI induced by flg22 against different pathogens.

To examine whether bsk7 and bsk8 mutants are affected in their responsiveness to other PAMPs in addition to flg22, we examined susceptibility of bsk7, bsk8, and bsk7,8 mutant plants to Pst and *B. cinerea*.
after treatment with elf18, which is recognized by the EFR PRR (Zipfel et al., 2006), and pep1, which is recognized by the PEPR1 and PEPR2 PRRs (Krol et al., 2010). In all the mutant genotypes tested, the effect of elf18 or pep1 pretreatment on Pst bacterial growth and disease lesions induced by B. cinerea infection was very similar to that observed in wild-type plants, while the efr and pepr1/pepr2 mutants were insensitive to elf18 and pep1, respectively (Krol et al., 2010; Zipfel et al., 2006) (Figure S5a–d). These observations suggest that BSK7 and BSK8 are involved in PTI triggered by flg22, but not by elf18 and pep1.

2.5 | bsk7, bsk8, and bsk7,8 mutants display a reduction in flg22-induced ROS production, callose deposition, and PR1 gene expression

To test the involvement of BSK7 and BSK8 in the activation of PTI-associated responses, wild-type plants, bsk7, bsk8, and bsk7,8 mutants, and bsk7/BSK7-HA and bsk8/BSK8-HA complemented plants were treated with flg22, elf18, or pep1 and monitored for accumulation of ROS and callose deposition at the cell wall. On treatment with flg22, bsk7, bsk8, and bsk7,8 mutant plants produced less ROS and accumulated less callose compared with wild-type and complemented plants (Figure 5a,b). Noteworthy, the double mutant bsk7,8 was more severely affected in these phenotypes than single bsk7 and bsk8 mutants (Figure 5a,b). Conversely, on treatment with elf18 and pep1, levels of ROS and callose accumulation in bsk7, bsk8, and bsk7,8 mutant plants were very similar to wild-type and complemented plants (Figures S6a,b and S7a,b).

Monitoring of MAPK phosphorylation in bsk7, bsk8, and bsk7,8 mutant plants revealed that the MAPKs MPK3 and MPK6 were rapidly phosphorylated in response to flg22, elf18, and pep1 in the mutants as well as in wild-type plants (Figures 5c, S6c, and S7c). We hypothesized that a role of BSK7 and BSK8 in MAP kinase phosphorylation is masked by functional overlap between BSK family members. To investigate this possibility, we monitored MAPK phosphorylation triggered by flg22 and elf18 in higher-order bsk mutants. These included triple (bsk3,4,8), quadruple (bsk3,4,7,8), quintuple (bsk3,4,6,7,8), sextuple (bsk1,3,4,6,7,8), and septuple (bsk1,3,4,5,6,7,8) mutants generated by Sreeramulu et al. (2013). In all mutants tested, MPK3 and MPK6 were phosphorylated in response to PAMPs as in wild-type plants (Figure S8a,b).

We then tested the expression pattern of defence-related genes in bsk7, bsk8, and bsk7,8 mutant plants treated with flg22, elf18, or pep1. The genes included in this analysis were FRK1 and WRKY29, which act downstream of MPK3 and MPK6 and are induced at early time points after PAMP treatment (Asai et al., 2002), and PR1, which is induced by salicylic acid and pathogen infection at later time points (Lebel et al., 1998). Quantitative reverse transcription PCR (RT-qPCR) analysis revealed that FRK1 and WRKY29 mRNA accumulated similarly in bsk7, bsk8, and bsk7,8 mutant, and wild-type plants treated with flg22, elf18, or pep1 (Figure 5d,e; Figures S6d,e and S7d,e). However, on flg22 treatment, the PR1 mRNA level was significantly lower in bsk7, bsk8, and bsk7,8 mutant plants than in wild-type and complemented plants (Figure 5f). Conversely, all the mutants accumulated PR1 mRNA levels similar to wild-type plants on elf18 or pep1 treatment (Figures S6f and S7f). Taken together, these results suggest that BSK7 and BSK8 play a role in PTI signalling pathways activated by flg22 that control ROS production, callose deposition, and expression of PR1, but not MAPK phosphorylation and FRK1 or WRKY29 gene expression.
2.6 Localization to the cell periphery is required for the function of BSK7 and BSK8 in immunity

To investigate BSK7 and BSK8 subcellular localization, the BSK7 and BSK8 coding region, either in the wild-type form or carrying a mutation (G2A) in the putative myristoylation site, were fused upstream to the yellow fluorescent protein (YFP). The BSK7-YFP, BSK8-YFP, BSK7<sup>G2A</sup>-YFP, and BSK8<sup>G2A</sup>-YFP fusions were transiently expressed in leaves of N. benthamiana plants via A. tumefaciens and their localization was monitored by fluorescence microscopy. A cyan fluorescent protein (CFP), which localizes to cytoplasm and nucleus, was used as control. As shown in Figure 6a,
BSK7 and BSK8 are anchored to the plasma membrane through myristoylation. (a) The indicated fusion proteins were coexpressed with the cyan fluorescent protein (CFP) in Nicotiana benthamiana leaves via Agrobacterium tumefaciens. After 36 hr, fluorescence was monitored in epidermal cells by confocal microscopy. Yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), and merged fluorescence images are shown. Regions of interest marked in the merged images by dotted squares are magnified and shown in the fourth column of panels. Fluorescence intensity was measured in the YFP and CFP channels along the dotted lines indicated in the magnified regions and plotted in the fifth column of panels. Scale bars in images represent 50 μm, except for the magnified images, where they represent 25 μm. (b) Plants of the indicated genotypes were treated with 1 μM flg22 or water, and 24 hr later were inoculated by infiltration with a suspension of Pseudomonas syringae pv. tomato (Pst) (10^5 cfu/ml). Bacterial growth was measured at 0 and 4 days postinoculation (dpi). Data are means ± SE of three biological replicates each consisting of five plants. PAMP-triggered immunity (PTI) was measured by subtracting bacterial growth at 4 dpi in flg22-treated plants from that in water-treated plants. Data were analysed for significant differences between the means based on Duncan’s multiple range test using analysis of variance (p < .05). Different letters indicate significant differences in PTI between genotypes.
the BSK7-YFP and BSK8-YFP fusion proteins localized exclusively to the cell periphery, while BSK7<sup>G2A</sup>-YFP and BSK8<sup>G2A</sup>-YFP were distributed in the cytoplasm and nucleus. To confirm that a G2A mutation alters the subcellular distribution pattern of BSK7 and BSK8, we measured fluorescence intensity across sections of cells expressing CFP and either wild-type or G2A mutant BSK7-YFP and BSK8-YFP. Similar profiles of fluorescence intensity were observed for the cytoplasm-localized CFP and either wild-type or G2A mutant BSK7-YFP and BSK8-YFP. These results suggest that BSK7 and BSK8 anchor to the plasma membrane through N-terminal myristoylation. To investigate if BSK7 and BSK8 are released from the plasma membrane on PAMP activation, leaves of <i>N. benthamiana</i> transiently expressing the BSK7-YFP and BSK8-YFP were treated with flg22 and examined by fluorescence microscopy 30 min later. BSK7-YFP and BSK8-YFP distribution was unaltered by flg22 treatment (Figure S9), indicating that BSK7 and BSK8 are not released from the plasma membrane on PTI activation.

To further study the role of myristoylation in the BSK7 and BSK8 biological function, we generated <i>Arabidopsis</i> transgenic plants expressing BSK7<sup>G2A</sup>-HA and BSK8<sup>G2A</sup>-HA under the control of the BSK7 and BSK8 native promoters in the respective mutant background (<i>bsk7</i>/BSK7<sup>G2A</sup>-HA and <i>bsk8</i>/BSK8<sup>G2A</sup>-HA). Two independent transgenic lines were selected that expressed either BSK7<sup>G2A</sup>-HA or BSK8<sup>G2A</sup>-HA, as detected by western blot analysis using anti-HA antibodies (Figure S4b). We then tested whether BSK7<sup>G2A</sup>-HA and BSK8<sup>G2A</sup>-HA were able to complement the respective PTI phenotype of <i>bsk7</i> and <i>bsk8</i> mutants, respectively. To this aim, wild-type, <i>bsk7</i>, <i>bsk8</i>, <i>bsk7</i>/BSK7-HA, and <i>bsk8</i>/BSK8-HA plants, and two independent <i>bsk7</i>/BSK7<sup>G2A</sup>-HA and <i>bsk8</i>/BSK8<sup>G2A</sup>-HA lines were pretreated with flg22 or water, and infected with <i>Pst</i> after 24 hr. As observed above, bacterial populations at 4 dpi were lower in wild-type plants pretreated with flg22 as compared to water, while in <i>bsk7</i> and <i>bsk8</i> mutant plants the effect of flg22 pretreatment on bacterial growth was significantly reduced (Figure 6b). As opposed to BSK7-HA and BSK8-HA, BSK7<sup>G2A</sup>-HA and BSK8<sup>G2A</sup>-HA failed to complement the phenotype observed in the respective <i>bsk7</i> and <i>bsk8</i> mutant plants (Figure 6b), suggesting that membrane localization is required for the BSK7 and BSK8 immune function.

### 2.7 The Putative ATP Binding Sites of BSK7 and BSK8 Play a Role in their Immune Function

Next, we investigated the importance of BSK7 and BSK8 kinase activity for their immune function. To this aim, we used BSK7 and BSK8 variants carrying mutations at lysine residues conserved in the ATP binding site of protein kinases and critical for their activity (Lys87; Hanks et al., 1988). BSK7<sup>K87E</sup>-HA and BSK8<sup>K87E</sup>-HA were then tested for their ability to complement the defective PTI response of the respective <i>bsk7</i> and <i>bsk8</i> mutant plants to flg22. For these experiments, we generated transgenic plants expressing BSK7<sup>K87E</sup>-HA and BSK8<sup>K87E</sup>-HA driven by their native promoters in the respective mutant background (<i>bsk7</i>/BSK7<sup>K87E</sup>-HA and <i>bsk8</i>/BSK8<sup>K87E</sup>-HA). Two independent transgenic lines were selected that expressed either BSK7<sup>K87E</sup>-HA or BSK8<sup>K87E</sup>-HA, as detected by western blot analysis using anti-HA antibodies (Figure S4c). Wild-type, <i>bsk7</i>, <i>bsk8</i>, <i>bsk7</i>/BSK7-HA, and <i>bsk8</i>/BSK8-HA plants and two independent <i>bsk7</i>/BSK7<sup>K87E</sup>-HA and <i>bsk8</i>/BSK8<sup>K87E</sup>-HA lines were pretreated with flg22. Twenty-four hours later, plants were infected with <i>Pst</i> and bacterial populations were determined at 4 dpi. Bacterial growth was lower in wild-type plants pretreated with flg22 compared to water, while in <i>bsk7</i> and <i>bsk8</i> mutant plants the effect of flg22 on bacterial growth was significantly reduced (Figure 7). The phenotype observed in <i>bsk7</i> and <i>bsk8</i> mutant plants was completely complemented by BSK7-HA and BSK8-HA, respectively, but not by BSK7<sup>K87E</sup>-HA and BSK8<sup>K87E</sup>-HA (Figure 7). These results suggest that BSK7 and BSK8 kinase activity play an important role in their immune function.

### 3 DISCUSSION

The physical interaction of the <i>Arabidopsis</i> BSK7 and BSK8 RLCKs with the FLS2 PRR in yeast, in planta and in vitro, was the first indication for a possible role of these proteins in plant immunity. Their involvement in signalling pathways that activate immune responses was then confirmed by analysis of <i>bsk7</i> and <i>bsk8</i> T-DNA insertion mutants that were impaired in PTI induced by the flg22 PAMP and are more susceptible to <i>Pst</i> bacteria and to the fungus <i>B. cinerea</i>. The involvement of signalling components in pathways that positively regulate defence responses to pathogens with different lifestyles is not unprecedented, for example <i>Arabidopsis</i> plants carrying a mutation in the gene encoding the ERECTA RLK display enhanced susceptibility to a diverse array of pathogens including the biotrophic bacterium <i>Ralstonia solanacearum</i> (Godiard et al., 2003), the hemibiotrophic fungus <i>Verticillium longisporum</i> (Haffner et al., 2014), and the necrotrophic fungus <i>Plectosphaerella cucumerina</i> (Llorente et al., 2005). BSK7 and BSK8 appear to be important components of signalling initiated by FLS2, but not by EFR and PEPR1 PRRs, as <i>bsk7</i> and <i>bsk8</i> mutant plants were defective in PTI responses activated by flg22, but not by elf18 and pep1. This is similar to BSK1, which mediates PTI responses activated by flg22, but not by elf18 (Shi et al., 2013), and different from BSK5, which is required for PTI induced by multiple PAMPs/DAMPs (Majhi et al., 2019). However, the evidence that <i>bsk7</i> and <i>bsk8</i> mutants are more susceptible to the fungus <i>B. cinerea</i>, which does not contain flagellin, suggests that BSK7 and BSK8 play a role not only in FLS2 signalling, but also downstream of PRRs that recognize fungal PAMPs. A signalling role downstream of either a single or multiple PRRs was previously observed for members of other immunity-associated RLCK families (Rao et al., 2018). For example, BIK1, which is a member of RLCK family VII, interacts with multiple PRRs (i.e., FLS2, EFR, PEPR1, and CERK1) and regulates flg22-, elf18-, pep1-, and chitin-mediated responses (Liu et al., 2013; Lu
et al., 2010; Zhang et al., 2010). Similarly, PBL1, PBS1, and PCRK1 of the same RLCK family are required for PTI responses induced by multiple PAMP/DAMPs (Sreekanta et al., 2015; Zhang et al., 2010). Conversely, PBL27 physically interacts with the chitin receptor CERK1 and contributes to the regulation of chitin-induced immunity, but not to flg22 signalling (Shinya et al., 2014).

A mutation in the BSK1, BSK5, BSK7, and BSK8 genes affects PTI responses initiated by FLS2 (this report; Majhi et al., 2019; Shi et al., 2013). In support of the concept that BSKs participate with distinct functions in signalling initiated by the same PRR, analysis of bsk7,8 double mutant plants revealed enhanced susceptibility to pathogens and reduced flg22-induced PTI responses in the double mutant as compared to bsk7 and bsk8 single mutants. The molecular function(s) of BSK family members that interact with the same PRR remain to be elucidated. It is possible that different BSKs play a function independently of each other or in cooperation, possibly forming dimers or multiprotein complexes. In support of the latter possibility, physical interactions were observed between BSK1, BSK5, and BSK8 (Sreeramulu et al., 2013). As previously proposed (Majhi et al., 2019), it is possible that BSKs play a scaffolding function and each of them associates with a specific client component of PTI signalling and brings it in proximity of the FLS2 PRR for the assembly of a common or multiple immune complex(es).

As opposed to other flg22-induced PTI responses examined (i.e., ROS production, callose deposition, and PR1 gene induction), activation of MPK3 and MPK6 and the expression of the FRK1 and WRKY29 genes, which act downstream of MPK3 and MPK6 (Asai et al., 2002), were not affected in either single bsk7 and bsk8 mutants or in the double bsk7,8 mutant. We hypothesized that the role of BSK7 and BSK8 in MAPK activation may be masked by functional redundancy with other BSK family members, as observed for RLCKs of family VII (Kong et al., 2016). To assess this hypothesis, we analysed Arabidopsis T-DNA insertion lines carrying different mutant combinations including up to seven BSK genes (bsk3,4,8, bsk3,4,7,8, bsk3,4,6,7,8, bsk1,3,4,6,7,8, and bsk1,3,4,5,6,7,8). However, none of the mutants showed reduction in flg22- or elf18-induced MAPK activation compared with wild-type plants. Interestingly, BSK1, which associates with FLS2, was shown to interact with and phosphorylate MAPKK5, and to be required for disease resistance to bacterial and fungal pathogens (Yan et al., 2018). We cannot completely rule out the possibility that the remaining five BSKs (BSK2, BSK9, BSK10, BSK11, and BSK12), which were not included in our mutant lines and tested in our study, are still sufficient for MAPK activation.

In line with their interaction with the FLS2 transmembrane receptor, BSK7 and BSK8 were found to localize to the cell periphery. A similar localization was observed for BSK1, BSK5, and BSK8 (Sreeramulu et al., 2013). As previously proposed (Majhi et al., 2019), it is possible that BSKs play a scaffolding function and each of them associates with a specific client component of PTI signalling and brings it in proximity of the FLS2 PRR for the assembly of a common or multiple immune complex(es).

Kinase assays performed in vitro did not detect phosphorylation of BSK7 or BSK8 by FLS2. Similarly, we were not able to detect BSK7 and BSK8 autophosphorylation in kinase assays carried out in the presence of Mg2+ and Mn2+ ions, indicating that unlike the previously
reported BSK1 and OsBSK3 (Shi et al., 2013; Yan et al., 2018; Zhang et al., 2016), BSK7 and BSK8 do not show kinase activity under our experimental conditions. A protein structure analysis of BSK8 suggested that BSK8 is a pseudokinase (Grütter et al., 2013). However, these results do not rule out that BSK7 and BSK8 have kinase activity in vivo. In support of this hypothesis, mutations in the putative ATP binding sites of BSK7 and BSK8 led to a reduced function in PTI signalling. Considering the structural studies of the BSK8 kinase domain (Grütter et al., 2013), we hypothesize that binding of BSK7 and BSK8 to other plant proteins may change their structural autoinhibitory state to a catalytically active state. Alternatively, in vivo phosphorylation of BSK7 and BSK8 by FLS2 may activate their kinase activity. This hypothesis is supported by the finding that OsBSK3 phosphorylation by OsBRI1 appears to enhance OsBSK3 autophosphorylation activity in vitro (Zhang et al., 2016).

In conclusion, our findings shed light onto the functional role of BSK7 and BSK8 in plant innate immunity, demonstrating they associate with FLS2 and play a role in concert with additional BSK family members in flg22-induced PTI signalling. Future research will be aimed at elucidating BSK7 and BSK8 mechanisms of activation, interacting partners, and mode of cooperation with other BSK family members in PTI signal transduction pathways.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials and growth conditions

Plant cultivars were A. thaliana ecotype Col-0 and N. benthamiana (Goodin et al., 2008). The Arabidopsis T-DNA insertion mutants used were bsk7 (WiscDsLox413-416; Sreeramulu et al., 2013), bsk8 (Salk_077982; Sreeramulu et al., 2013), fhs2 (Salk_141277; Xiang et al., 2008), efr (Salk_044334; Zipfel et al., 2006), and pep1/pepr2 (kindly provided by Jian-Min Zhou; Liu et al., 2013). Mutants were obtained from the Arabidopsis Biological Resource Center. Arabidopsis transgenic lines containing the constructs ProBSK7:BSK7-HA, ProBSK7:BSK7K87E-HA, and ProBSK7:BSK7K87E-CA in the bsk7 mutant background or ProBSK8:BSK8-CA in the bsk8 background were generated by A. tenuifolius-mediated transformation of homozygous bsk7 or bsk8 mutant lines, respectively. Arabidopsis plants were grown in phytochambers at 20–22 °C with 40%–60% humidity and approximately 120 μmol·m⁻²·s⁻¹ light intensity in short day conditions (8 hr light/16 hr dark) for PTI assays or in long day conditions (16 hr light/8 hr dark) for seed set. N. benthamiana plants were grown in a growth room in long day conditions at 25 °C.

4.2 | Bacterial, yeast, and fungal strains and growth conditions

The strains were E. coli DH5α (Invitrogen) and Rosetta (Merck), P. syringae pv. tomato DC3000 (Pst) (Guo et al., 2009), A. tenuifolius GV2260 (Deblaere et al., 1985), Saccharomyces cerevisiae Y2HGold (Clontech), and B. cinerea B05.10 (Ma et al., 2017). Bacterial, yeast, and fungal strains were grown with the appropriate antibiotics as follows: E. coli in Luria-Bertani (LB) medium at 37 °C, Pst and A. tenuifolius in LB medium at 28 °C, S. cerevisiae at 30 °C in selective synthetic complete medium supplemented with 2% glucose, and B. cinerea in potato dextrose broth (PDB) at 20 °C.

4.3 | Peptide elicitors

Peptides of flg22 (QRLSTGSRINSKDDAAGLQIA; Krol et al., 2010), elf18 (Ac-SKEKFTERKPHVNYGTIG; Kunze et al., 2004), and pep1 (ATKVAKQRGKEKVSSGRPQHN; Krol et al., 2010) were obtained from EZBiolab, dissolved in water to stock solutions of 1 mM, and diluted to the working concentration.

4.4 | Construction of vectors

For yeast two-hybrid assays, genes encoding BSK7 (At1g63500) and BSK8 (At5g41260), and cytoplasmic domains of PEPR1 (amino acids 781–1,123; PEPR1-CD), EFR (amino acids 680–1,031; EFR-CD), and FLS2 (amino acids 828–1,173; FLS2-CD) were amplified from Arabidopsis Col-0 cDNA using Phusion DNA polymerase (Thermo Fisher Scientific) and cloned into the pAS1 (bait) or pGADT7 (prey) vectors (Clontech Laboratories) in frame with the GAL4 DNA-binding domain or GAL4 activation domain, respectively. Kinase-deficient PEPR1-CD and EFR-CD were generated by site-directed mutagenesis using the QuickChange II kit (Agilent Technologies).

For split luciferase complementation assays in N. benthamiana leaves, the BSK7, BSK8, PEPR1, EFR, FLS2, and GFP genes were cloned into pCAMBIA1300:C-LUC fused to the C-terminal 398–550 amino acids of firefly luciferase (C-LUC) or into pCAMBIA1300:N-LUC fused to the N-terminal 2–416 amino acids of firefly luciferase (N-LUC) and driven by the CaMV 35S promoter (Chen et al., 2008). For split luciferase complementation assays in Arabidopsis protoplasts, the BSK7 and BSK8 genes fused to C-LUC and PEPR1, EFR, FL2, and GFP genes fused to N-LUC were excised using restriction enzymes or PCR-amplified from the pCAMBIA vectors and inserted into the pTEX vector under the control of CaMV 35S promoter (Frederick et al., 1998).

For protein expression in E. coli, BSK7, BSK8, and the kinase domain of FLS2 (amino acids 828–1,173; FLS2-KD) were fused to the C-terminus of glutathione-S-transferase in the pGEX-4T-1 vector (GE Healthcare) and the kinase domain of BR1 (amino acids 814–1,196; BR1-KD) was fused to the C-terminus of the maltose-binding protein (MBP) in the pMAL-c2x vector (New England Biolabs). PEPR1-CD, EFR-CD, and FLS2-CD were cloned into the pET-16b vector with a 10 × His tag (Novagen).

For subcellular localization, BSK7, BSK8, BSK7G2A, and BSK8G2A coding sequences were fused upstream to the gene encoding the yellow fluorescence protein (YFP) in the pBTEX binary vector under the control of the CaMV 35S promoter (Frederick et al., 1998).
For transgene complementation, BSK7 or BSK8 genomic fragments containing their promoter and terminator regions (ProBSK7:BSK7-HA or ProBSK8:BSK8-HA) were amplified from Col-0 genomic DNA and cloned into the binary vector pCAMBIA3300 (Cambia) with a haemagglutinin (HA) tag.

The mutant BSK7 and BSK8 genomic fragments ProBSK7:BSK7G2A-HA, ProBSK8:BSK8G2A-HA, ProBSK7:BSK7K87E-HA, and ProBSK8:BSK8K87E-HA were generated by site-directed mutagenesis and cloned into the binary vector pCAMBIA3300 for complementation assays. Sequences of oligonucleotides are available upon request.

4.5 | Protoplast preparation and transfection

Protoplasts were prepared from leaves of 5-week-old Arabidopsis plants and transfected with plasmid DNA as described previously (Popov et al., 2016).

4.6 | Agrobacterium-mediated transient expression

For transient expression, A. tumefaciens overnight cultures were pelleted, washed three times with 10 mM MgCl₂, resuspended in induction medium (10 mM MgCl₂, 10 mM MES [pH 5.6], and 200 μM acetoxyrinone), and incubated at 28 °C with shaking for 3–4 hr. A. tumefaciens cultures were diluted to OD₆₀₀ = 0.2 and syringe-infiltrated into leaves of 6-week-old N. benthamiana plants.

4.7 | Arabidopsis transformation

Arabidopsis plants were transformed using the floral dip method with A. tumefaciens GV2260 (Zhang et al., 2006). Transformant seeds were germinated on plates (one-half-strength Murashige and Skoog salts with vitamins supplemented with MES [pH 5.7], 1% wt/vol sucrose, 0.8% wt/vol agar) supplemented with Basta (10 μg/ml) and timentin (150 μg/ml). After 2 weeks, resistant plants were transferred to soil.

4.8 | Expression and purification of GST and MBP fusion proteins in E. coli

BSK7, BSK8, and FLS2-KD were cloned into the pGEX-4T-1 vector, while BRI1-KD was cloned into the pMAL-c2x vector. Proteins were expressed in the E. coli Rosetta strain and purified as described (Majhi et al., 2019).

4.9 | Yeast two-hybrid assay

Yeast two-hybrid interactions were conducted as described (Sreeramulu et al., 2013).

4.10 | Split luciferase complementation assay

Gene fragments encoding full-length BSK7, BSK8, PEPR1, EFR, FLS2, and GFP were cloned in frame to firefly luciferase fragments in the binary vectors pCAMBIA1300:N-LUC or pCAMBIA1300:C-LUC. The obtained vectors were transformed into A. tumefaciens and coexpressed in N. benthamiana leaves. Split luciferase complementation assays were performed as described (Majhi et al., 2019).

4.11 | In vitro pull-down assay

GST, GST-BSK7, and GST-BSK8 were expressed in E. coli and purified as described (Majhi et al., 2019). Purified proteins were incubated with glutathione agarose beads (Sigma-Aldrich) at 4 °C for 2 hr. After three washes with binding buffer, beads were incubated with equal amounts of bacterial lysate containing kinase-deficient PEPR1-CD-His, EFR-CD-His, and FLS2-CD-His recombinant protein with constant rotation at 4 °C for 3 hr. The beads were washed five times with wash buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 mM EDTA) and the bound protein was eluted with elution buffer (25 mM Tris- HCl pH 7.5, 150 mM NaCl and 1 mM EDTA containing 10 mM reduced glutathione). Input and pulled-down proteins were fractionated by 10% (wt/vol) SDS-PAGE and detected by western-blot analysis with anti-His antibodies.

4.12 | Protein extraction

Protein extraction from yeast and leaves, and in vitro kinase assays were performed as described (Majhi et al., 2019).

4.13 | In vitro kinase assay

Kinase assays were performed as described by Zhao et al. (2019) with minor modifications. GST and MBP fusion proteins (0.1–0.5 μg) were incubated at 25 °C for 1 hr in a kinase assay solution containing 25 mM Tris- HCl (pH 7.5), 1 mM dithiothreitol, 10 mM MgCl₂, 10 mM CaCl₂, 10 mM MnCl₂, 20 μM ATP, and 10 μCi [γ³²P]ATP (3,000 Ci/ mmol; PerkinElmer). Reactions were stopped by the addition of SDS-sample buffer. Half of the reaction volume was fractionated by SDS-PAGE and stained with Coomassie blue. The second half was fractionated by SDS-PAGE, transferred onto a PVDF membrane, and the membrane was exposed to autoradiography.

4.14 | Subcellular localization and image analysis

To visualize BSK7 and BSK8 subcellular localization, the BSK7-YFP, BSK8-YFP, BSK7G2A-YFP, and BSK8G2A-YFP fusion proteins were expressed via A. tumefaciens in leaves of 6-week-old N. benthamiana plants. Protein localization was visualized by a confocal laser...
scanning microscope (LSM 780; Zeiss) as described by Majhi et al. (2019). Fluorescence intensities of regions of interest were measured using the Plot Profile tool in the Fiji software (https://fiji.sc/).

4.15 Bacterial growth assay

Four-week-old Arabidopsis plants were inoculated by syringe-infiltration with a suspension (10^5 cfu/ml) of Pst in a 10 mM MgCl2 solution. Three 1-cm diameter leaf discs were sampled at 0 and 4 dpi from five inoculated plants and ground in 1 ml of 10 mM MgCl2. Samples were then 10-fold serially diluted and plated on LB supplemented with 25 µM rifampicin. The colony counts were recorded 2 days after incubation at 28 °C.

4.16 B. cinerea inoculation

B. cinerea spores were diluted to 5 × 10^5 spores/ml in 0.5 × potato dextrose broth. Droplets (10 µl) of 0.5 × potato dextrose broth with B. cinerea spores were deposited on leaf surfaces of 4-week-old Arabidopsis plants (three leaves per plant). After incubation of the inoculated plants at high humidity for 3 days, the size of the disease lesion was measured. At least 15 lesion diameters were evaluated for each independent treatment (five plants).

4.17 PTI assays

ROS, callose deposition, and MAPK phosphorylation assays were carried out as described by Majhi et al. (2019).

4.18 RNA isolation and RT-qPCR

Total RNA was isolated from leaves (60 mg) using the SV total RNA isolation system (Promega). RNA samples (1 µg) were reverse-transcribed with oligo(dT) primers employing a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and subjected to RT-qPCR using gene-specific primers (available on request). cDNAs were amplified using the SYBR Premix Ex Taq II (Clontech) and the Mx3000P System (Agilent Technologies). The ACTIN2 gene was used for normalization and gene expression was calculated by the comparative C_t method (Pfaffl, 2001).

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COMPETING INTERESTS

The authors declare no competing financial or nonfinancial interests.

AUTHOR CONTRIBUTIONS

G.Se. and B.B.M. conceived and designed the experimental plan. G.Se., B.B.M., and G.So. wrote the article. B.B.M., S.G., G.So., and S.S. conducted the experiments, analysed the data, and drafted the figures.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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