

Short communication

Physical association of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) immune receptors in Arabidopsis

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SUMMARY

Plants possess two distinct types of immune receptor. The first type, pattern recognition receptors (PRRs), recognizes microbe-associated molecular patterns (MAMPs) and initiates pattern-triggered immunity (PTI) on recognition. FLS2 is a PRR, which recognizes a part of bacterial flagellin. The second type, resistance (R) proteins, recognizes pathogen effectors and initiates effector-triggered immunity (ETI) on recognition. RPM1, RPS2 and RPS5 are R proteins. Here, we provide evidence that FLS2 is physically associated with all three R proteins. Our findings suggest that signalling interactions occur between PTI and ETI at very early stages and/or that FLS2 forms a PTI signalling complex, some components of which are guarded by R proteins.

INTRODUCTION

Plants can sense microbial organisms by detecting microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) with pattern recognition receptors (PRRs) (Ausubel, 2005; Medzhitov and Janeway, 1997; Tsuda and Katagiri, 2010), which are often leucine-rich repeat receptor kinases (LRR-RKs). One well-studied PRR in Arabidopsis is FLS2, which is an LRR-RK and recognizes a conserved 22-amino-acid fragment (flg22) of bacterial flagellin (Chinchilla *et al.*, 2006; Gomez-Gomez and Boller, 2000; Zipfel *et al.*, 2004). flg22 perception by FLS2 triggers a chain of signalling events that eventually results in induced immunity called pattern-triggered immunity (PTI) (Chinchilla *et al.*, 2007a; Jones and Dangl, 2006; Tsuda and Katagiri, 2010; Zipfel *et al.*, 2004).

Another mode of induced immunity in plants is called effector-triggered immunity (ETI) (Jones and Dangl, 2006), also known as gene-for-gene resistance (Flor, 1971). ETI signalling is initiated on

the basis of the perception of pathogen effectors by plant disease resistance (R) proteins, either directly or indirectly (Dangl and Jones, 2001; Jones and Dangl, 2006). Most R proteins are members of the nucleotide-binding site/leucine-rich repeat (NB-LRR) family (Dangl and Jones, 2001). Examples of well-studied R proteins in Arabidopsis include RPM1, RPS2 and RPS5 (Bent *et al.*, 1994; Grant *et al.*, 1995; Mindrinos *et al.*, 1994; Warren *et al.*, 1998). Indirect recognition of effectors by R proteins is explained by the 'guard hypothesis', in which R proteins 'guard' certain host proteins ('guardees') that are manipulated by pathogen effectors (Dangl and Jones, 2001). One well-known 'guardee' is RIN4, which is targeted by at least three bacterial effector proteins (AvrRpm1, AvrB and AvrRpt2) and 'guarded' by two R proteins (RPM1 and RPS2) (Axtell and Staskawicz, 2003; Mackey *et al.*, 2002, 2003). Another 'guardee' is PBS1, which is targeted by the bacterial effector protein AvrPphB and 'guarded' by RPS5 (Shao *et al.*, 2003). Accumulated evidence suggests an arms race between pathogens and plants, in which pathogens interfere with plant PTI using effectors and plants evolve to mount strong ETI responses on recognition of effectors (Boller and He, 2009; Chisholm *et al.*, 2006; Jones and Dangl, 2006). However, not much is known about how PTI and ETI interplay to coordinate plant immunity. Here, we show evidence that the PTI and ETI receptors can reside in the same protein complex, which raises the possibility that PTI and ETI signalling interact at the very beginning.

RESULTS

Previously, we have developed a method to purify RPS2 protein complexes using a biotin tag called the 'HPB' tag and identified putative complex components by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Qi and Katagiri, 2009). Among eight replicate assays, we identified peptides of BSK1 (At4g35230, IPI00530358; two peptide hits) and BSK8 (At5g41260, IPI00529310; one peptide hit) at a 95% probability level, once each in the samples pulled down with RPS2, but not in negative control samples even at a 50% confidence level

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(Fig. S1, see Supporting Information) (Qi and Katagiri, 2009). The BSK family consists of 12 members (Tang *et al.*, 2008), including BSK1 and BSK8, and the family members belong to the receptor-like cytoplasmic kinase (RLCK) subfamily XII (Shiu *et al.*, 2004). Some BSKs, including BSK1, positively participate in brassinosteroid signalling through a physical association with the BRI1 receptor (Tang *et al.*, 2008). Both FLS2 and BRI1 are LRR-RKs and physically associate with an LRR-RK, BAK1 (Chinchilla *et al.*, 2007b; Li *et al.*, 2002; Nam and Li, 2002). Furthermore, BSK8 was phosphorylated on flg22 treatment (Benschop *et al.*, 2007). These lines of evidence prompted us to investigate the possibility that BSKs physically associate with FLS2.

First, the subcellular localization of BSK8 was examined using Arabidopsis plants expressing the *BSK8::YFP::HA* transgene and confocal microscopy. Full-length BSK8::YFP::HA protein was detected (Fig. S2, see Supporting Information) and clearly localized to the plasma membrane (PM) (Fig. 1A), which is the same as the localization of other BSK members (Tang *et al.*, 2008). As the PM localization of BSK8 was consistent with the PM localization of FLS2 (Gohre *et al.*, 2008; Robatzek *et al.*, 2006), we next examined a possible physical association between BSK8 and FLS2 by a pulldown assay. FLS2::HPB (Qi and Katagiri, 2009) and BSK8::GFP were transiently expressed in *Nicotiana benthamiana*. HPB-tagged proteins are biotinylated in plant cells and thus readily captured by streptavidin beads (Qi and Katagiri, 2009). Indeed, BSK8::GFP was pulled down by FLS2::HPB with streptavidin beads from the sample in which both proteins were co-expressed, but not from the negative control sample where only BSK8::GFP was expressed (Fig. 1B). In addition, N-terminus Myc-tagged BSK8 (Myc::BSK8) was pulled down by FLS2::HPB (Fig. S3, see Supporting Information).

The finding that BSK8 appears to physically associate with both FLS2 and RPS2 led us to hypothesize that RPS2 and FLS2 may reside in the same protein complex. To test this idea, we performed a pulldown assay using a plant line transgenically expressing *RPS2::HPB* from the *RPS2* promoter in the *rpm1 rps2* double-mutant background (Qi and Katagiri, 2009). FLS2 was pulled down by *RPS2::HPB* from *rpm1 rps2 RPS2::HPB* plants, but not from negative control *rpm1 rps2* plants (Fig. 2A). Therefore, FLS2 and RPS2 are physically associated. Next, we determined whether FLS2 forms protein complexes with RPM1 and RPS5, using a pulldown assay with transgenic plants expressing *RPM1::HPB* and *RPS5::HPB* (Qi and Katagiri, 2009). FLS2 was also pulled down by *RPM1::HPB* and *RPS5::HPB* (Fig. 2A). The anti-FLS2 antibody recognized a band of ~175 kDa in Col wild-type plants, but none in *fls2* mutants (Fig. 2B), indicating that the antibody is specific to FLS2, as shown previously (Chinchilla *et al.*, 2006). RIN4, which is known to physically interact with RPS2 and RPM1 (Axtell and Staskawicz, 2003; Mackey *et al.*, 2002, 2003), served as a positive control and was clearly pulled down by both *RPS2::HPB* and *RPM1::HPB* (Fig. 2A). All of the

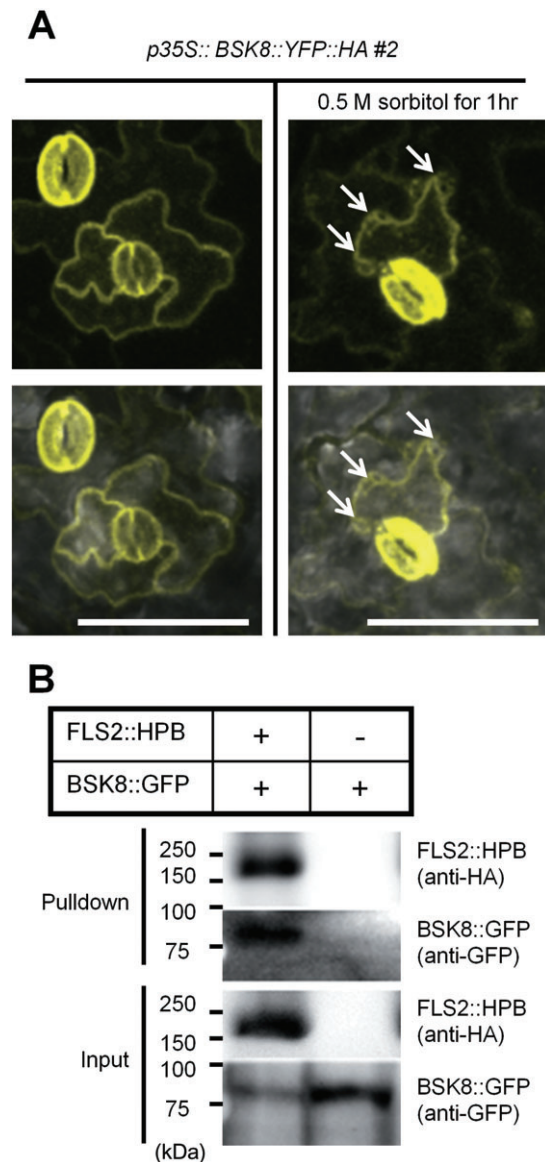


Fig. 1 Plasma membrane (PM)-localized BSK8 is physically associated with FLS2 *in planta*. (A) BSK8 is localized to PM. Leaves of 5-week-old T3 homozygous *p35S::BSK8::YFP::HA* line #2 plants were employed for the detection of yellow fluorescent protein (YFP) signal in epidermal cells using confocal microscopy. The top panel shows the YFP channel signal. The bottom panel shows a merged photograph of both the YFP channel and the bright field channel. Pretreatment with 0.5 M sorbitol for 1 h resulted in the detachment of PM from the cell wall (shown by white arrows), indicating that BSK8 is localized to PM, not to the cell wall. Bar, 100 μ m. (B) BSK8::GFP forms a protein complex with FLS2::HPB in *Nicotiana benthamiana*. BSK8::GFP and FLS2::HPB were transiently expressed in *N. benthamiana* leaves, and FLS2::HPB was pulled down with streptavidin beads. *Nicotiana benthamiana* leaves expressing only BSK8::GFP were used as a negative control. Both pulldown samples and input samples were analysed by immunoblotting using anti-haemagglutinin (HA) and anti-green fluorescent protein (GFP) antibodies. Both experiments were performed twice with similar results.

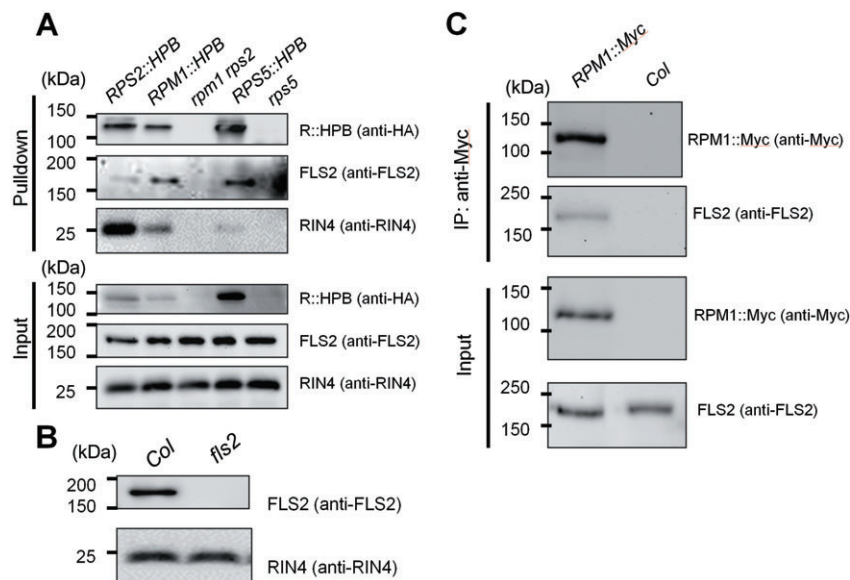


Fig. 2 FLS2 was pulled down by three distinct R proteins in Arabidopsis. (A) The pull-down assays were performed using solubilized membrane proteins prepared from 5-week-old Arabidopsis plants of *RPS2::HPB rpm1 rps2* (*RPS2::HPB*), *RPM1::HPB rpm1 rps2* (*RPM1::HPB*), *rpm1 rps2* (control), *RPS5::HPB rps5* (*RPS5::HPB*) and *rps5* (control). The HPB-tagged proteins were pulled down with streptavidin beads. The pull-down and input samples were examined by immunoblotting with anti-haemagglutinin (HA) and anti-FLS2 antibodies. (B) Anti-FLS2 antibody specifically recognizes FLS2, but not other proteins. Detection of RIN4 by an anti-RIN4 antibody was used as a loading control. (C) The pull-down assay was performed using solubilized membrane proteins prepared from 4-week-old Arabidopsis plants of *RPM1::Myc* and Col (control). *RPM1::Myc* was pulled down with an anti-Myc antibody. Both pull-down and input proteins were examined by immunoblotting with anti-Myc and anti-FLS2 antibodies. The experiments shown in (A) and (B) were performed three times with similar results, and that in (C) was performed twice with similar results.

Arabidopsis lines used in the study showed high expression of the wild-type 3-methylcrotonyl CoA carboxylase (MCCA), from which the biotinylation site of the HPB tag was derived (Qi and Katagiri, 2009). As FLS2 was not pulled down from the plant lines without *R::HPB* transgenes (although a large amount of MCCA was present in the samples), FLS2 did not physically associate with the biotinylation site of the HPB tag. In addition, as the amount of FLS2 pulled down did not correlate with the amount of *R::HPB* expressed (*RPM1::HPB* pulled down more FLS2 than *RPS2::HPB*, whereas *RPS2::HPB* accumulated more than *RPM1::HPB*; Fig. 2A), it is very unlikely that FLS2 was pulled down through a physical association with the HPB tag. To prove that the FLS2–R protein complex formation does not depend on the HPB tag, we immunoprecipitated *RPM1::Myc*, which does not contain any part of the HPB tag, from extracts of an Arabidopsis *RPM1::Myc* transgenic line using an anti-Myc antibody to determine whether FLS2 would be pulled down by *RPM1::Myc*. As shown in Fig. 2C, FLS2 was indeed pulled down by *RPM1::Myc*. We thus conclude that the FLS2–R protein complexes are not artefacts arising from the HPB tag.

To further confirm the R protein–FLS2 association *in vivo*, a reciprocal pull-down assay was conducted between *RPM1* and FLS2. The *FLS2::HPB* plant line (Qi and Katagiri, 2009) was crossed to a plant line expressing *RPM1::Myc* (Boyes *et al.*,

1998). Pull-down was then conducted using F1 plants which express both tagged proteins and control plants expressing only *RPM1::Myc*. *RPM1::Myc* was pulled down by *FLS2::HPB* from F1 plants, but not from the *RPM1::Myc* control plant (Fig. 3). Thus, we demonstrated *RPM1*–*FLS2* association in a reciprocal manner.

It has been shown that RIN4 interacts with both RPS2 and RPM1 (Fig. 2A) (Axtell and Staskawicz, 2003; Mackey *et al.*, 2002, 2003; Qi and Katagiri, 2009) and participates in FLS2 signalling (Kim *et al.*, 2005). We therefore examined whether RIN4 was also residing in the FLS2–protein complex. We conducted a pull-down assay with *FLS2::HPB* plants and used an *fls2* mutant as a control. We were able to pull down RIN4 with *FLS2::HPB* when the cross-linker dithiobis(succinimidyl propionate) (DSP) was applied (Fig. 4). It should be noted that the cross-link was cleaved before resolving the pulled down proteins by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). As DSP cross-linking greatly increased the pull-down of RIN4 by both RPS2 (Qi and Katagiri, 2009) and RPM1 (data not shown), the results presented here indicate that RIN4 is probably associated with FLS2, but its associations with RPS2, RPM1 or FLS2 are relatively weak and mostly disrupted by the stringent wash conditions used in the pull-down assay.

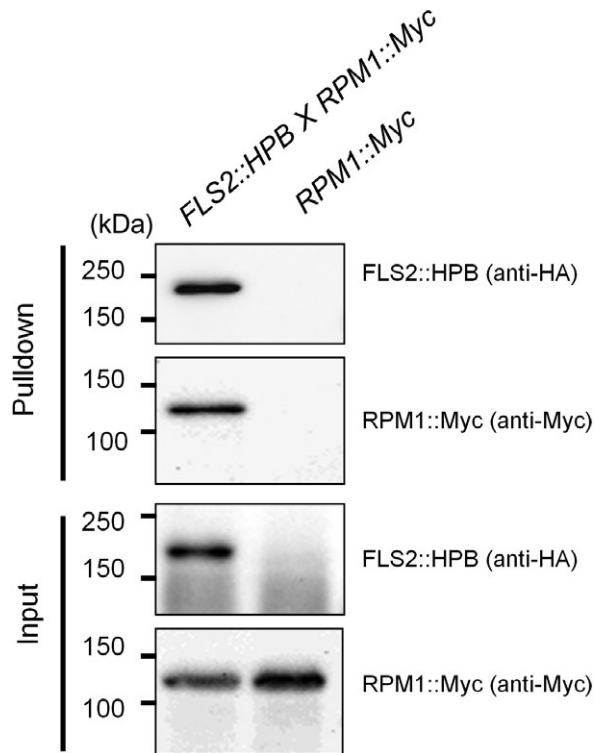


Fig. 3 RPM1 was pulled down by FLS2 in Arabidopsis. The pull-down assays were performed using solubilized membrane proteins prepared from 5-week-old Arabidopsis plants of genotypes *FLS2::HPB* × *RPM1::Myc* (F1 generation) and *RPM1::Myc* (control). *FLS2::HPB* was pulled down with streptavidin beads. The pull-down and input samples were examined by immunoblotting with anti-haemagglutinin (HA) and anti-Myc antibodies. This experiment was performed three times with similar results.

DISCUSSION

We have demonstrated that the PRR FLS2 forms a complex with the R proteins RPS2, RPM1 and RPS5. For the pull-down assay results shown in Figs 2A,C, 3 and 4, the tagged proteins were transgenically expressed from their own promoters (except for *RPM1::HPB*, for which the *RPS2* promoter was used), which substantially reduces the possibility of artefactual associations as a result of misexpression of the proteins. We also used stringent wash conditions in the pull-down assay: 1% Nonidet P40 (NP40), which is known to solubilize the protein-rich microdomains of membranes called lipid rafts (Rixon *et al.*, 2004), and 0.5% sodium deoxycholate, which is an ionic detergent, in two different salt concentrations (150 and 50 mM NaCl). The fact that the membrane-integrated protein RIN4 was barely pulled down by FLS2 without cross-linking (Fig. 4) strongly suggests that such membrane microdomains were efficiently disrupted in our standard pull-down assay procedure, which does not include protein cross-linking. It is likely that the protein complexes containing FLS2 and R proteins observed here were formed through strong physical associations.

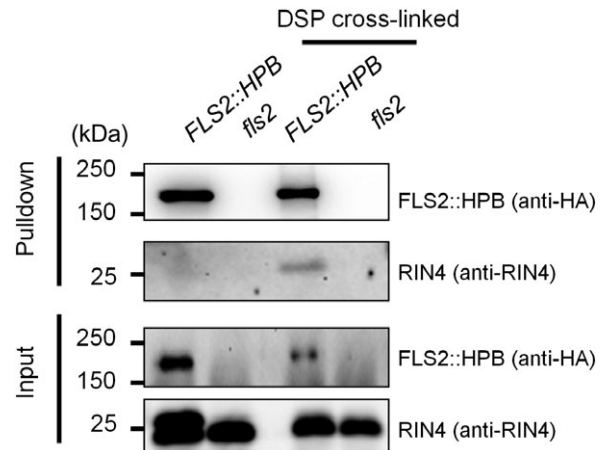


Fig. 4 FLS2 and RIN4 may reside in the same protein complex. The pull-down assays were performed using solubilized membrane proteins [either without or with dithiobis(succinimidyl propionate) (DSP) cross-linking] prepared from 5-week-old Arabidopsis plants of *FLS2::HPB fls2* (*FLS2::HPB*) and *fls2* (control). *FLS2::HPB* was pulled down with streptavidin beads. The pull-down and input samples were examined by immunoblotting with anti-haemagglutinin (HA) and anti-RIN4 antibodies. This experiment was performed three times with similar results.

RPS2, RPM1 and RPS5 are all PM-localized R proteins of the coiled-coil (CC)-NB-LRR class (Axtell and Staskawicz, 2003; Boyes *et al.*, 1998; Dangl and Jones, 2001; Holt *et al.*, 2005). We have discovered another characteristic common to these three R proteins: the ability to associate with the PTI receptor FLS2. Whether the physical association between two types of immune receptor is a general phenomenon is an intriguing question. Although FLS2 was not included among the RPS2 complex component candidates, one receptor-like kinase (At4g08850) was (Qi and Katagiri, 2009). At4g08850 might be an unidentified PRR and, if so, PRRs other than FLS2 might form complexes with RPS2 and other R proteins.

What is the biological significance of PTI and ETI receptors residing in the same protein complex(es)? There are at least two hypotheses, which are not mutually exclusive. In the first hypothesis, such an association may be a result of the fact that PTI and ETI share signalling components. For example, recent work has shown that PTI and ETI share a highly overlapping signalling network (Tsuda *et al.*, 2009). Residence in the same protein complex may simply be a fortuitous consequence, as both types of receptor physically associate with the common signalling components. Alternatively, there could be functional significance in having two types of receptor in a single protein complex: PTI and ETI signalling may interact with each other through this protein complex. For example, the activation of PTI signalling might sensitize ETI signalling. This is an attractive hypothesis, and it is likely that PTI and ETI signalling interact in some ways. However, without knowing the exact composition and functional

modes of this presumptive protein complex, it is very difficult to pinpoint such signalling interactions occurring within the protein complex. Therefore, it will be important to elucidate details of the protein complex to evaluate its functional significance.

The second hypothesis is that different R proteins are physically associated with FLS2 because the R proteins 'guard' particular components in the FLS2–protein complex. It has been shown recently that RLCKs, including BIK1, PBS1 and other close members, are involved in FLS2 signalling by physically associating with FLS2 (Lu *et al.*, 2010; Zhang *et al.*, 2010). Thus, in the case of RPS5, it 'guards' PBS1 (Shao *et al.*, 2003), which participates in FLS2 signalling (Zhang *et al.*, 2010). The physical association between RPS5 and FLS2 suggests that RPS5, PBS1 and FLS2 may reside in the same protein complex, which is consistent with the 'guard hypothesis'. In the case of RPS2 and RPM1, both 'guard' RIN4 (Axtell and Staskawicz, 2003; Mackey *et al.*, 2002, 2003), which has been shown to be involved in the flg22-induced response (Kim *et al.*, 2005). Our results suggest the existence of 'guard' complexes containing FLS2, its signalling partners (PBS1 and RIN4) and R proteins 'guarding' the partners.

EXPERIMENTAL PROCEDURES

Constructs and plants

All the plants used in this study had the genetic background of accession Col-0.

The BSK8 cDNA sequence without a stop codon was amplified with the primers At5g41260-5 (ATGGGTTGTGAGGTTTCAAAGT TATCTGCA) and At5g41260-3 (CAAAGGGTTCTTTGCTTTCAA GCAT) by reverse transcriptase-polymerase chain reaction (RT-PCR), cloned into pCR®8/GW/TOPO® (Invitrogen, Carlsbad, CA, USA) and recombined into the Gateway® destination vector pEG101 (Earley *et al.*, 2006) to yield BSK8::YFP::HA expressed from the cauliflower mosaic virus (CaMV) 35S promoter or pMDC83 (Curtis and Grossniklaus, 2003) to yield BSK8::GFP expressed from the CaMV 35S promoter. *Agrobacterium tumefaciens* strain GV3101/pMP90 was transformed with both constructs. The transformed *A. tumefaciens* strain with pEG101-BSK8 was used to transform a *bsk8* mutant (SALK_090812) (Alonso *et al.*, 2003) using the floral dip method (Clough and Bent, 1998). T1 transgenic plants were selected by spraying LIBERTY 200 SL herbicide (18.19% glufosinate ammonium; Bayer Cropscience, Kansas City, MO, USA) at a 1 : 2000 dilution in water. T3 homozygous plants were selected in the same manner.

The RPM1::Myc plant was prepared by Boyes *et al.* (1998). The following genotypes have been described previously in Qi and Katagiri (2009): *RPS2::HPB line #1*, *RPM1::HPB line #2*, *FLS2-HPB line #19*, *rpm1 rps2*, *fls2* and *rps5*.

Pulldown assay

Pulldown assay using Arabidopsis material

The procedure (either with or without DSP cross-linking) was conducted as described previously, except that the amount of plant material was scaled down to 5 g per sample, except for the experiment in Fig. 2C (Qi and Katagiri, 2009).

The co-immunoprecipitation experiment in Fig. 2C was performed similarly with the following exceptions. Ten grams per sample of plant material were used without cross-linking. Fifty microlitres of anti-c-Myc monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; clone 9E10) were added to each solubilized microsomal protein sample (2 mL) and incubated at 4 °C on a rotator for 2 h. Then, 50 µL of protein A/G agarose (Santa Cruz Biotechnology) was added to each sample, which was incubated for another 4 h. All the samples were spun down at 1000 *g* for 2 min, washed with RIPA buffer 1 [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% NP40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulphonylfluoride (PMSF)] twice and then RIPA buffer 2 [same as RIPA buffer 1, except that 50 mM (instead of 150 mM) NaCl was used] twice. The precipitated proteins were eluted by heating agarose in 50 µL of 1 × SDS sample buffer at 99 °C for 10 min.

Pulldown assay using N. benthamiana material

Equal amounts of cultured *A. tumefaciens* strain GV3101/pVP90 carrying different expression plasmids were suspended with 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (10 mM MES-KOH, 10 mM MgCl₂, 150 µM acetosyringone, pH 5.6) to a final optical density at 600 nm (OD₆₀₀) of 0.4, so that the concentration of each strain was OD₆₀₀ = 0.2. For the negative control, the final OD₆₀₀ was 0.2 as only one construct was used. The bacterial suspension was infiltrated into 4-week-old *N. benthamiana* leaves for transient expression. Two days later, 1 g of each infiltrated leaf sample was collected and quickly frozen in liquid nitrogen, and then ground with a mortar and pestle in 2 mL of extraction buffer [50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES)-KOH, pH 7.6, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM dithiothreitol (DTT)] with protease inhibitors (1 tablet/10 mL Complete Mini® and 1 mM PMSF, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 µg/mL E64; all from Roche, Indianapolis, IN, USA). The ground sample in extraction buffer (~2 mL) was incubated at 4 °C on a rotator for 30 min for solubilization, followed by centrifugation at 16 000 *g* at 4 °C for 30 min to obtain the supernatant as a protein extract. Meanwhile, 100 µL per sample of Dynabeads® M-280 (Invitrogen) were washed with extraction buffer three times and resuspended in 100 µL per sample of extraction buffer. Each protein extract was mixed with 100 µL prewashed Dynabeads® M-280 and incubated on a rotator at 4 °C for 2 h. The streptavidin beads were then washed three times with RIPA buffer 1 and three

times with RIPA buffer 2. The bead-captured proteins were eluted by heating the beads in 60 μ L of 1 \times SDS sample buffer at 99 °C for 10 min.

Immunoblot

Protein samples of equal volume were resolved by 10% SDS-PAGE, and then transferred to poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad, Hercules, CA, USA) by semi-dry electrophoretic transfer using the TRANS-BLOT® SD (Bio-Rad) device. For detection of the proteins on the blot, the blots were cut into strips according to the expected molecular weight of the proteins, and the strips of the blots were probed with the corresponding antibodies separately. The following antibodies or reagents were used: anti-c-Myc monoclonal antibody (Santa Cruz Biotechnology, clone 9E10) at 1 : 200 dilution and goat anti-mouse horseradish peroxidase (HRP) conjugate (Pierce, Rockford, IL, USA) at 1 : 5000 dilution; anti-haemagglutinin (HA) high-affinity monoclonal antibody (Roche, clone 3F10) at 1 : 500 dilution and goat anti-rat immunoglobulin G (IgG) (H+I) HRP conjugate (Bethyl, Montgomery, TN, USA) at 1 : 5000 dilution; anti-green fluorescent protein (GFP) polyclonal antibody (Invitrogen), anti-FLS2 (Chinchilla *et al.*, 2006) at 1 : 500 dilution, anti-RIN4 (Mackey *et al.*, 2002) at 1 : 2000 dilution and goat anti-rabbit HRP conjugate (Pierce). SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce) was used for detection and images were recorded using a chilled CCD-camera.

Confocal microscopy

Rosette leaves from 5-week-old *Arabidopsis* plants were cut into approximately 5 \times 5 mm² squares and mounted between slides and cover glasses with water. The samples were then excited with a 514-nm laser and signals were filtered through a yellow fluorescent protein (YFP) (543-nm) filter, using an Eclipse C1si Spectral Imaging Confocal Microscope (Nikon, Melville, NY, USA). The images were collected using EZ-C1 software (Nikon).

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REFERENCES

- Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C. and Ecker, J.R. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.
- Ausubel, F.M. (2005) Are innate immune signaling pathways in plants and animals conserved? *Nat. Immunol.* **6**, 973–979.
- Axtell, M.J. and Staskawicz, B.J. (2003) Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. *Cell*, **112**, 369–377.
- Benschop, J.J., Mohammed, S., O'Flaherty, M., Heck, A.J., Slijper, M. and Menke, F.L. (2007) Quantitative phosphoproteomics of early elicitor signaling in *Arabidopsis*. *Mol. Cell Proteomics*, **6**, 1198–1214.
- Bent, A.F., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R., Giraudat, J., Leung, J. and Staskawicz, B.J. (1994) RPS2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. *Science*, **265**, 1856–1860.
- Boller, T. and He, S.Y. (2009) Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science*, **324**, 742–744.
- Boyes, D.C., Nam, J. and Dangl, J.L. (1998) The *Arabidopsis thaliana* RPM1 disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. *Proc. Natl. Acad. Sci. USA*, **95**, 15 849–15 854.
- Chinchilla, D., Bauer, Z., Regenass, M., Boller, T. and Felix, G. (2006) The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell*, **18**, 465–476.
- Chinchilla, D., Boller, T. and Robatzek, S. (2007a) Flagellin signalling in plant immunity. *Adv. Exp. Med. Biol.* **598**, 358–371.
- Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J.D., Felix, G. and Boller, T. (2007b) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature*, **448**, 497–500.
- Chisholm, S.T., Coaker, G., Day, B. and Staskawicz, B.J. (2006) Host–microbe interactions: shaping the evolution of the plant immune response. *Cell*, **124**, 803–814.
- Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.
- Curtis, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* **133**, 462–469.
- Dangl, J.L. and Jones, J.D. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, **411**, 826–833.
- Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K. and Pikaard, C.S. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* **45**, 616–629.
- Flor, H.H. (1971) Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* **9**, 275–296.
- Gohre, V., Spallek, T., Haweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J.W. and Robatzek, S. (2008) Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. *Curr. Biol.* **18**, 1824–1832.
- Gomez-Gomez, L. and Boller, T. (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cells*, **5**, 1003–1011.
- Grant, M.R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., de Torres, M., Mansfield, J.W. and Robatzek, S. (1995) Structure of the *Arabidopsis* RPM1 gene enabling dual specificity disease resistance. *Science*, **269**, 843–846.

- Holt, B.F., 3rd, Belkadir, Y. and Dangl, J.L. (2005) Antagonistic control of disease resistance protein stability in the plant immune system. *Science*, **309**, 929–932.
- Jones, J.D. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323–329.
- Kim, M.G., Cunha, L., McFall, A.J., Belkadir, Y., DebRoy, S., Dangl, J.L. and Mackey, D. (2005) Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in Arabidopsis. *Cell*, **121**, 749–759.
- Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E. and Walker, J.C. (2002) BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell*, **110**, 213–222.
- Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L. and He, P. (2010) A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proc. Natl. Acad. Sci. USA*, **107**, 496–501.
- Mackey, D., Holt, B.F., Wiig, A. and Dangl, J.L. (2002) RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. *Cell*, **108**, 743–754.
- Mackey, D., Belkadir, Y., Alonso, J.M., Ecker, J.R. and Dangl, J.L. (2003) Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell*, **112**, 379–389.
- Medzhitov, R. and Janeway, C.A., Jr (1997) Innate immunity: the virtues of a nonclonal system of recognition. *Cell*, **91**, 295–298.
- Mindrinis, M., Katagiri, F., Yu, G.L. and Ausubel, F.M. (1994) The *A. thaliana* disease resistance gene RPS2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. *Cell*, **78**, 1089–1099.
- Nam, K.H. and Li, J. (2002) BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell*, **110**, 203–212.
- Qi, Y. and Katagiri, F. (2009) Purification of low-abundance Arabidopsis plasma-membrane protein complexes and identification of candidate components. *Plant J.* **57**, 932–944.
- Rixon, H.W., Brown, G., Aitken, J., McDonald, T., Graham, S. and Sugrue, R.J. (2004) The small hydrophobic (SH) protein accumulates within lipid-raft structures of the Golgi complex during respiratory syncytial virus infection. *J. Gen. Virol.* **85**, 1153–1165.
- Robatzek, S., Chinchilla, D. and Boller, T. (2006) Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. *Genes Dev.* **20**, 537–542.
- Shao, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J.E. and Innes, R.W. (2003) Cleavage of Arabidopsis PBS1 by a bacterial type III effector. *Science*, **301**, 1230–1233.
- Shiu, S.H., Karlowski, W.M., Pan, R., Tzeng, Y.H., Mayer, K.F. and Li, W.H. (2004) Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. *Plant Cell*, **16**, 1220–1234.
- Tang, W., Kim, T.W., Osés-Prieto, J.A., Sun, Y., Deng, Z., Zhu, S., Wang, R., Burlingame, A.L. and Wang, Z.Y. (2008) BSKs mediate signal transduction from the receptor kinase BRI1 in Arabidopsis. *Science*, **321**, 557–560.
- Tsuda, K. and Katagiri, F. (2010) Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* **13**, 459–565.
- Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J. and Katagiri, F. (2009) Network properties of robust immunity in plants. *PLoS Genet.* **5**, e1000772.
- Warren, R.F., Henk, A., Mowery, P., Holub, E. and Innes, R.W. (1998) A mutation within the leucine-rich repeat domain of the Arabidopsis disease resistance gene RPS5 partially suppresses multiple bacterial and downy mildew resistance genes. *Plant Cell*, **10**, 1439–1452.
- Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S., Mengiste, T., Zhang, Y. and Zhou, J.M. (2010) Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. *Cell Host Microbe*, **7**, 290–301.
- Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G. and Boller, T. (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature*, **428**, 764–767.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Identification of BSK1 (A) and BSK8 (B) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the RPS2-HPB pulldown samples. This figure shows original database search results viewed in the Scaffold viewer (http://www.proteomesoftware.com/Scaffold/Scaffold_viewer.htm).

BSK1 protein was identified with 100% probability and BSK8 protein was identified with 94% probability (top left panel) based on the identification of two BSK1 peptides and one BSK8 peptide at a 95% probability level (top right panel).

Fig. S2 Detection of BSK8::YFP::HA protein from the *p35S::BSK8::YFP::HA* transgenic Arabidopsis line #2. Leaf tissues of a control *bsk8* plant (SALK_090812) and a *p35S::BSK8::YFP::HA* #2 plant (in SALK_090812 background) were flash-frozen in liquid nitrogen and ground to a fine powder. Then, 2 × Laemmli buffer [4% sodium dodecylsulphate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8] was added at a ratio of 1 g tissue to 2 mL buffer. The samples were boiled for 6 min and centrifuged at 16 000 g for 10 min. The supernatant was employed for immunoblotting using anti-green fluorescent protein (GFP) antibody. This experiment was performed twice with similar results.

Fig. S3 Myc::BSK8 forms a protein complex with FLS2::HPB in *Nicotiana benthamiana*. Myc::BSK8 and FLS2::HPB were transiently expressed in *N. benthamiana* leaves and a streptavidin bead-based pulldown assay was performed. FLS2::HPB was pulled down with streptavidin beads. *Nicotiana benthamiana* leaves expressing only Myc::BSK8 were used as a negative control. Both pulldown samples and input samples were analysed by immunoblotting using anti-haemagglutinin (HA) and anti-Myc antibodies. This experiment was performed twice with similar results.

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