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The CDG1 Kinase Mediates Brassinosteroid Signal Transduction from BRI1 Receptor Kinase to BSU1 Phosphatase and GSK3-like Kinase BIN2

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SUMMARY

The brassinosteroid (BR) signaling pathway includes two receptor-like kinases (BRI1 and BAK1), a plasma membrane-associated kinase (BSK1), two phosphatases (BSU1 and PP2A), a GSK3-like kinase (BIN2), and two homologous transcription factors (BZR1 and BES1/BZR2). But the mechanisms of signal relay are not fully understood. Here, we show that a receptor-like cytoplasmic kinase named CDG1 mediates signal transduction from BRI1 to BSU1. Transgenic experiments confirm that CDG1 and its homolog CDL1 positively regulate BR signaling and plant growth. Mass spectrometry analysis identified BRI1 phosphorylation sites in CDG1 and CDG1 phosphorylation sites in BSU1. Mutations of these phosphorylation sites compromised the BR signaling functions. The results demonstrate that BRI1 phosphorylates S234 to activate CDG1 kinase, and CDG1 in turn phosphorylates S764 to activate BSU1, which inactivates BIN2 by dephosphorylating Y200 of BIN2. This study thus demonstrates a complete phosphorylation/dephosphorylation cascade linking a steroid-activated receptor kinase to a GSK3-like kinase in plants.

INTRODUCTION

Signal transduction mediated by membrane-bound receptor-like kinases (RLKs) is an important regulation mechanism for all cellular organisms, particularly for plants, which rely on signal transduction for development and adaptation to the environment. There are over 400 genes encoding RLKs in *Arabidopsis* and over 600 in rice (Shiu et al., 2004). Studies of a small number of these RLKs demonstrated important functions in a wide range of processes, including hormone responses, development, cell-cell communication, and defense against pathogens (De Smet et al., 2009; Tör et al., 2009). One of the best-studied RLK signaling pathways in plants is the BRI1 receptor kinase pathway, which transduces the brassinosteroid (BR) signal (Kim and Wang, 2010).

BR is a steroidal hormone that regulates a wide range of developmental processes in plants (Clouse and Sasse, 1998). BR-deficient and BR-insensitive mutants show pleiotropic defects including extreme dwarfism, male sterility, delayed senescence, and constitutive photomorphogenesis in the dark (Li et al., 1996). In contrast to animal steroid receptors, which are nuclear transcription factors, the BR receptor BRI1 is one of the 230 members of the leucine-rich repeat receptor-like kinases (LRR-RLK) in *Arabidopsis* (Li and Chory, 1997). BRI1 perceives BR through its extracellular domain and transduces the signal through its cytoplasmic kinase domain (Kinoshita et al., 2005; Wang et al., 2001). In the absence of BR, BRI1 kinase is inactive, while BIN2, a GSK3-like kinase (Li and Nam, 2002), phosphorylates and inactivates two homologous nuclear transcription factors called BZR1 and BZR2 (also named BES1) to repress BR-responsive gene expression (Gampala et al., 2007; He et al., 2002; Vert and Chory, 2006; Wang et al., 2002; Yin et al., 2002). BR binding to BRI1's extracellular domain activates its kinase activity by inducing association and transphosphorylation with the coreceptor BAK1, another LRR-RLK (Li et al., 2002; Nam and Li, 2002; Wang and Chory, 2006; Wang et al., 2008). Activation of BRI1 also involves dissociation of *B*RI1 *k*inase *i*nhibitor *1* (BKI1) caused by tyrosine phosphorylation (Jaillais et al., 2011; Wang and Chory, 2006). Activated BRI1 kinase initiates a signaling cascade that leads to inactivation of BIN2 and dephosphorylation of BZR1 and BZR2 by the protein phosphatase 2A (Tang et al., 2011). Dephosphorylated BZR1 and BZR2 accumulate in the nucleus and regulate the expression of BR target genes (He et al., 2005; Sun et al., 2010; Yin et al., 2005; Yu et al., 2011).

Signal transduction from BRI1 to BIN2 involves the *B*R-*s*ignaling *k*inase *1* (BSK1) (Tang et al., 2008) and the *B*RI1 *su*ppressor *1* (BSU1) phosphatase (Kim et al., 2009; Mora-García et al., 2004), but the mechanisms of signal relay are not fully understood. BSK1 is a member of the receptor-like cytoplasmic kinase (RLCK) XII family (Tang et al., 2008). RLCKs are homologous to transmembrane RLKs but contain no extracellular or transmembrane domains (Shiu et al., 2004). BSK1 is localized at the plasma membrane and is phosphorylated at S230 by BRI1 upon BR treatment (Tang et al., 2008). S230 phosphorylation increases BSK1 binding to BSU1 (Kim et al., 2009; Tang et al., 2008). BSU1 inactivates BIN2 by dephosphorylating its Y200 residue, and BR treatment enhances BSU1 dephosphorylation of BIN2, but the mechanism of BSU1 activation remains unclear (Kim et al., 2009).

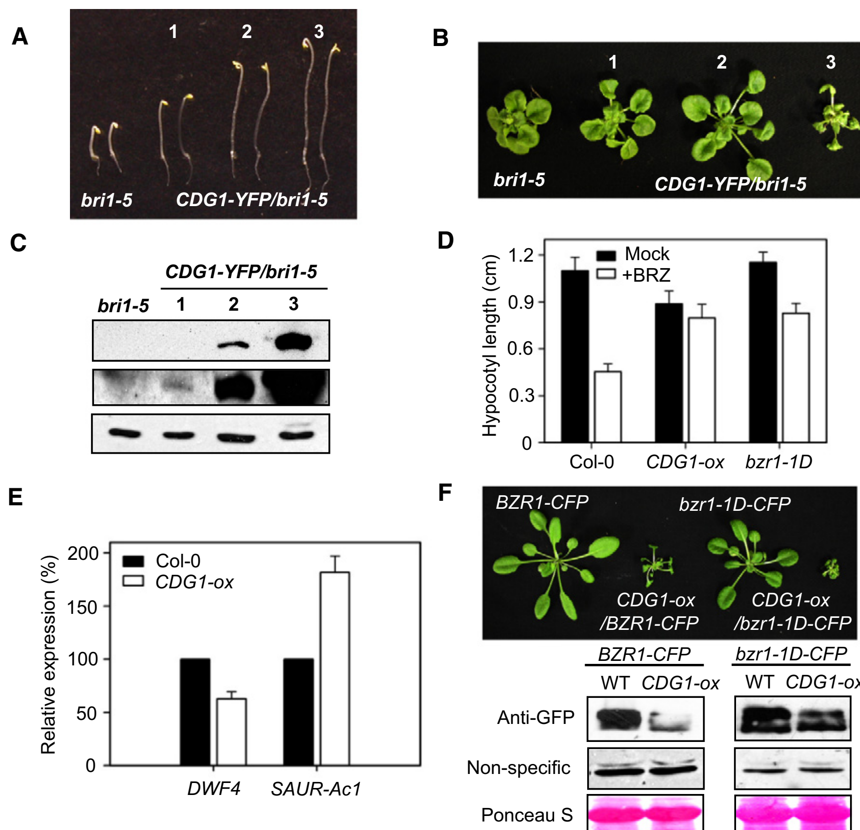

Figure 1. CDG1 Positively Regulates BR Signaling

(A–C) Three independent *35S::CDG1-YFP* transgenic lines are marked as numbers 1 to 3. Seedlings were grown in the dark for 5 days (A) or for 3 weeks in the light (B), and the expression levels of CDG1-YFP in plants shown in panel (B) were detected by immunoblotting using anti-GFP antibody (C). Upper and middle panels of (C), respectively, show short and long exposure, and the bottom panel shows a nonspecific band as loading control.

(D) Hypocotyl length of wild-type (Col-0), CDG1-overexpression plant (*CDG1-ox*), and *bzr1-1D* seedlings grown in the dark for 5 days on MS medium supplemented with mock solution (Mock) or 2 μM BRZ. Error bars are ± SEM.

(E) RNA levels of *DWF4* and *SAUR-AC1* were analyzed by quantitative real time-PCR in Col-0 and *CDG1-ox* plant. Error bars are ± SEM.

(F) CDG1 overexpression causes accumulation of dephosphorylated BZR1. Upper panel shows the phenotype of transgenic plants expressing BZR1-CFP or bzr1-1D-CFP from the BZR1 promoter and overexpressing CDG1. Lower panel shows immunoblot analysis of BZR1-CFP using anti-GFP antibody. Ponceau S staining of the blot is shown as a loading control.

A previous study suggested that *c*onstitutive *d*ifferential *g*rowth 1 (CDG1), a member of the RLCK VIIc family, is involved in activation of BR signaling (Muto et al., 2004). The *cdg1-D* mutant, overexpressing CDG1 due to activation tagging mutagenesis, showed insensitivity to BR biosynthetic inhibitor brassinazole (BRZ) and altered expression of BR-responsive genes, consistent with activation of the BR signaling pathway (Muto et al., 2004). However, the biochemical function of CDG1 in BR signaling has not been studied.

In this study, we show that CDG1 and its homolog CDL1 mediate signal transduction from BRI1 to BSU1. CDG1 phosphorylates the S764 residue in BSU1's phosphatase domain to increase BSU1's ability to dephosphorylate Y200 residue of BIN2. CDG1 is phosphorylated by BRI1 at S44, S47, and S234, and mutations of these phosphorylation sites compromised CDG1's ability to activate BR signaling in vivo. In vitro assays demonstrate that BSU1 and either CDG1 or BSK1 are the minimum components required for signal transduction from BRI1 to BIN2. Our results therefore establish a complete phosphorylation/dephosphorylation cascade that mediates the inactivation of a GSK3-like kinase by a steroid-activated receptor kinase in plants (Figure S1).

RESULTS

CDG1 Positively Regulates BR Signal Transduction

To elucidate the functional role of CDG1 in BR signaling, we first transformed BR-deficient and BR-insensitive mutants with a CDG1 overexpression (CDG1-ox) construct. CDG1-ox partially suppressed the phenotypes of the BR-deficient *det2* mutant (Figure S2A) and the BR receptor *bri1-5* mutant (Figures 1A–1C). Similarly to the *bzr1-1D* mutant, in which the bzr1-1D protein is activated by increased interaction with PP2A (Tang et al., 2011; Wang et al., 2002), the *CDG1-ox* plants showed reduced sensitivity to the BR biosynthetic inhibitor BRZ (Figure 1D), reduced expression of the BR-repressed BZR1-target gene *DWF4*, increased expression of BR-induced BZR1-target gene *SAUR-AC1* (Figure 1E), and accumulation of dephosphorylated BZR1 proteins (Figure 1F). Light-grown plants overexpressing high levels of CDG1 showed smaller sizes with curled leaves and elongated petioles (Figures 1B and 1F), reminiscent of plants grown on high concentrations of BR or overexpressing the dominant allele of *bzr1-1D*. These results demonstrate that CDG1-ox activates the BR signal transduction pathway in *Arabidopsis*.

CDG1 and CDL1 are Plasma Membrane Proteins that Positively Regulate BR Signaling

When expressed in either stably transformed *Arabidopsis* plants or transiently transformed tobacco cells, CDG1 fused to the yellow fluorescence protein (CDG1–YFP) was only observed at the cell surface, independent of BR treatment (Figures 2A and S2B). CDG1 has two cysteine residues in its N-terminal region predicted to be palmitoylation sites (Figure 2A). Substitution of these cysteine residues to alanine (CDG1-C4,6A-YFP) resulted in nuclear localization of the CDG1 protein in both *Arabidopsis* and tobacco cells (Figures 2A and S2B), suggesting that CDG1 is anchored to the plasma membrane by palmitoylation. The

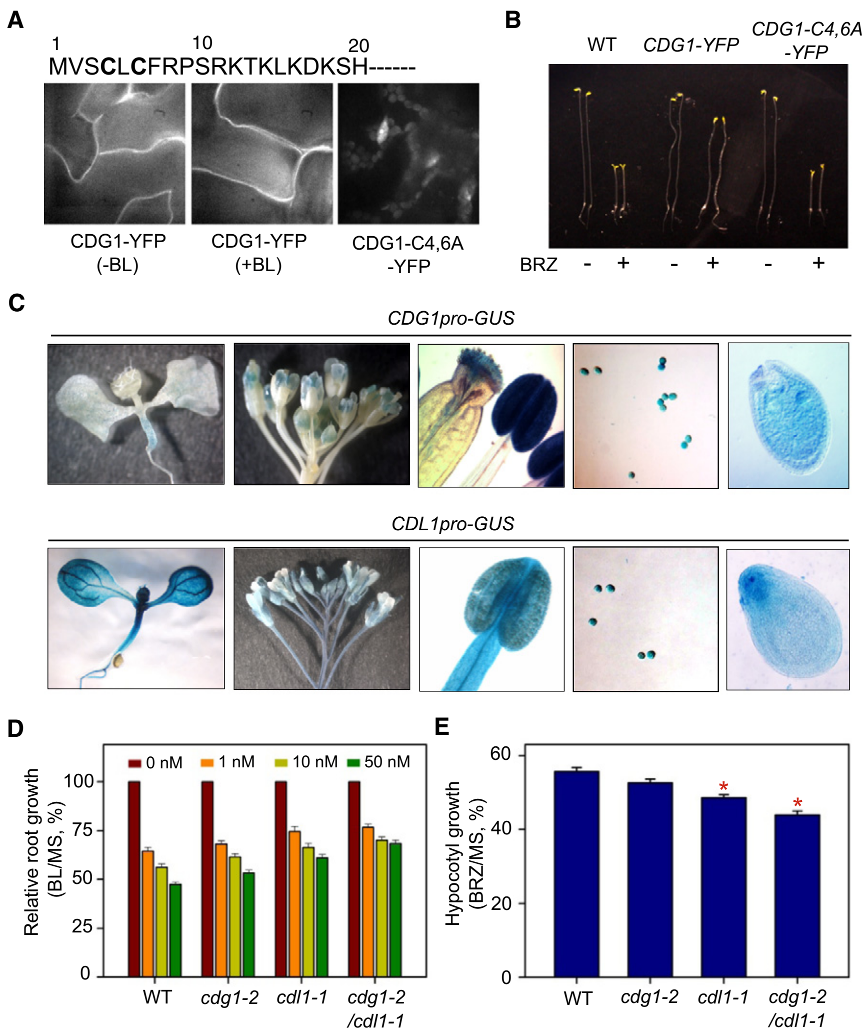

Figure 2. Subcellular Localization, Tissue-Specific Expression, and Phenotype of Knockout Mutant of CDG1 and Its Homolog, CDL1

(A) Plasma membrane localization of CDG1-YFP anchored by palmitoylation. Amino acid sequence of the N-terminal region of CDG1 shows the two cysteine residues (bold) as putative palmitoylation sites. Confocal images show localization of CDG1-YFP or CDG1-C4,6A-YFP in leaf epidermal cells of transgenic *Arabidopsis*. A CDG1-YFP sample was treated with 200 nM BL for 30 min.

(B) Mislocalized CDG1 failed to activate BR signaling. Seedlings of wild-type (Col-0) and transgenic plant expressing CDG1-YFP or CDG1-C4,6A-YFP were grown in the dark for 5 days on MS medium with or without 2 μM BRZ.

(C) Gene expression pattern of CDG1 and CDL1. GUS staining images show expression of the CDG1 promoter-GUS and CDL1 promoter-GUS in transgenic plants. From left to right are 5-day-old seedlings, flowering buds, stamens and stamen filaments, pollen, and immature seeds.

(D) BR sensitivity of *cdg1-2*, *cdl1-1*, and *cdg1-2/cdl1-1* mutant. Seedlings were grown under light for 5 days on MS medium containing indicated concentration of brassinolide (BL). Error bars are ± SEM.

(E) BRZ sensitivity of *cdg1-2*, *cdl1-1*, and *cdg1-2/cdl1-1* mutant. Seedlings were grown in the dark for 5 days on MS or 2 μM BRZ medium, and hypocotyl lengths were measured. Asterisk, $p < 0.0001$. Error bars are ± SEM.

transgenic plants overexpressing CDG1-C4,6A-YFP showed no phenotypes (Figure 2B), indicating that plasma membrane localization is required for CDG1 to function in BR signal transduction.

The closest *Arabidopsis* homolog of CDG1 (CDG1-like1, CDL1) shares 65% amino acid sequence identity with CDG1. Like CDG1, CDL1-YFP was also detected in the plasma membrane (Figure S2C), and overexpression of CDL1 suppressed the *bri1-5* mutant phenotype (Figure S2D), suggesting a similar function in activating BR signaling. A CDG1 promoter-GUS reporter gene indicates that CDG1 is expressed at a very low level in vegetative tissues but at high levels in the stamen and pollen of flowers (Figure 2C). By contrast, CDL1 promoter-GUS expression was ubiquitously detected in reproductive and vegetative tissues (Figure 2C). Consistent with their expression patterns, the *cdg1* loss-of-function mutant (*cdg1-2*), which was isolated as an intragenic suppressor of *cdg1-D* (Muto et al., 2004), showed no obvious phenotypes, whereas the *cdl1-1* knockout mutant showed reduced sensitivity to BR and enhanced sensitivity to BRZ (Figures 2D and 2E). In addition, the *cdg1-2/cdl1-1* double mutant showed stronger BL insensitivity and enhanced BRZ sensitivity compared to the *cdg1-2* or *cdl1-1* single mutant (Figures 2D and 2E). However, the phenotypes of *cdg1-2/cdl1-1* are still very subtle compared to typical BR-insensitive mutants, such as *bri1-5* or *bin2-1*, likely due to redundant functions of other homologous RLCKs. Interestingly, the expression pattern of CDG1 is similar to that of BSU1, and CDL1 is expressed in a way broadly similar to the BSU1 homolog, BSL1 (BSU1-like 1) (Figure S2E).

CDG1 Acts Upstream of BIN2 in the BR Signaling Pathway

To analyze the genetic interaction of CDG1 with BR signaling components, we crossed the CDG1-ox plant into the BR-insensitive mutants *bri1-116* (null allele) and *bin2-1* (gain-of-function allele). CDG1-ox partly suppressed the phenotype of *bri1-116* but did not suppress the phenotype of the *bin2-1* homozygous mutant (Figures 3A and 3B), indicating that CDG1 acts upstream of BIN2 and downstream of BRI1. Notably, the phenotypes of CDG1 overexpressors were very similar to those of BSU1-ox plants, such as bending in the stem-branch junction, the flattened shape of the silique, and pale green and slender leaves (Figures 3C–3E, and S3A). In addition, plants overexpressing both CDG1-YFP and BSU1-myc showed dramatically enhanced phenotypes of leaf twisting and BRZ resistance compared to plants expressing either CDG1-YFP or BSU1-myc alone (Figures

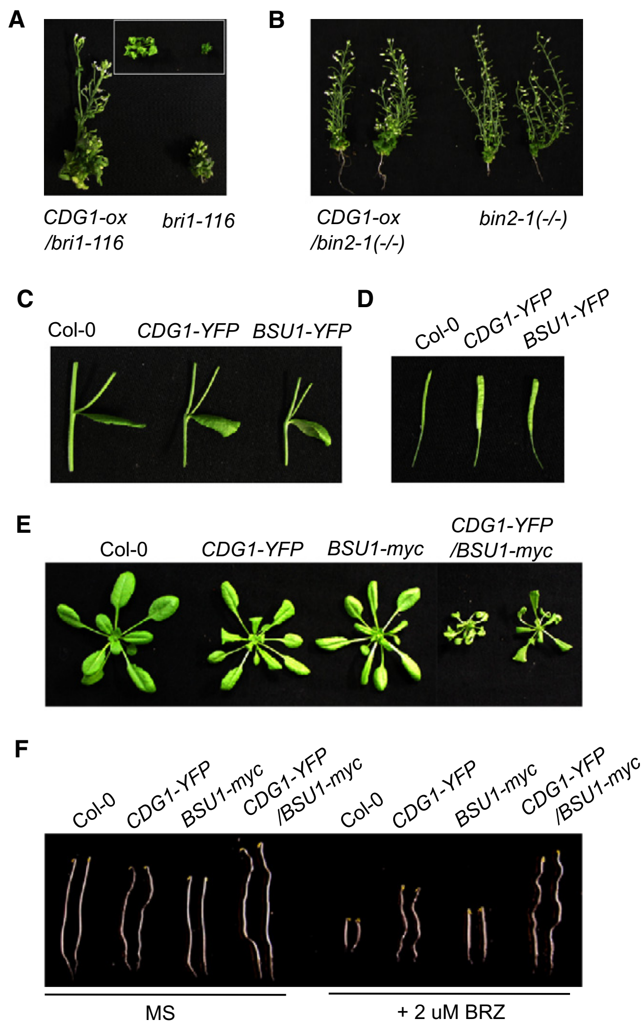

Figure 3. Phenotypes Caused by CDG1 Overexpression in Various Genetic Backgrounds

(A and B) Overexpression of CDG1 partially suppresses the dwarf phenotype of *bri1-116* (A) but not *bin2-1* (B). Plants were grown for 7 weeks. Inset of (A) shows phenotype of 4-week-old plants.

(C–F) Overexpression of *CDG1* and *BSU1* causes similar phenotypes and is synergistic. Both CDG1-ox and BSU1-ox plants display bending in the stem-branch junction region (C), flattening and rectangular shape of the silique (D), pale green leaves (E), and slightly reduced sensitivity to BRZ (F). Plants overexpressing both CDG1-YFP and BSU1-myc show enhanced phenotypes (E, F). Seedlings were grown in the dark for 5 days on MS medium with or without 2 μM BRZ (F).

3E, 3F, and S3B), suggesting that CDG1 and BSU1 function together in BR signaling.

CDG1 Interacts with BSU1 In Vitro and In Vivo

We tested whether CDG1 and BSU1 directly interact with each other. In vitro overlay assays showed that GST-CDG1 in a gel blot can be detected by MBP-BSU1 and HRP-conjugated anti-MBP antibody (Figure 4A). In yeast two-hybrid assays, CDG1 strongly interacts with BSU1 and weakly with BSL1. However, CDL1 strongly interacts with both BSU1 and BSL1 (Figure 4B). Furthermore, *bi*molecular *f*luorescence *c*omplementation (BiFC) assays showed a strong fluorescence signal in tobacco epidermal cells coexpressing CDG1 or CDL1 fused to the N-terminal half of YFP (nYFP) and BSU1 or BSL1 fused to the C-terminal half of YFP (cYFP), whereas tobacco cells coexpressing BIN2-cYFP and CDG1-nYFP or CDL1-nYFP showed no fluorescence signal (Figure 4C), indicating that CDG1/CDL1 interacts with BSU1/BSL1 but not BIN2. Consistent with the yeast two-hybrid data, in vivo interaction between CDG1 and BSL1 in tobacco cells was very weak, whereas CDL1 strongly interacted with both BSU1 and BSL1 (Figures 4B and 4C). We further confirmed in vivo interaction between BSU1 and CDG1 or CDL1, using coimmunoprecipitation assays. BSU1-myc protein was immunoprecipitated by anti-GFP antibody from tobacco cells coexpressing BSU1-myc and CDG1-YFP or CDL1-YFP (Figure S3C) or *Arabidopsis* coexpressing BSU1-myc and CDG1-YFP (Figure 4D). BR treatment slightly increased the amount of BSU1-myc coimmunoprecipitated with CDG1-YFP in transgenic *Arabidopsis* (Figure 4D), suggesting that BR signaling increases the interaction between CDG1 and BSU1.

CDG1 Activates BSU1 by Phosphorylating its S764 Residue

Both CDG1 and CDL1 showed strong autophosphorylation activity in vitro (Figures 5A, S4A, and S4E). We created kinase-inactive CDG1 harboring a mutation (Y147G) in the ATP-binding pocket of CDG1 kinase. This mutation abolished CDG1 kinase activity in vitro and the typical CDG1-ox phenotypes in transgenic plants (Figures S4A–S4D). These results indicate that the kinase activity of CDG1 is required for CDG1 to effectively activate BR response. Thus, we hypothesized that CDG1 modulates BSU1 activity through phosphorylation. Indeed, in vitro kinase assays showed that CDG1 phosphorylated BSU1 (Figure 5A). Consistent with the different strength of interactions observed in yeast two-hybrid and BiFC assays (Figures 4B and 4C), CDG1 only weakly phosphorylated three other BSU1 homologs (BSL1, BSL2 and BSL3) (Figure 5A), and CDL1 phosphorylated both BSU1 and BSL1 equally well in vitro (Figure S4E).

We next examined whether BR treatment induces phosphorylation of BSU1 in plant cells. The BSU1-myc transgenic *Arabidopsis* plants were treated with BR, and the BSU1-myc protein was analyzed by two-dimensional gel electrophoresis followed by immunoblotting. The results show that the acidic spots of BSU1-myc protein were increased and the basic spots were decreased after BR treatment, suggesting that BSU1 is phosphorylated upon BR signaling (Figure 5B). We then performed mass spectrometric analysis of the in vivo phosphorylation sites of BSU1 using proteins immunoprecipitated from transgenic *Arabidopsis*, and we identified three in vivo phosphorylation sites (S395, S444, and S764) of BSU1 (Figures 5C and S5A–S5C). The S444 and S764 residues were also identified in the three BSU1 homologs (BSL1, BSL2 and BSL3) by a recent proteomic study (Benschop et al., 2007; Sugiyama et al., 2008). Substitution of S764 to alanine (BSU1-S764A) greatly reduced the phosphorylation of BSU1 by both CDG1 and CDL1 (Figures

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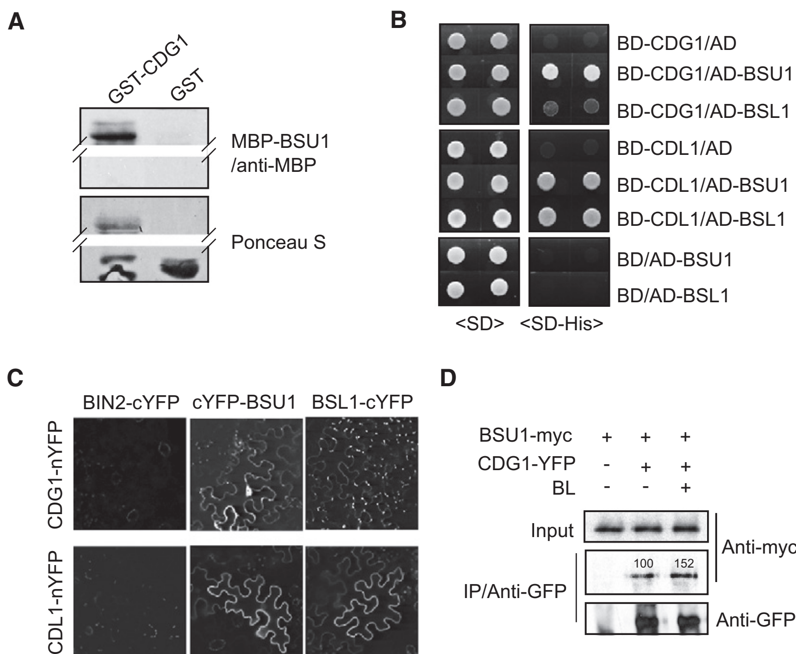


Figure 4. CDG1 Interacts with BSU1 In Vitro and In Vivo

(A) A gel blot separating GST and GST-CDG1 was probed with MBP-BSU1 followed by HRP-conjugated anti-MBP antibody. Ponceau S staining shows the total protein on blot.

(B) Yeast two-hybrid assay between CDG1 or CDL1 and BSU1 or BSL1. Pairs of indicated bait (BD) and prey (AD) vectors were transformed into yeast cells. Yeast growth on the SD-His medium indicates interaction.

(C) BiFC assays of in vivo interactions between CDG1 or CDL1 and BSU1 or BSL1. The indicated constructs were cotransformed into tobacco leaf epidermal cells. Bright spots are autofluorescence derived from chloroplast.

(D) Coimmunoprecipitation of CDG1 and BSU1. Leaves of transgenic plants coexpressing CDG1-YFP and BSU1-myc were treated with mock solution or 200 nM BL for 30 min. The CDG1-YFP proteins were immunoprecipitated with anti-GFP antibody, and the immunoblot was probed with anti-myc and anti-GFP antibody. Numbers indicate relative BSU1-myc signal level normalized to the CDG1-YFP signal.

5D and S5D), whereas mutations of S395 and S444 had little effect, indicating that both CDG1 and CDL1 specifically phosphorylate the S764 residue of BSU1.

BSU1 contains an N-terminal Kelch-repeat domain and a C-terminal phosphatase domain, and the S764 residue is in the C-terminal region of the phosphatase domain (Mora-García et al., 2004). In vitro overlay assay indicated that BIN2 interacts with the C-terminal phosphatase domain but not with the N-terminal Kelch-repeat domain of BSU1 (Figure S5E). CDG1 phosphorylation increased BSU1 binding to BIN2, but had little effect on BSU1-S764A binding to BIN2 (Figure 5E). Furthermore, CDG1 phosphorylation strongly enhanced the ability of BSU1 but not of BSU1-S764A to dephosphorylate Y200 of BIN2 (Figure 5F). However, CDG1 did not increase BSU1 dephosphorylation of the artificial substrate phosphomyelin basic protein (Figure S5F). These results indicate that CDG1 phosphorylates BSU1 S764 to promote BSU1 dephosphorylation of BIN2, most likely by enhancing BSU1-BIN2 interaction rather than increasing the catalytic activity of BSU1.

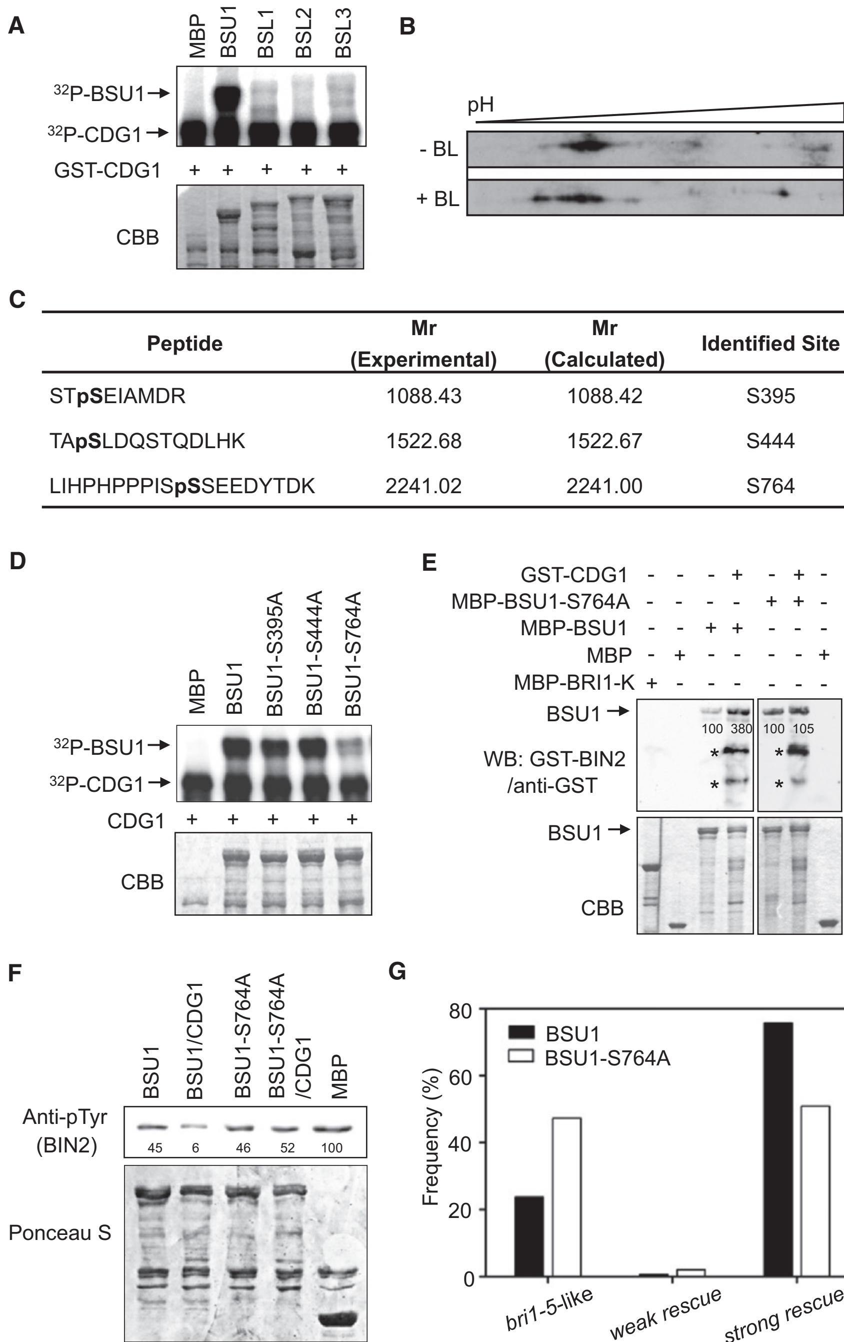
To further confirm the functional importance of S764 phosphorylation of BSU1, we overexpressed wild-type BSU1 and BSU1-S764A mutant in *bri1-5*. The percentage of transgenic plants showing phenotypic suppression was significantly lower in the T1 population transformed with BSU1-S764A than that transformed with the wild-type BSU1 (Figures 5G and S5G). While the S764A mutation significantly reduced the efficiency of BSU1 to suppress *bri1-5*, some transgenic plants showed strong phenotype suppression, suggesting that unphosphorylated BSU1 has some basal activity. Taken together, our results strongly suggest that CDG1 phosphorylation of S764 of BSU1 increases its interaction with and dephosphorylation of BIN2, which inactivates BIN2 and stops the phosphorylation of BZR1.

CDG1 is Activated by BRI1 Phosphorylation

The plasma membrane localization and action upstream of BSU1 suggest that CDG1 may act directly downstream of BRI1. We thus tested BRI1-CDG1 interaction. As shown in Figure 6A, a strong BiFC fluorescence signal was observed in the tobacco epidermal cells coexpressing BRI1-nYFP and CDG1-cYFP, whereas no signal was detected in cells coexpressing nYFP fusion of a receptor kinase unrelated to BR signaling (At5g49760) and CDG1-cYFP. In yeast two-hybrid assays, CDG1 interacted with the BRI1 kinase domain, but not with the BAK1 kinase domain, whereas BRI1 kinase domain interacted with neither BSU1 nor BSL1 (Figure S6A). In addition, CDG1-myc was coimmunoprecipitated by anti-GFP antibody from the microsomal fraction of transgenic *Arabidopsis* coexpressing BRI1-GFP and CDG1-myc (Figure 6B). These results indicate that BRI1 directly interacts with CDG1.

In vitro kinase assay demonstrated that a kinase-inactive CDG1 mutant protein (CDG1-Y147G) was strongly phosphorylated by the BRI1 kinase domain but not by the BAK1 kinase domain, relative to their autophosphorylation activity (Figure 6C). Thus, we performed mass spectrometry analysis of the kinase-inactive CDG1-Y147G phosphorylated by BRI1 kinase, and we identified two serine residues (S44 and S47) in the N-terminal region and one serine residue (S234) in the activation loop domain of CDG1 as BRI1-phosphorylation sites (Figures 6D and S6B–S6D). All three residues are also conserved in CDL1 protein. In particular, S234 of CDG1 corresponds to the BRI1 phosphorylation site (S230) of BSK1 (Figure S7A). Mutations of both S44 and S47 (CDG1-S44,47A) or only S234 (CDG1-S234A) to alanine significantly reduced BRI1 transphosphorylation of CDG1 (Figures 6E and S7B), confirming that BRI1 phosphorylates CDG1 at S44, S47, and S234.

To evaluate the biological importance of BRI1 phosphorylation of CDG1 in BR signaling, we overexpressed wild-type CDG1 and



Peptide	Mr (Experimental)	Mr (Calculated)	Identified Site
ST**pS**EIAMDR	1088.43	1088.42	S395
TA**pS**LDQSTQDLHK	1522.68	1522.67	S444
LIHPHPPPIS**pS**SEEDYTDK	2241.02	2241.00	S764

Figure 5. CDG1 Specifically Phosphorylates the S764 Residue of BSU1

(A) CDG1 phosphorylates BSU1 but not the BSU1 homologs in vitro. MBP or MBP-fused BSU1, BSL1, BSL2, and BSL3 were incubated with GST-CDG1 and ^{32}P-γ-ATP. Upper panel shows autoradiography and bottom panel Coomassie brilliant blue (CBB) staining of the gel.

(B) Total protein of *BSU1-myc* transgenic plants treated with mock solution (−BL) or 200 nM BL (+BL) for 30 min were analyzed by two-dimensional electrophoresis followed by immunoblotting using anti-myc antibody.

(C) Identification of three in vivo phosphorylation sites of BSU1. Phosphopeptide sequences identified by LC-MS/MS analysis of BSU1-YFP immunoprecipitated from transgenic plants using anti-GFP antibody.

(D) In vitro kinase assay shows CDG1 phosphorylates an S764 residue of BSU1. Equal amounts of MBP-fused BSU1, BSU1-S395A, BSU1-S444A, and BSU1-S764A were incubated with GST-CDG1 and ^{32}P-γ-ATP. Upper panel shows autoradiography, and bottom shows Coomassie brilliant blue (CBB)-stained gel.

(E) BSU1 phosphorylated by CDG1 more effectively binds to BIN2 in vitro. A gel blot separating MBP fusion proteins of the kinase domains of BRI1 (MBP-BRI1-K), MBP, MBP-BSU1, MBP-BSU1-S764A, or MBP-BSU1 and MBP-BSU1-S764A preincubated with GST-CDG1 and ATP, was sequentially probed with GST-BIN2 and anti-GST antibody. Asterisks indicate the signal derived from GST-CDG1. Numbers indicate relative signal level normalized to the CBB staining of MBP-BSU1.

(F) CDG1 phosphorylation of BSU1 increases BSU1 activity of dephosphorylating BIN2. MBP-BSU1 or MBP-BSU1-S764A immobilized on amylose beads were preincubated with GST or GST-CDG1. The beads were washed and further incubated with GST-BIN2. Dephosphorylation of Y200 residue of BIN2 was detected by immunoblotting using the anti-tyrosine 279/216 GSK3α/β (anti-pTyr) antibody. The blot was also stained with Ponceau S to show protein loading. Numbers indicate relative signal levels normalized to the loading control.

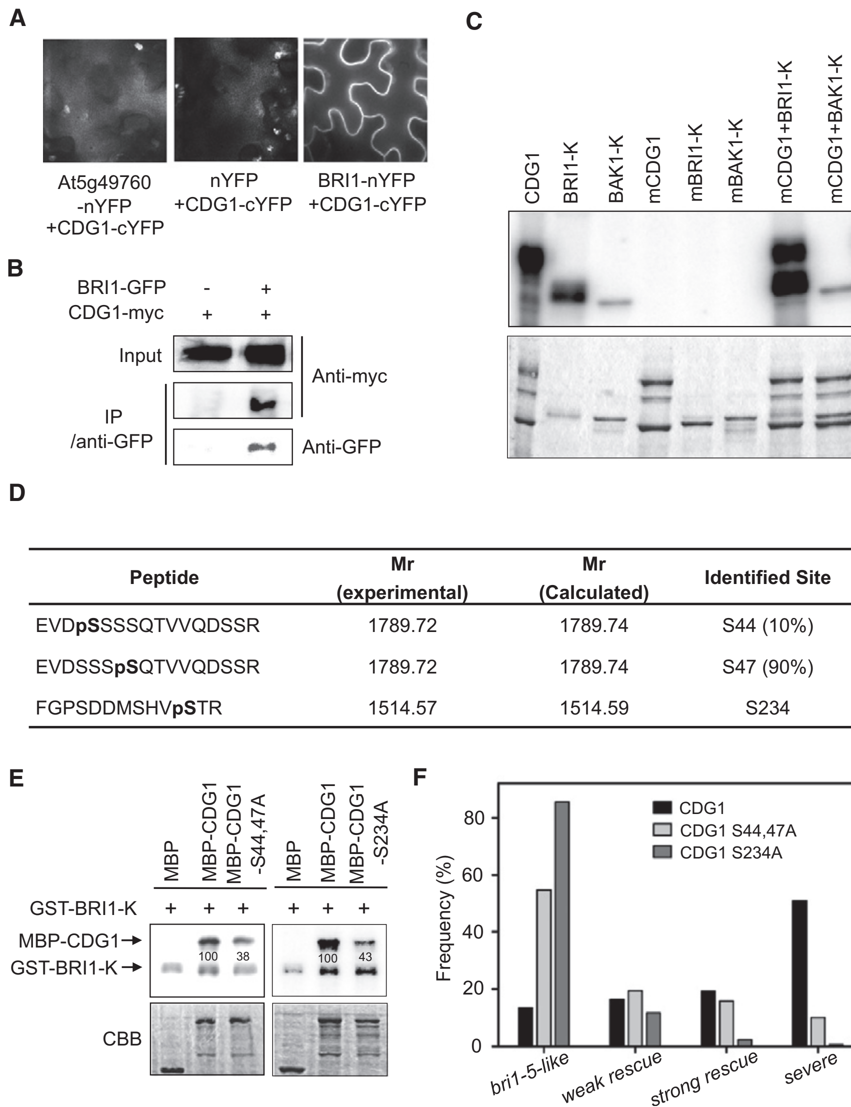
(G) Phenotypic frequency of transgenic *bri1-5* overexpressing wild-type BSU1 or BSU1-S764A. A total of 321 and 264 T1 transgenic plants were counted, respectively.

mutant CDG1 containing mutations of the BRI1 phosphorylation sites in *bri1-5*. The frequency for phenotypic suppression of the *bri1-5* mutant was greatly reduced in transgenic plants transformed with the *35S::CDG1-S234A* construct and slightly reduced in plants transformed with the *35S::CDG1-S44,47A* construct, compared to those overexpressing wild-type CDG1 (Figures 1B and 6F). The results suggest that BRI1 phosphorylation of CDG1 at S234 is a major mechanism of CDG1 activation, whereas phosphorylation in the N-terminal region also contributes to the activation.

The Signaling Cascade of Phosphorylation and Dephosphorylation from BRI1 to BIN2

To understand the mechanism of CDG1 activation by BRI1, we further tested the effect of BRI1 phosphorylation on CDG1 binding to BSU1. Preincubation of CDG1 with BRI1 and ATP increased CDG1 binding to and phosphorylation of BSU1 in vitro (Figures 7A and 7B), suggesting that BRI1 phosphorylation of CDG1 activates CDG1 phosphorylation of BSU1.

The above results demonstrate key steps of BR signal transduction, including BRI1 phosphorylation/activation of CDG1,



Peptide	Mr (experimental)	Mr (Calculated)	Identified Site
EVD**pS**SSSQTVVQDSSR	1789.72	1789.74	S44 (10%)
EVDSSS**pS**QTVVQDSSR	1789.72	1789.74	S47 (90%)
FGPSDDMSHV**pS**TR	1514.57	1514.59	S234

Figure 6. BRI1 Interacts with and Phosphorylates CDG1

(A) BiFC assay of BRI1-CDG1 interaction. Tobacco leaf epidermal cells were transformed with indicated constructs. An LRR-RLK (At5g49760) unrelated to BR signaling was used as a negative control. Bright spots are autofluorescence from chloroplast.

(B) Coimmunoprecipitation between BRI1-GFP and CDG1-myc. BRI1-GFP was immunoprecipitated with anti-GFP antibody from transgenic *Arabidopsis* expressing CDG1-myc or coexpressing CDG1-myc and BRI1-GFP, and the gel blot was probed with anti-myc and anti-GFP antibodies.

(C) BRI1 kinase domain (BRI1-K), but not BAK1 kinase domain (BAK1-K), strongly phosphorylates CDG1 in vitro. Kinase-inactive mutant proteins (mBRI1, mBAK1, and mCDG1) were phosphorylated by the indicated wild-type kinases using ^{32}P-γ-ATP. Upper panel is an autoradiography image, and lower panel shows the gel stained by CBB.

(D) BRI1 phosphorylates three serine residues of CDG1 in vitro. Kinase-inactive GST-CDG1-Y147G was incubated with GST-BRI1-K and subjected to trypsin digestion followed by LC-MS/MS analysis. The percentage in parenthesis indicates relative abundance of phosphopeptide containing phospho S44 and phospho S47 residues.

(E) BRI1 phosphorylation of CDG1 is significantly reduced by S44,47A and S234A mutations of CDG1. Upper panel shows autoradiography and lower panel CBB-stained gel. Numbers indicate relative signal level of phosphorylation normalized to loading control.

(F) Phenotypic frequency of 172, 139, and 351 T1 transgenic *bri1-5* plants overexpressing wild-type CDG1, CDG1-S44,47A, or CDG1-S234A, respectively. The categories of phenotypes were classified based on the phenotype 1, 2, and 3, respectively, as indicated in Figure 1B.

CDG1 phosphorylation of BSU1, and BSU1 dephosphorylation/inactivation of BIN2. To confirm such a signaling cascade in the test tube, we performed in vitro BIN2 Y200 dephosphorylation assays using BSU1 in the presence of CDG1, BSK1 or BRI1 (Figure 7C). Under our experimental conditions, incubation with BSU1 reduced the Y200 phosphorylation of BIN2 by about 20%. When wild-type BRI1 kinase was added without CDG1 or BSK1, there was no significant effect on BSU1 dephosphorylation of BIN2. BSK1 also failed to enhance BIN2 dephosphorylation without active BRI1. However, when BRI1 kinase was added together with BSK1, BIN2 dephosphorylation increased to about 50%. However, BSK1 preincubated with BRI1 kinase and ATP still failed to phosphorylate BSU1 (Figure S7C). When CDG1 was added without active BRI1, BIN2 Y200 phosphorylation was reduced by about 60%, and the dephosphorylation was further enhanced when active BRI1 kinase was added together (Figure 7C). However, when CDG1 and BSK1 were added together, no further increase of BIN2 dephosphorylation was observed; possibly, BSU1 was fully activated under this condition. These results demonstrate that BSU1 can be activated by BRI1 through either BSK1 or CDG1. BSU1 activation by BSK1 requires BRI1 kinase activity, whereas activation by CDG1 does not require BRI1 but is enhanced by BRI1. The results identify BSU1 and either CDG1 or BSK1 as the minimum set of components for transducing the signal from the receptor kinase BRI1 to the GSK3-like kinase BIN2.

We further examined the relationship between CDG1 and BSKs. Of BSK family members, the *bsk3* mutant is known to display strong BL insensitivity and BRZ hypersensitivity (Tang et al., 2008). Therefore, we introduced CDG1-ox into the *bsk3* mutant to test if CDG1 and BSK3 are functionally redundant or act independently of each other. In the root inhibition assay by BL, *CDG1-ox* plants were more sensitive than WT while the *bsk3* mutant showed strong BL insensitivity. Interestingly, CDG1-ox decreased BL insensitivity of the *bsk3* mutant, as it did in WT (Figure S7D). However, importantly, *CDG1-ox/bsk3*

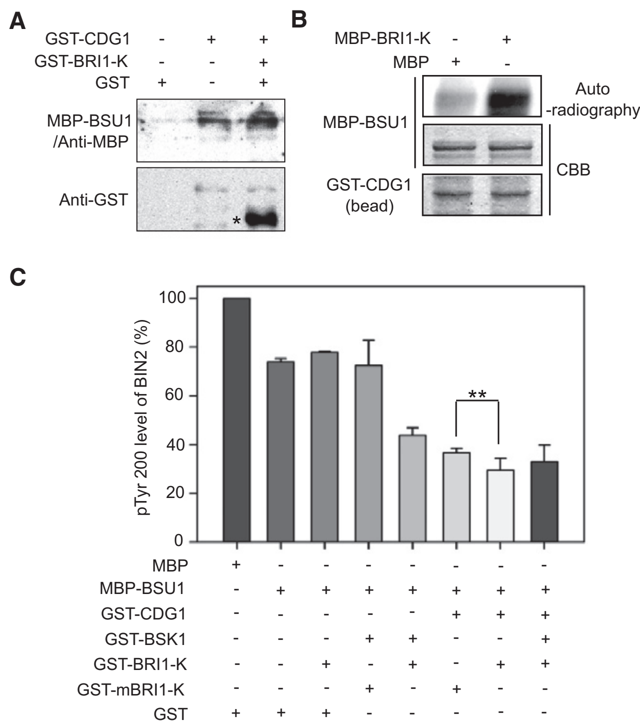


Figure 7. BRI1 Regulation of BIN2 Y200 Dephosphorylation In Vitro through CDG1 or BSK1 and BSU1

(A) BRI1 phosphorylation of CDG1 promotes CDG1 binding to BSU1 in vitro. A gel blot separating GST, GST-CDG1, and GST-CDG1 preincubated with GST-BRI1-K and ATP was sequentially probed with MBP-BSU1 and HRP-conjugated anti-MBP antibody and reprobed with anti-GST antibody. Asterisk (*) indicates GST-BRI1.

(B) Preincubation of CDG1 and BRI1 enhances CDG1 phosphorylation of BSU1 in vitro. GST-CDG1 was preincubated with MBP or MBP-BRI1 in the kinase buffer and then recovered by glutathione beads. Equal amounts of the beads were incubated with MBP-BSU1 and ^{32}P-γ-ATP, and the proteins were analyzed by SDS-PAGE and autoradiography after CBB staining.

(C) BRI1 promotes BSU1 dephosphorylation of BIN2 in the presence of CDG1 and BSK1 in vitro. GST-CDG1, GST-BSK1, GST-BRI1-K, and GST-mBRI1-K were preincubated with MBP or MBP-BSU1 in the presence of ATP for 1.5 hr, and then BIN2 protein was added and further incubated. The gel blot was sequentially probed with anti-pTyr297/216 GSK3α/β antibody and anti-BIN2 antibody, and the ratios between the two signals were quantified as levels of Y200 phosphorylation. Double asterisk (**) indicates a significant difference ($p < 0.08$). Error bars are ± SEM.

double mutant still showed considerable BL insensitivity compared to WT (Figure S7D), suggesting that CDG1-ox is not able to fully complement the phenotype of the *bsk3* mutant. The same phenomenon was also observed in hypocotyl elongation of seedlings grown on BRZ medium in the dark (Figure S7E). These results suggest that CDG1 and BSK3 may act independently.

DISCUSSION

Combinations of genetic and proteomic studies have established the BR signaling pathway as one of the best-understood signal transduction pathways in plants. In particular, the BR signaling pathway serves as a model for understanding the large numbers of receptor kinase pathways in plants. In this study, we further complete this signaling pathway by showing the function of CDG1 in transducing the BR signal from the BRI1 receptor kinase to the BSU1 phosphatase and the BIN2 kinase. Our results reveal mechanistic details of signaling between these components. Together with previous studies (Kim and Wang, 2010; Tang et al., 2011), this study provides the BR signaling pathway with detailed mechanisms of each step of signal relay: BR binding to the extracellular domain of BRI1 induces dimerization between BRI1 and BAK1 and disassociation of BKI1, causing full activation of the BRI1 kinase; BRI1 phosphorylates BSK1 at S230 or CDG1 at S234 to promote interaction with BSU1; CDG1 phosphorylates BSU1 at S764 to increase BSU1 binding to BIN2; BSU1 dephosphorylates BIN2 at Y200 to inactivate BIN2 and stop the phosphorylation of BZR1. In the mean time, PP2A directly interacts with and dephosphorylates phospho-BZR1 (Figure S1) (Tang et al., 2011). Dephosphorylated BZR1 translocates to the nucleus and regulates thousands of BR target genes (Sun et al., 2010).

CDG1 Mediates BRI1 Activation of BSU1

Our analysis of genetic interactions indicated that CDG1 acts downstream of BRI1 and upstream of BIN2. We previously showed that BSU1 also acts upstream of BIN2. The direct interactions of CDG1 with BRI1 and BSU1 indicate that CDG1 acts immediately downstream of BRI1 and thus upstream of BSU1. We further identified three phosphorylated residues of BSU1 (S395, S444, and S764) in vivo and determined that S764 is a major site for CDG1 phosphorylation and activation of BSU1. CDG1 phosphorylation of the wild-type BSU1 increased its binding and dephosphorylation of BIN2, and this effect of CDG1 is abolished by the S764A mutation of BSU1 (Figures 5E and 5F). Consistent with the in vitro data, expression of the mutant BSU1-S764A was less effective in suppressing the *bri1-5* phenotypes compared with the wild-type BSU1 (Figure 5G). These results demonstrate that CDG1 activates BSU1 by phosphorylation of BSU1 S764. Interestingly, CDG1 phosphorylation of BSU1 had no significant effect on BSU1 dephosphorylation of an artificial substrate, suggesting that CDG1 promotes BIN2 tyrosine dephosphorylation by increasing BSU1-BIN2 binding rather than BSU1's catalytic activity.

While phosphorylation of S764 seems essential for CDG1 activation of BSU1, BSU1 seems to have a basal level of BIN2-binding and dephosphorylation activity without CDG1 phosphorylation. The mutant BSU1-S764A showed basal levels of in vitro binding and dephosphorylation of BIN2 independent of CDG1 treatment. Expression of BSU1-S764A also suppressed the phenotype of the *bri1-5* mutant, although at a reduced efficiency compared to wild-type BSU1 (Figures 5E–5G). On the other hand, BSU1 is also phosphorylated in vivo at two additional residues, S395 and S444, which might contribute to regulation of BSU1 in vivo. Unlike S764, mutations of S399 and S444 to alanine did not significantly reduce CDG1 phosphorylation of BSU1 in vitro, suggesting that these residues are not phosphorylated by CDG1. What kinase mediates the phosphorylation of these residues and how they affect BSU1 function remain to be studied.

We showed that BRI1 directly phosphorylates CDG1. Mass spectrometry analysis identified three BRI1 phosphorylation sites of CDG1 (S44, S47, and S234). Mutations of these sites (CDG1-S44,47A or CDG1-S234A) decreased the in vitro phosphorylation by BRI1, as well as in vivo phenotypic suppression of *bri1-5* by CDG1-ox (Figure 6D). The different severities of *bri1*-suppression phenotype suggest that phosphorylation of S234 plays a major role in activating CDG1, whereas S44 and S47 have a smaller contribution. In vitro assays indicated that BRI1 phosphorylation increases CDG1 binding to BSU1. BRI1 phosphorylation may also increase the kinase activity of CDG1, but this is difficult to distinguish experimentally from BRI1's own kinase activity.

Parallel Functions of CDG1, BSK1, and Their Homologs

BSK1 and CDG1 are members of two distinct RLCK subfamilies (RLCK XII and VIIc, respectively), but they seem to play a similar role in BR signaling. The S234 of CDG1 is equivalent to S230 of BSK1, which is phosphorylated by BRI1 to promote binding to BSU1 (Tang et al., 2008). However, while CDG1 activates BSU1 by phosphorylating S764, it is unclear how BSK1 activates BSU1. BSK1 lacks some of the invariant residues considered essential for kinase activity, such as the aspartate of the canonical DFG motif in domain VII, and no kinase activity has been detected for BSK1 or its homologs in vitro (Bayer et al., 2009; Castells and Casacuberta, 2007; Tang et al., 2008). Atypical kinases defective in catalytic activity can mediate signal transduction through protein-protein interaction. For example, the ErbB3 receptor, a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine-protein kinases, is kinase-impaired, but is phosphorylated by other members of the EGFR proteins and acts as a docking site for downstream signaling components (Kim et al., 1998). The BRI1-phosphorylated BSK1 may function as a scaffold or docking platform that brings BSU1 together with BRI1 or CDG1, for phosphorylation, or together with BIN2, to facilitate dephosphorylation and inactivation of BIN2. Alternatively, BSK1 may acquire kinase activity upon phosphorylation by BRI1 or other kinases in vivo, although BRI1 phosphorylation of BSK1 did not confer BSK1 phosphorylation activity of BSU1 in vitro (Figure S7C).

While BSK1 requires BRI1 activation, CDG1 itself shows kinase activity and considerable ability to activate BSU1, independent of BRI1, though this ability is further enhanced by BRI1 (Figures 5F and 7C). Recombinant CDG1 could autophosphorylate S234 (Figure S7F), and it bound to BSU1 more strongly than BSK1 did (Figure S7G). Thus, CDG1 may provide a high basal BR signaling level, whereas BSK1 would provide a more effective switch controlled by the level of BR. It is possible that BSK1 and CDG1 function in different cells to provide different BR sensitivities, or they may also act as two independent parallel mechanisms or act together cooperatively in certain cells. Parallel components mediating the same step of signal transduction has been observed in mammals. For example, insulin signaling activates protein kinase B/Akt (PKB) to suppress GSK3 activity. PKB is activated by both 3-phosphoinositide-dependent protein kinase 1 (PDK1) and mammalian target of rapamycin protein kinase c2 (mTORc2) through phosphorylation on different amino acids of PKB (Cybulski and Hall, 2009).

Different members of each of the CDG1, BSK1, and BSU1 families appear to perform similar biochemical, functions but act in different tissues and have different interaction specificities. CDG1 and BSU1 are highly expressed in pollen but very weakly in vegetative tissues, whereas BSKs, CDL1, and BSL1 showed more ubiquitous expression patterns (Figure S2E), raising a possibility that pairs of members of the two families form tissue-specific signaling modules. Although overexpression of CDG1 and BSU1 causes strong phenotypes during vegetative development, their functions might be restricted to reproductive development in wild-type plants. This possibility is further supported by the higher interaction affinity between coexpressed homologs. The weak phenotype of the *cdg1/cdl1* double mutant suggests that additional homologs may have redundant functions. CDG1 and CDL1 belong to the RLCK VIIc subfamily with 15 members that share 54%–65% amino acid sequence identity (Shiu et al., 2004), among which *At5g13160* (64% sequence identity with CDG1) is expressed broadly like CDL1 and *At5g18610* (63% identity) is expressed highly in pollen similar to CDG1 (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). The additive effects of CDG1-ox and the *bsk3* mutant suggest that they may act in independent parallel pathways, possibly activating different members of the BSU family. We hypothesize that BSKs and CDG1 families may provide not only tissue-specific functions but also may act through independent mechanisms, whereas members within each family can also have distinct or redundant functions.

RLCKs Mediate Signal Output from Plant RLKs

The functions of BSK1 and CDG1 may represent a general role of RLCKs in transducing signals downstream of transmembrane RLKs. About 150 genes in *Arabidopsis* encode RLCKs (Shiu et al., 2004). In addition to BSK1, CDG1, and their close homologs that function in BR signaling, a small number of other RLCKs have been studied in plants. One of them is the short suspensor (SSP) (Bayer et al., 2009), which is a distant member of the BSK family (BSK12) (Tang et al., 2008). Genetic analysis suggests that SSP acts upstream of the YDA MAP kinase cascade in controlling embryonic development (Bayer et al., 2009). Recently, several RLCK VII members, including PBS1, PBS1-like proteins (PBLs), and *b*otrytis-*i*nduced *k*inase *1* (BIK1), were shown to act downstream of multiple immune receptor kinases that detect pathogen-associated molecular patterns (PAMPs) (Lu et al., 2010; Zhang et al., 2010). In particular, BIK1 and PBL1 appear to act downstream of FLS2, the receptor-like kinase perceiving flagellin, in a manner similar to BRI1 regulation of BSK1 and CDG1. BIK1 and PBL1 interact with unstimulated FLS2, and are phosphorylated upon flagellin stimulation and then disassociate from FLS2 (Zhang et al., 2010), which is similar to the BSK1 phosphorylation and dissociation from BRI1 upon BR signaling. CDG1, BIK1, and PBS1 are all members of the RLCK VII subfamily. As such, FLS2 and BRI1 share not only the same coreceptor kinase, BAK1 (Li, 2010), but also members of the same RLCK family for downstream signal transduction. Future studies will clarify whether different RLKs use similar or distinct downstream signaling mechanisms in plants.

EXPERIMENTAL PROCEDURES

In Vitro Kinase and Phosphatase Assays

Recombinant proteins were expressed in *E. coli* and purified by affinity tags. To test phosphorylation of BSU1 and BSU1 variants by CDG1 or CDL1, 0.5 μg of GST-CDG1 or GST-CDL1 was incubated with 2.5 μg of MBP-BSU1 or mutant MBP-BSU1 in the kinase buffer (20 mM Tris [pH 7.5], 1 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT) containing 100 μM ATP and 10 μCi ^{32}P-γ-ATP at 30°C for 3 hr. To examine whether BRI1 phosphorylates CDG1, GST-CDG1-Y147G, GST-mBRI1-K (Y956G), and GST-mBAK1-K (K317E) were incubated with wild-type kinases according to Tang et al. (2008). Protein phosphorylation was analyzed by SDS-PAGE followed by autoradiography.

To test BSU1 activation by CDG1, MBP-BSU1 protein bound to amylose beads was first incubated with GST-CDG1 and ATP or buffer without GST-CDG1 as control, and GST-CDG1 was removed by washing. The pretreated MBP-BSU1 beads were then incubated with GST-BIN2, and BIN2 phospho-Tyr200 was analyzed by immunoblotting using the anti-pTyr297/216 GSK3α/β antibody, as described previously (Kim et al., 2009). For the experiment shown in Figure 7C, 0.5 μg of GST-CDG1, GST-BSK1, GST-BRI1-K, or GST-mBRI1-K were preincubated with MBP or MBP-BSU1 (1 μg) in the presence of ATP for 1.5 hr. Then, 0.5 μg of BIN2 protein, purified using Profinity eXact fusion-tag system (Bio-Rad, Hercules, CA), was added, and the proteins were further incubated for 4 hr. The proteins were separated on SDS-PAGE gel, and the blot was sequentially probed with anti-pTyr297/216 GSK3α/β antibody and anti-BIN2 antibody. The chemiluminescence images were acquired using the digital FluorChem Q analyzer (Alpha Innotech Corp., San Leandro, CA), and the signal of each band was quantified by ImageQuant 5.2 software (GE Healthcare, Sunnyvale, CA).

Determination of Phosphorylation Sites

Proteins were extracted from leaf tissues of transgenic *Arabidopsis* overexpressing BSU1-YFP using IP buffer (50 mM Tris [pH 7.5], 50 mM NaCl, 300 mM Sucrose, 1% Triton X-100, 1 mM PMSF, 1 μM E-64, 1 μM bestatin, 1 μM pepstatin and 2 μM leupeptin) and incubated with anti-GFP antibody bound to protein A beads for 1 hr. Beads were washed five times with IP buffer containing 0.2% Triton X-100. The proteins were eluted with 2% SDS and separated by SDS-PAGE. After Coomassie blue staining, the BSU1-YFP gel band was excised and subjected to in-gel trypsin digestion followed by LC-MS/MS analysis.

To identify the BRI1 phosphorylation site on CDG1, GST-BRI1-K (5 μg), and GST-CDG1-Y147G protein (20 μg) were incubated with 100 μM ATP in the kinase buffer for 12 hr at 30°C. The protein solution was subjected to in-solution alkylation/tryptic digestion followed by LC-MS/MS analysis as described previously (Gampala et al., 2007).

Coimmunoprecipitation

Coimmunoprecipitations were performed as described previously (Kim et al., 2009). The microsomal fraction was prepared by centrifugation at 20,000 g and 100,000 g for 1 hr. The resulting pellet was resuspended in extraction buffer containing 1% Triton X-100. After centrifugation at 14,000 rpm for 10 min, solubilized proteins were incubated with anti-GFP antibody bound to protein A beads for 1 hr. The beads were washed four times with extraction buffer containing 0.1% Triton X-100 and eluted with SDS sample buffer.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2011.05.037.

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