A Brassinosteroid-Signaling Kinase Interacts with Multiple Receptor-Like Kinases in *Arabidopsis*

Dear Editor,

Higher plants have evolved hundreds of genes encoding receptor-like kinases (RLKs), which function as cell surface receptors perceiving developmental and environmental signals (Shiu et al., 2004). Many RLKs have been shown to play specific roles in hormone responses, developmental regulation, defense against pathogen infection, and adaptation to abiotic stresses (Chae et al., 2009; Antolin-Llovera et al., 2012). The mechanisms that ensure specific signal transduction from each RLK to target cellular responses remain poorly understood. Recent studies revealed that many RLKs transduce signals by phosphorylating receptor-like cytoplasmic kinases (RLCKs), which lack the transmembrane domains but are anchored at the plasma membrane through lipid modification (Tang et al., 2008; Zhang et al., 2010; Shi et al., 2013). There are over 400 RLKs and only about 150 RLCKs in *Arabidopsis* (Shiu et al., 2004). One outstanding question is whether each RLCK mediates signaling downstream of a specific RLK, participates in multiple RLK pathways, or mediates crosstalk between RLK pathways.

One of the best-studied plant RLKs is the leucine-rich repeat receptor-like kinase (LRR–RLK) Brassinosteroid Insensitive 1 (BRI1), which mediates plant responses to the steroid hormone brassinosteroid (BR) (Wang et al., 2012). Upon activation by BR binding, BRI1 phosphorylates several members of the BR-Signaling Kinases (BSKs, including BSK1, BSK2, and BSK3) (Tang et al., 2008), to transduce the signal to the downstream cytoplasmic component BRI1 Suppressor1 (BSU1) phosphatase (Wang et al., 2012). BSKs belong to the RLCK subfamily XII (Shiu et al., 2004), which includes 12 members that all contain an N-terminal kinase domain and a C-terminal tetratricopeptide motif (Tang et al., 2008).

Multiple members of the BSK family appear to play redundant roles in BR signaling. Mutation of BSK3 slightly reduced hypocotyl and root sensitivity to BR, whereas mutation of other BSKs did not cause any obvious phenotype (Tang et al., 2008). In contrast, the *bsk3,4,7,8* quadruple and *bsk3,4,6,7,8* pentuple mutants displayed severely reduced BR sensitivity and growth phenotypes. In addition, multiple BSKs interacted with BRI1 *in vivo* and served as its phosphorylation substrates *in vitro*. These genetic and biochemical data support a redundant function of multiple BSKs in the BR-signaling pathway (Sreeramulu et al., 2013). On the other hand, there is an increasing body of evidence for additional functions of BSKs outside the BR pathway. For instance, BSK1 was recently shown to physically associate with the pathogen associated molecular pattern (PAMP) receptor kinase FLAGELLIN SENSING2 (FLS2) and to mediate a subset of flagellin-induced responses (Shi et al., 2013).

To understand the functions of BSKs and the mechanisms underlying RLCK-mediated RLK signaling specificity, we performed a proteomics study of BSK3-interacting proteins in *Arabidopsis*. Microsomal proteins were extracted from the BR-deficient mutant *de-etiolated 2* (*det2*) expressing the BSK3–YFP (Yellow Fluorescence Protein) protein, or the non-transgenic *det2* as negative control, and BSK3–YFP and associated proteins were immunoprecipitated using an anti-GFP antibody. Liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis identified a large number of proteins co-immunoprecipitated in the BSK3–YFP sample but not in the non-transgenic *det2* control. These include three BSK homologs (BSK4, BSK7, and BSK8) and 12 transmembrane receptor-like kinases of the leucine-rich repeat family (LRR–RLKs) (Supplemental Table 1). These LRR–RLKs include Impaired Oomycete Susceptibility 1 (IOS1), which is involved in defense against mildew infection (Hok et al., 2011). Additional LRR–RLKs have not been studied previously and we named them BSK3-interacting RLKs (BSRs): BSR050 (At2G37050, a member of LRR-1c subfamily), BSR650 (At1G07650, a LRR-VIII-2), BSR430 (At1G53430, a LRR-VIII-2), BSR440 (At1G53440, a LRR-VIII-2), BSR840 (At3G14840, a LRR-VIII-2), BSR850 (At4G08850, a LRR-XII), and BSR880 (At3G02880, a LRR-III). BSR650, BSR430, BSR440, and BSR840 are four close homologs in the subfamily LRR-VIII-2 (Shiu et al., 2004), suggesting a conserved function of BSK3 interaction in this subfamily. The 12 BSK3-interacting LRR–RLKs identified in this study represent six RLK subfamilies that potentially regulate distinct processes.

To confirm the interaction between BSK3 and the identified LRR–RLKs, we performed co-immunoprecipitation experiments by transient co-expression in *Nicotiana benthemiana* leaves. BSU1–YFP, which is known to interact with BSK3 (Wang et al., 2012), was included as a positive control, and leaves infiltrated with BSK3-7Myc-6His alone was a

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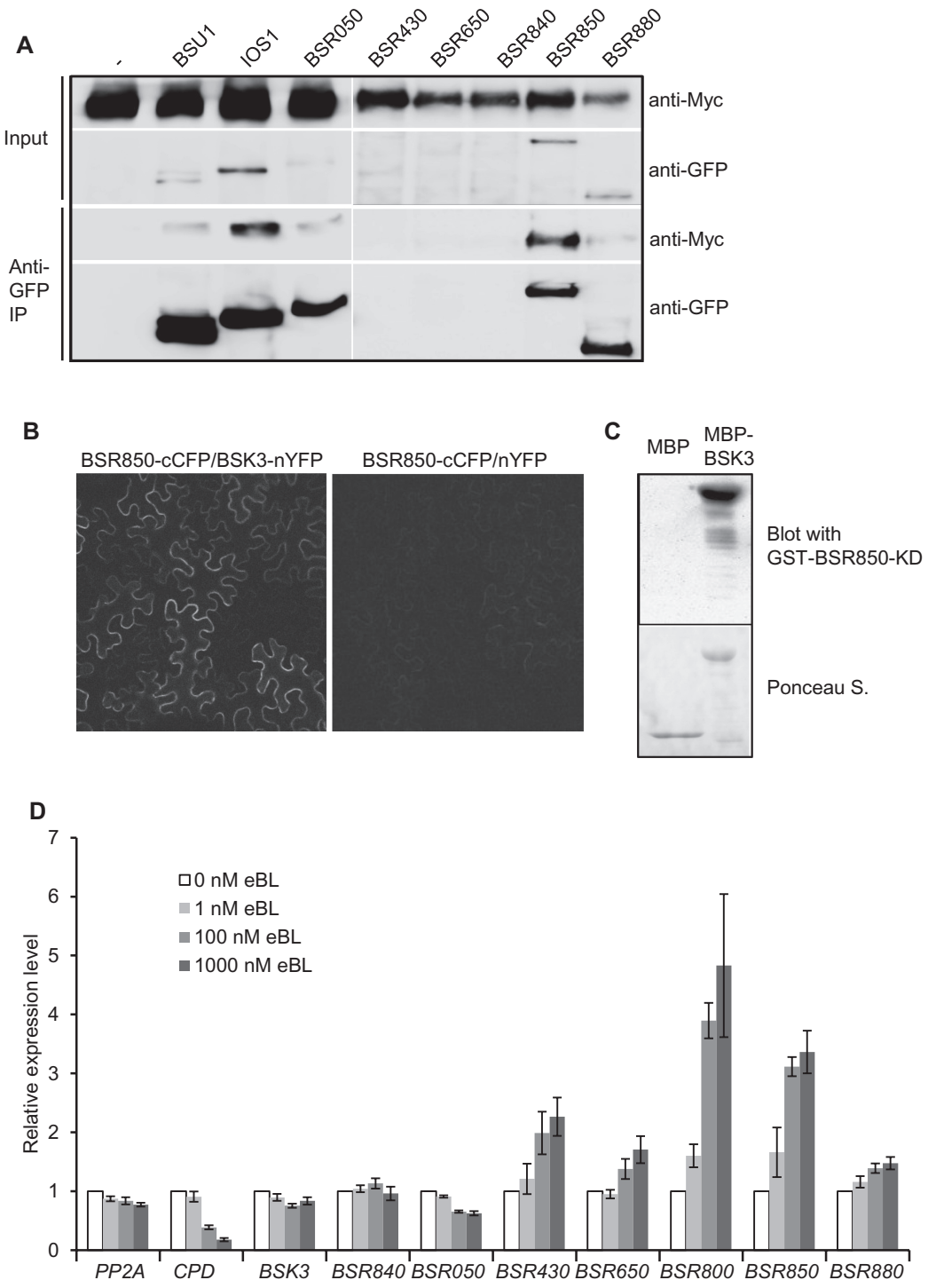

Figure 1. BSK3 Interacts with Multiple Receptor-like Kinases.

(A) Confirmation of interactions between BSK3 and LRR–RLKs using co-immunoprecipitation. The BSK3–Myc–His fusion protein was co-expressed with the indicated BSK3-interacting proteins tagged by GFP, using transient transformation of tobacco leaves. The GFP fusion proteins were immunoprecipitated using an anti-GFP antibody, and the immunoblots was probed with anti-Myc antibody first and then anti-GFP antibody.

(B) Confirmation of direct interactions between BSK3 and BSR850 using BiFC.

(C) Confirmation of direct interactions between BSK3 and BSR850 using the overlay assay. Gel blot of indicated proteins was probed with GST–BSK3 and anti-GST antibody.

(D) BR increases the expression levels of several BSK3-interacting LRR–RLKs. Total RNAs were extracted from 7-day-old Col-0 seedlings grown on half-strength MS media containing indicating concentrations of eBL. Expression levels of the indicated genes were analyzed using quantitative RT–PCR and normalized to the *UBQ5* gene. Error bars are standard errors of three biological replicates.

negative control. The three LRR–RLKs of the LRR-VII-2 subfamily, BSR430, BSR650, and BSR840, failed to express at a detectable level in tobacco leaves (Figure 1A). After immunoprecipitation by anti-GFP antibodies, BSK3-7Myc-6His was detected when co-expressed with BSU1–YFP, IOS1–GFP, BSR050–GFP, BSR850–GFP, and BSR880–GFP, but not in the negative control or in samples that expressed undetectable levels of BSRs (Figure 1A), indicating that these BSR proteins associated with BSK3 *in vivo*.

We performed bimolecular florescence complementation (BiFC) assays by transient co-expression of BSK3–nYFP and BSR850–cCFP in *Nicotiana benthamiana* leaves. As shown in Figure 1B, fluorescence signals were detected on the plasma membrane of *Nicotiana benthamiana* leaves co-expressing BSK3–nYFP and BSR850–cCFP, whereas no signal was observed when BSR850–cCFP was co-expressed with non-fusion nYFP. In addition, we also performed an *in vitro* overlay assay (Figure 1C). The kinase domain of BSR850 showed binding to maltose-binding protein (MBP)–BSK3, but not to MBP, on a gel blot. These *in vivo* and *in vitro* results demonstrate that BSR850 kinase directly interacts with BSK3 at the plasma membrane.

Two previously reported BSK interactors, BRI1 and BSU1 (Wang et al., 2012), were not detected in our proteomic experiment. However, the BSU1–BSK3 interaction was detected in the co-immunoprecipitation assay (Figure 1A), indicating that our proteomic analysis did not detect all the BSK3-interacting proteins. Some interactions might be undetected because the interaction is dynamic and unstable, or the interactor is of low abundance in the tissues used in this study. Therefore, it is possible that BSK3 interacts with even more RLKs than those that we have detected.

To test whether the BSK3-interacting RLKs are involved in BR response, we analyzed the RNA expression of these LRR–RLK genes in wild-type seedlings grown on media containing different concentrations of epi-brassinolide (eBL). As shown in Figure 1D, the expression level of *PP2A* was unaffected while that of *CPD* was significantly decreased by increasing concentrations of eBL, consistent with previous observations (Wang et al., 2012). The expression levels of *IOS1*, *BSR850*, *BSR430*, *BSR650*, and *BSR800* were increased by BR treatment in a concentration-dependent manner. In particular, the expression levels of *IOS1* and *BSR850* increased over three-fold after growing on 100 nM eBL, whereas the other three genes showed milder responses. The expression levels of *BSR050* decreased slightly, whereas the levels of *BSK3* and *BSR840* were unaffected by BR. The BR-responsive expression suggests a role for these BSK3-interacting RLKs in BR-regulated responses.

In addition to BR response, several BSK3-interacting RLKs seem to be involved in immunity. *IOS1* has been shown to be induced by oomycete and to play a role in defense against fungal infection (Hok et al., 2011). Furthermore, expression levels of *BSR840* and the closest homolog of *BSR850* (At1g35710) were also increased by oomycete (Hok et al., 2011), whereas *BSR850* was repressed by bacterial infection (Chae et al., 2009), suggesting a role for these additional BSK3-interacting RLKs and thus BSK3 itself in immunity. Our results support a broad role of BSK3 in BR, immunity, and possibly additional RLK-mediated processes.

Together with our initial finding of BSKs as a BR-signaling component (Tang et al., 2008) and a recent finding that BSK1 interacts with the flagellin receptor kinase FLS2 (Shi et al., 2013), this study reveals extensive sharing of a RLCK by multiple transmembrane RLKs with distinct functions. Such sharing may provide a mechanism for beneficial crosstalk between RLKs, or allow independent RLKs to use the same downstream component in distinct pathways. Crosstalk has been observed between BRI1 and the immunity RLK pathways, which share the BAK1 co-receptor (Wang, 2012), as well as the substrate RLCKs as demonstrated in this study. On the other hand, the flagellin receptor kinase FLS2 is known to interact with not only BSK1, but also other RLCKs including BIK1, PBS1, and PBLs (Zhang et al., 2010), whereas BRI1 also phosphorylates not only members of the BSK family, but also a distantly related RLCK named CDG1 (Wang et al., 2012). As such, it appears that different RLKs transduce signals through overlapping sets of RLCKs, and each RLCK mediates signaling from multiple RLKs. The interaction of BSK3 with other BSK members raises the possibility of higher-order complexes containing multiple RLKs and RLCKs. Further functional and biochemical studies of these newly identified BSK3-interacting RLKs will shed light on the structure of the RLK signaling network and the mechanisms underlying RLK signaling specificity and crosstalk in plants.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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Peng Xu[a,b,2], *Shou-Ling Xu*[b,c,2], *Zi-Jing Li*[a], *Wenqiang Tang*[a], *Alma L. Burlingame*[c] and *Zhi-Yong Wang*[b,1]

a Institute of Molecular and Cell Biology, Hebei Key Laboratory of Molecular and Cellular Biology, College of Life Sciences, Hebei Normal University, Shijiazhuang, Hebei 050024, China
b Department of Plant Biology, Carnegie Institution for Science, Stanford, CA 94305, USA
c Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143, USA
[1] To whom correspondence should be addressed. E-mail zywang24@stanford.edu, tel. +01 650 739 4205, fax 650 325 6857.
[2] These authors contributed equally to this work.

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