Crystal structures of the phosphorylated BRI1 kinase domain and implications for brassinosteroid signal initiation

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SUMMARY

Brassinosteroids, which control plant growth and development, are sensed by the membrane receptor kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1). Brassinosteroid binding to the BRI1 leucine-rich repeat (LRR) domain induces heteromerisation with a SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK)-family co-receptor. This process allows the cytoplasmic kinase domains of BRI1 and SERK to interact, trans-phosphorylate and activate each other. Here we report crystal structures of the BRI1 kinase domain in its activated form and in complex with nucleotides. BRI1 has structural features reminiscent of both serine/threonine and tyrosine kinases, providing insight into the evolution of dual-specificity kinases in plants. Phosphorylation of Thr1039, Ser1042 and Ser1044 causes formation of a catalytically competent activation loop. Mapping previously identified serine/threonine and tyrosine phosphorylation sites onto the structure, we analyse their contribution to brassinosteroid signaling. The location of known genetic missense alleles provide detailed insight into the BRI1 kinase mechanism, while our analyses are inconsistent with a previously reported guanylate cyclase activity. We identify a protein interaction surface on the C-terminal lobe of the kinase and demonstrate that the isolated BRI1, SERK2 and SERK3 cytoplasmic segments form homodimers in solution and have a weak tendency to heteromerise. We propose a model in which heterodimerisation of the BRI1 and SERK ectodomains brings their cytoplasmic kinase domains in a catalytically competent arrangement, an interaction that can be modulated by the BRI1 inhibitor protein BKI1.

Keywords: hormone signaling, brassinosteroid receptor, protein phosphorylation, receptor kinase, Arabidopsis thaliana, growth control, plant development, protein crystallography.

INTRODUCTION

Plants have evolved a unique set of membrane receptor kinases, which contain an extracellular LRR ligand binding domain, a single membrane spanning helix and a cytoplasmic kinase domain (Shiu and Bleecker, 2001) (LRR-RKs). LRR-RKs sense diverse ligands, ranging from small molecules (Wang et al., 2001) to peptides (Chinchilla et al., 2006; Ogawa et al., 2008) and entire proteins (Yang et al., 2003; Zipfel et al., 2006) to, for example, regulate growth (Li and Chory, 1997), development (Clark et al., 1997; Shpak et al., 2005) and interactions with the environment (Gómez-Gómez and Boller, 2000; Nishimura et al., 2002). Many LRR-RKs in Arabidopsis remain orphan receptors, but selected members have been studied extensively. The LRR-RK BRI1 senses brassinosteroids, a class of polyhydroxylated steroid hormones (Fujioka and Yokota, 2003). A complete signaling pathway that connects brassinosteroid sensing by BRI1 to nuclear transcription factors has been uncovered (Zhu et al., 2013). BRI1 constantly cycles between the plasma membrane and endosomes (Geldner et al., 2007). In the absence of steroid hormone, BRI1 is kept in a basal state by several mechanisms, which include auto-inhibition by its C-terminal tail (CT, residues 1161–1196, Figure 1a) (Wang et al., 2005a), auto-phosphorylation on Thr872 in the kinase domain (Wang et al., 2005b) and interaction with the BRI1 KINASE INHIBITOR protein BKI1 (Wang and Chory, 2006; Jaillais et al., 2011b). BKI1 contains an N-terminal targeting motif for the plasma membrane and a C-terminal sequence
that binds the BRI1 kinase domain, where it inhibits the association of BRI1 with SERKs, a protein family of smaller LRR-RKs essential for BR signal initiation (Li et al., 2002a; Nam and Li, 2002; Karlova et al., 2006; Gou et al., 2012).

The initial events in brassinosteroid signaling have been described in molecular detail. Brassinolide (BL), a potent brassinosteroid in Arabidopsis, is sensed directly by BRI1 (Li and Chory, 1997; Wang et al., 2001), specifically by its extracellular LRR domain (Kinoshita et al., 2005; Hothorn et al., 2011; She et al., 2011). BL-binding to the BRI1 LRR domain causes ordering of a approximately 70-residue island domain (Hothorn et al., 2011; She et al., 2011), creating a docking platform for the smaller and shape-complementary LRR domain of a SERK co-receptor (Santiago et al., 2013; Sun et al., 2013a). The hormone itself acts as a ‘molecular glue’, holding the BRI1 and SERK ectodomains together and bringing their C-termini in close proximity (Hothorn et al., 2011; Sun et al., 2013a).

On the cytoplasmic side of signaling events, it has been established that the kinase domains of BRI1 and SERK3 (BAK1) can physically interact (Li et al., 2002a; Nam and Li, 2002; Oh et al., 2010), and trans-phosphorylate each other in vitro (Li et al., 2002a) and in vivo, following BL stimulus (Wang et al., 2008). Full-length BRI1 and SERK3 have been shown to interact in yeast cells (Nam and Li, 2002) and in planta, in a BL-dependent manner (Wang et al., 2008). Also, BL application induces BRI1-catalysed phosphorylation of the BKI1 membrane targeting motif on Tyr211.

Figure 1. Overall structure of the active BRI1 kinase domain and features of the nucleotide binding site.
(a) Schematic overview of the BRI1 kinase domain constructs used in this study with construct borders included. The JM, KD and CT segments have been previously assigned using a BRI1 homology model (Jaillais et al., 2011b).
(b) Ribbon diagram of the BRI1 kinase domain. The N-lobe (residues 865–956) is shown in light-blue, the hinge region (residues 957–959) in orange, the activation loop (residues 1027–1056) in yellow and the C-lobe (960–1160) in dark-blue, respectively. Four phosphorylation sites present in the structure are highlighted in bonds representation (with phosphorus coloured in cyan).
(c) View of the adenine nucleotide binding pocket in BRI1 occupied by the non-hydrolysable ATP analogue AppNHp (gray, in bonds representation). The two Mn2+ ions are highlighted as magenta spheres, residues contacting the nucleotide are shown in yellow (in bonds representation). Hydrogen-bonding interactions of AppNHp with BRI1 are denoted as dotted lines (in black). The gatekeeper Tyr956 in the back pocket of the binding site makes a hydrogen-bond with Glu927, which in turn salt-bridges to Lys911 to keep the kinase domain in its active conformation. The genetic alleles bri1-1 (Ala909–Thr) and bri1-115 (Gly1048–Asp) in close proximity to the nucleotide binding site are highlighted as green spheres.
(d) A complex structure with ATP (gray, in bonds representation) identifies the γ-phosphate of the nucleotide facing outwards, away from the catalytic Asp1009. An omit 2Fo-Fc electron density map contoured at 1.5 σ is shown alongside (blue mesh). A similar non-catalytic conformation of the nucleotide has been observed in a SERK3–AppNHp complex (in yellow, bonds representation, Protein Data Bank identifier (PDB-ID): 3uim).
(e) The BRI1–ADP complex with ADP in grey (in bonds representation). Hydrogen-bonding interactions of the adenine base and ribose with the BRI1 hinge region (in orange) main chain atoms and with two water molecules (red spheres) are shown as dotted lines (in grey). An omit 2Fo-Fc electron density map contoured at 1.5 σ is shown alongside (blue mesh).
thereby releasing BKI1 from BRI1 at the plasma membrane into the cytosol and allowing BRI1 and SERK3 to interact (Jailais et al., 2011b). Sequential auto- and trans-phosphorylation of the BRI1 and SERK3 kinase domains enable BRI1 to phosphorylate immediate downstream signaling components, such as the BRI1 SUBSTRATE KINASES (BSKs) (Tang et al., 2008; Sreeramulu et al., 2013) and the receptor-like cytoplasmic kinase CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1) (Kim et al., 2011).

It is presently not well understood how the BRI1 kinase domain switches from its basal to its activated state, making it capable of phosphorylating BK1, SERKs and downstream components. BRI1 was first described as a canonical Ser/Thr kinase (Friedrichsen et al., 2000; Oh et al., 2000) and its auto-phosphorylation sites have been mapped (Wang et al., 2005a). Ser/Thr trans-phosphorylation has been demonstrated for brassinosteroid signaling components including SERK3, BSK1 and CDG1 (Tang et al., 2008; Wang et al., 2008; Kim et al., 2011). BRI1 is a dual-specificity kinase as it also harbours significant tyrosine kinase activity and can both autophosphorylate on tyrosine residues within the kinase and juxtamembrane domains (JM; Figure 1a) (Oh et al., 2009) as well as trans-phosphorylate Tyr211 in BK1 (Jailais et al., 2011b) and tyrosines in other proteins (Wu et al., 2012).

In this study we set out to obtain a three-dimensional map of the complex phosphorylation events taking place in BRI1. High resolution crystal structures of an active, phosphorylated BRI1 kinase domain now provide detailed insight into its kinase mechanism. Further, our structures rationalise the effects of previously characterised genetic missense alleles in the BRI1 kinase domain and define a surface area that could mediate interactions with early components in the brassinosteroid signaling pathway.

RESULTS

Overall structure of the BRI1 kinase domain

We systematically screened for crystallisable fragments of the BRI1 cytoplasmic domain (Figure 1a) and obtained crystals for a catalytically active mutant (Thr872–Ala, (Wang et al., 2005a) which omits the JM (residues 814–865) (see Experimental Procedures). It is of note that the JM is crucial for full BRI1 kinase activity (Wang et al., 2005a; Oh et al., 2009, 2012a), and thus some aspects of BRI1’s catalytic function cannot be fully rationalised using our crystallographic model (see below).

We improved the lattice packing of this crystal form by reductive protein methylation (Shaw et al., 2007) and collected data extending to 2.48 Å resolution (Table 1). We determined the crystal structure of BRI1865–1160 using the molecular replacement method. The solution comprises one BRI1 kinase domain per asymmetric unit. BRI1 folds into the canonical bilobal kinase architecture (Figure 1b).

Residues 865–1160 are well defined in the electron density map, including the activation loop (Figure 1b, see below). The entire C-terminal tail however appears disordered and likely occupies large solvent channels in our hexagonal crystals (Figure S1). We speculate that the BRI1 CT is auto-phosphorylated (Wang et al., 2005a,b) and thus released from its auto-inhibitory conformation (Wang et al., 2005b). Consistently, we obtained well-diffracting crystals of a construct comprising only the BRI1 catalytic domain (BRI1865–1160 Thr872–Ala), in its apo-form and in different nucleotide-bound states (Table 1).

The BRI1 nucleotide binding site

A 2.7 Å structure with the non-hydrolysable nucleotide analogue AppNHp and in the presence of Mn2+ ions reveals the substrate in the BRI1 nucleotide binding site, sandwiched between the N- and C-lobe (Figure 1c). There are no significant conformational changes comparing the BRI1865–1160 apo and AppNHp bound states (root mean square deviation (RMSD) is approximately 0.4 Å comparing 283 corresponding Cα atoms). The ATP analogue adopts an active conformation as previously seen for example in Akt/PKB (Yang et al., 2002), with two manganese ions bridging the γ- and β-phosphate of the nucleotide with Asn1014 and with Asp1027 of the DFG motif (Figure 1c). The γ-phosphate contacts the catalytic Asp1009 and the neighbouring Lys1011, the adenine base forms two hydrogen bonds with main chain atoms from the hinge region (Figure 1c). In contrast, a complex structure with ATP presents the nucleotide in a catalytically incompetent configuration with the γ-phosphate rotated outwards away from the catalytic Asp1009, very similar as recently observed in crystals of the SERK3 kinase domain (Yan et al., 2012) (Figure 1d). Finally, a complex structure with ADP at 1.98 Å resolution reveals a well ordered adenine base and ribose but a largely flexible diphosphate moiety of the reaction product (Figure 1e).

The kinase domain itself adopts an active conformation with a salt-bridge formed between Lys911 and Glu927 (Figure 1c). Glu927 in turn establishes a hydrogen bond with the conserved gatekeeper tyrosine, a residue in the centre of the ATP binding site which determines the size of the αC-helix. Asp1027 from the DFG motif (Figure 1e). It has been demonstrated previously that interfering with this interaction pattern (Lys911–Glu, Tyr956–Phe) renders the BRI1 kinase catalytically inactive (Oh et al., 2000, 2009, 2012a), as does mutation of Asp1027 from the DFG motif to asparagine (Jailais et al., 2011b). The strong genetic BRI1 missense allele bri1-1 (Clouse et al., 1996; Friedrichsen et al., 2000) closely maps to the nucleotide binding pocket and the corresponding mutation Ala909–Thr likely interferes with adenine nucleotide binding (green sphere in Figure 1c, compare with Figure S2). On the opposite site of the nucleotide binding pocket, the strong bri1-115 allele
Structural basis for BRI1’s dual-specificity kinase activity

Next, we performed three-dimensional homology searches with the program DALI (Holm et al., 2008). We identified BRI1865–1160 to be closely related to the human interleukin receptor-associated kinase 4 (IRAK-4) (Figure 3a) (Kuglstatter et al., 2007), a member of a group of kinases involved in animal host defence signaling (Li et al., 2002a). Similar to BRI1, IRAKs require two metal ions for catalysis (Hekmat-Nejad et al., 2010) (Figure 1c) and are the only members of the human kinome that use a tyrosine as gatekeeper residue (Figure 3b) (Wang et al., 2006). Indeed we find Tyr262 in IRAK-4 to engage in the same interactions as described above for the BRI1 gatekeeper Tyr956 (Figures 1c and 3b). Structural comparison with the gatekeeper arrangement in SERK3 (Yan et al., 2012) suggests, that hydrogen-bond interactions of the hydroxyl group of the gatekeeper residue (Figure 3b) (Wang et al., 2006) disrupt substrate binding (Li and Chory, 1997).

Our structure identifies that BRI1 is a canonical kinase with respect to its overall fold, structural motifs and catalytic residues. It has however been previously suggested that BRI1 and other plant receptor kinases contain a functional guanylate cyclase (GC) domain embedded in their kinase cores, enabling them to simultaneously exhibit kinase and guanylate cyclase (GC) activities (Kwezi et al., 2007, 2011; Qi et al., 2010). We mapped the putative GC domain suggested for BRI1 (BRI1 residues 1012–1134, yellow in Figure 2a) onto the structure of BRI1865–1160 (Kwezi et al., 2007). The assignment includes the proposed GC catalytic motif (BRI1 residues 1071–1084, red in Figure 2a as well as Asp1038 and 1087 suggested to be involved in magnesium ion coordination (Kwezi et al., 2007). In the structure we find the envisioned GC domain to comprise large parts of BRI1’s C-lobe, with the suggested GC catalytic core buried deep inside the hydrophobic core of the kinase domain and with Asp1038 and 1087 being positioned 35 Å apart from each other. Based on this analysis it is unlikely that a catalytically competent GC domain can form in BRI1. Consistently, we find that BRI1865–1160 cannot form either cGMP or cAMP in HPLC-based activity assays (Figure 2b,c).
the invariant gatekeeper tyrosine with the conserved Lys/Glu salt-bridge (residue 911/927 and 317/334 in BRI1 and SERK3, respectively) are hall-marks of activated plant receptor-like kinases (Figures 3b and S2).

The BRI1 activation loop adopts a conformation rather similar to that seen in the IRAK-4 structure (Figure 3a,c). It is of note that based on the conformation of its activation loop, IRAK-4 has been suggested to be a dual-specificity kinase (Wang et al., 2006). Since BRI1 auto- and trans-phosphorylates on Ser/Thr and on Tyr residues (Friedrichsen et al., 2000; Oh et al., 2000, 2009; Wang et al., 2005a; Jaillais et al., 2011b), we analysed the conformation of the BRI1 activation loop in detail: in planta, the activation loop is phosphorylated on Thr1039, Ser1042, Ser1044/Thr1045 and on Thr1049 (Wang et al., 2005a). A subset of these positions (Thr1039, Ser1042 and Ser1044) is found phosphorylated in our structures (Figures 3c and S2). Thr1039 and Ser1042 are located at the surface of the kinase domain and possibly stabilise the conformation of the activation loop via interaction with His1040 (Figure 3d). This network of interactions is extended by Ser1060 adjacent to the activation loop. The conserved Ser1060 is phosphorylated in our structures (Figures 3d and S2) and may also be phosphorylated in vivo (Wang et al., 2005a). Our analysis suggests that phosphorylation of Thr1039, Ser1042 and Ser1060 may affect the orientation of the activation loop and thus BRI1 kinase activity and its interaction with substrates. Consistently, Thr1039–Ala and Ser1042–Ala mutant plants exhibit intermediate brassinosteroid signaling phenotypes (Wang et al., 2005a).

In contrast, Ser1044 in the BRI1 activation loop displays a strong loss-of-function phenotype (Wang et al., 2005a). We find this residue folded back towards a positively charged pocket formed by Arg1008 from the HRD motif containing the catalytic aspartate, by Arg1032 in the activation loop and by N-lobe Arg922 (Figure 3e). None of the arginines however is in direct contact with the phosphate group of Ser1044, while one would expect to find several direct protein–phosphate interactions in canonical Ser/Thr kinases (Wang et al., 2006). Instead, the Ser1044 phosphate binding pocket in BRI1 is reminiscent of typical tyrosine kinases such as insulin receptor (Hubbard, 1997), as illustrated in Figure 3(e).

The P + 1 pocket in BRI1, which critically determines the kinase substrate specificity, is very similar to the one described for protein kinase A, a classical Ser/Thr kinase (Madhusudan et al., 1994) (Figure 3f). Importantly, Thr1049, which forms the core of the P + 1 pocket in BRI1,
can be differentially phosphorylated in vivo (Wang et al., 2005a). Thr1049 phosphorylation may thus control BRI1 specificity towards different substrates (Figure 3f). The strong phenotype associated with this mutant highlights the importance of the P + 1 pocket in brassinosteroid receptor function (Li and Chory, 1997).

Taken together, the BRI1 activation loop harbours structural features reminiscent of both Ser/Thr and tyrosine kinases, enabling it to act on both types of substrates.
A three-dimensional map of BRI1 phosphorylation sites

A complex pattern of Ser/Thr and tyrosine phosphorylation has been identified for BRI1 (Wang et al., 2005a; Oh et al., 2009). Many of the phosphorylation sites can be mapped onto the BRI1 kinase domain structure (Figure 4a), where they occupy three different functional areas in the enzyme. Not surprisingly, most phosphorylation events take place within the activation loop (see above, yellow in Figure 4a). Thr872, Thr880 and Ser887 form a second cluster in the BRI1 N-lobe, in a region N-terminal to the first β-strand (magenta in Figure 4a). This N-terminal extension is not structurally conserved among different kinase families (Wang et al., 2006), but is known to contribute to kinase function and in the case of BRI1 is essential for kinase activity (Oh et al., 2012a). In the human protein kinase Nek7, interactions between the corresponding segment and the conserved N-lobe region are essential for catalytic activity (Richards et al., 2009). Similar interactions as previously seen with Nek7 are present in BRI1 (Figure 4b). Phosphorylation of Thr872, Thr880 and S887 could disrupt these interactions, leading to reduced BRI1 activity, as previously demonstrated (Wang et al., 2005a). In line with this, we could only obtain crystals of BRI1 upon mutating Thr872 to alanine, thereby possibly stabilising the interaction of the N-terminal extension with the N-lobe β-sheet. It is of note that the N-terminal extension in BRI1 connects to the JM region (highlighted in blue in Figure 4a) which is again important for BRI1 activity (Wang et al., 2005b). Several Ser/Thr and tyrosine phosphorylation sites in the JM also negatively regulate brassinosteroid signaling (Wang et al., 2005a; Oh et al., 2009, 2011).

A third cluster of phosphorylation sites is formed by Ser891 and Tyr956, which are located in the active site of the kinase (red in Figure 4a). Phosphorylation of Ser891 is likely to affect the conformation of the glycine-rich loop in BRI1, which in turn could distort the triphosphate binding pocket for the nucleotide substrate (Figure 4c). Consistently, a phospho-mimicking mutation at position 891 yields severely dwarfed plants, while the corresponding alanine mutant acts as a gain-of-function allele (Oh et al., 2012a,b). Phosphorylation of the critical gatekeeper Tyr956 is likely to completely inactivate BRI1 (Figure 4c), based on its role in the formation of a catalytically competent enzyme (Figures 1c and 3b).

Figure 4. Phosphorylation sites in the BRI1 kinase domain.

(a) Ribbon diagram of the BRI1 kinase domain, coloured according to Figure 1(b). The known phosphorylation sites are highlighted by red spheres. They are grouped into three major clusters; the N-lobe of the kinase (in light-blue), the activation loop region (in yellow) and the kinase active site (in red). The region N-terminal of the first β-strand in BRI1 (residues 865–888) is highlighted in magenta.

(b) Analogous N-terminal regions in BRI1 and the human kinase Nek7. Shown are Cα traces of BRI1 (in blue) and Nek7 (PDB-ID 2qwm, gold) with the BRI1 phosphorylation sites depicted as red spheres, and selected residues shown in bonds representation. Note that the area surrounding Thr872 in BRI1 is very similar in Nek7 and that the N-terminal region needs to unfold to allow for Thr880 and S887 to become phosphorylated.

(c) Detailed view of the BRI1 kinase nucleotide binding site. pSer891 is located in the glycine-rich loop of BRI1 (residues 890–895, glycine residues shown as blue spheres). The gatekeeper Tyr956 is in hydrogen-bond contact with the critical Lys911/Glu927 pair and its phosphorylation is likely to inactivate BRI1.
A protein docking platform located in the BRI1 C-lobe

We next mapped the known BRI1 missense alleles onto the structure (Clouse et al., 1996; Li and Chory, 1997; Friedrichsen et al., 2000; Xu et al., 2008). Most of the alleles target residues in the BRI1 kinase N-lobe (bri1-1, bri1-202), C-lobe (bri1-108, bri1-301) and activation loop (bri1-103, bri1-115) that are likely involved in the structural stabilisation of the enzyme (Figure 5a). This finding is consistent with the strong loss-of-function phenotypes that have been reported for these mutants (Clouse et al., 1996; Li and Chory, 1997; Friedrichsen et al., 2000; Xu et al., 2008). We have previously mapped the binding surface of the inhibitor protein BK1, which binds to the BRI1 C-lobe, specifically to a region involving Ala1104 and Leu1106 (Jaillais et al., 2011b) (Figure 5b,c). Binding of the BK1 C-terminal tail to the C-lobe inhibits the interaction of BRI1 with SERKs, suggesting that the C-lobe could represent a docking platform for the co-receptor kinase domain (Jaillais et al., 2011b). Importantly, bri1-117 (Asp1139→Asn) closely maps to this envisioned interaction surface (Li and Chory, 1997; Friedrichsen et al., 2000) (Figure 5a-c). It is thus possible that in bri1-117 mutant plants, interaction between the BRI1 and SERK kinase domains is affected, which would be consistent with the strong loss-of-function phenotype (Li and Chory, 1997; Friedrichsen et al., 2000).

The isolated cytoplasmic parts of BRI1, SERK3 and SERK2 homodimerise

We noted during the purification of BRI1 domain fragments (Figure 1a) that a construct comprising the entire cytoplasmic portion (JM–KD–CT) eluted much earlier in size-exclusion chromatography experiments than expected for a monomer. We found the elution volume of BRI1814–1196 to be consistent with a homodimer of approximately 80 kDa (Figure 6c) (Jaillais et al., 2011b). We systematically tested different BRI1 fragments (Figure 1a) in analytical size-exclusion chromatography experiments and found that the JM and the CT appear to mediate the formation of BRI1 homodimers, as deletion of either segment renders the resulting kinase domain monomeric in our assays (Figures 6c and S4). This situation holds also true for the cytoplasmic portions of SERK2 and SERK3, which again appear to be homodimers when expressed in isolation in E. coli (Figure 6d and S4). Upon mixing recombinant BRI814–1196 and SERK3250–615, there appears to be only a weak tendency to form larger heterooligomers (Figure 6e,f).

We have previously reported that the spiral-shaped extracellular domain of BRI1 is exclusively monomeric in solution and has no tendency to homomerise upon BL-binding (Hotthorn et al., 2011). Instead we found that brassinosteroid binding creates a docking platform for the smaller and shape-complementary LRR-domain of SERKs (Santiago et al., 2013). The steroid hormone acts as a ‘molecular glue’ and promotes the tight association of the receptor and co-receptor ectodomains (Santiago et al., 2013). To assess the oligomeric state of the BRI1–SERK1 ectodomain complex, we performed analytical ultracentrifugation. Our experiments confirm that the BL-bound BRI1 ectodomain behaves as a monomer in solution, while addition of the SERK1 ectodomain results in the formation of a very stable heterodimer (Figure 6g,h). We conclude that the extracellular domains in BRI1 and SERKs form heterodimers upon sensing brassinolide, while their cytoplasmic segments can form homodimers when expressed in isolation.
DISCUSSION

Evolution of dual-specificity plant receptor kinases

It has been previously suggested based on sequence comparisons and structural homology modeling that plant receptor kinases including BRI1 and SERKs are closely related to the animal Pelle family (Shiu and Bleecker, 2001; Klaus-Heisen et al., 2011). The crystal structure of the BRI1 kinase domain further supports this hypothesis: First, there is a high degree of structural similarity between BRI1 and IRAK-4, the human orthologue of Drosophila Pelle (Figure 3a). Second, BRI1 and SERK3 contain an unusual gatekeeper tyrosine residue, which in animal kinases is only found in the Pelle family (Figure 3b) (Wang et al., 2005).
In our structure, residues involved in the binding of BKI1 and in IRAK-4, and BRI1 and IRAK-4 are dual-specificity kinases (Figure 3c). We thus speculate that the last common ancestor of Pelle/IRAKs and plant receptor kinases already had dual-specificity activity towards Ser/Thr and tyrosine substrates and that the plant kingdom preserved this feature. The BRI1 activation loop contains structural fingerprints from both Ser/Thr and tyrosine kinases (Figure 3e,f), enabling BRI1 to phosphorylate rather diverse substrates (Oh et al., 2000, 2009; Wang et al., 2005a; Tang et al., 2008; Jaillais et al., 2011b). It is interesting that Thr1049 in the P + 1 pocket of BRI1, which critically determines substrate specificity in other kinases, can be phosphorylated in planta (Wang et al., 2008) (Figure 3f). Thr1049 phosphorylation/de-phosphorylation could thus enable BRI1 to switch or modulate its substrate specificity. Alternatively, Thr1049 phosphorylation could be used to inactive the kinase (Wang et al., 2005a).

The BRI1 C-lobe surface is critical for brassinosteroid signaling

In our structure, residues involved in the binding of BKI1 cluster together with the known missense allele bri1-117 at the bottom of the BRI1 C-lobe (Li and Chory, 1997; Friedrichsen et al., 2000; Wang and Chory, 2006; Jaillais et al., 2011b) (Figures 5a-c and S2). We have previously demonstrated that a BK1-derived peptide can bind to this surface and inhibit the interaction of BRI1 with SERK3 (Jaillais et al., 2011b). It is thus possible that the BRI1 C-lobe provides an interaction surface with SERKs. In the case of animal epidermal growth factor receptor (EGFR), formation of an asymmetric kinase homodimer activates the receptor (Jura et al., 2009). In EGFR, this dimer is formed by the JM domain of one kinase partner interacting with the C-lobe of the second kinase partner (Jura et al., 2009; Endres et al., 2011). Interestingly, this interaction between two kinase domains can be modulated by the protein inhibitor MIG6 binding to the C-lobe of one of the partners (Zhang et al., 2007). Based on this structural analogy, one may speculate that in the case of BRI1 and SERKs similar asymmetric dimers (in this case heterodimers) might be formed upon receptor activation and that BKI1 binding to the BRI1 C-lobe could prevent this interaction (Wang and Chory, 2008; Jaillais et al., 2011b).

Kinase homodimers could represent a ground state of the receptor

There is structural and biochemical evidence that the initial extracellular events in LRR receptor kinase signaling involve ligand-dependent heterodimerisation of a receptor protein with a shape-complementary co-receptor (Figure 6g,h (Hothorn et al., 2011; She et al., 2011; Santiago et al., 2013; Sun et al., 2013a,b). Heterodimerisation of BRI1 and SERK1 or of FLAGELLIN SENSITIVE 2 (FLS2) and SERK3 brings the C-termini of receptor and co-receptor in close proximity in crystals (Santiago et al., 2013; Sun et al., 2013a,b). We can thus speculate that interaction of BRI1/FLS2 with SERK ectodomains may also affect the arrangement of their cytoplasmic parts in planta. Experimental evidence for BL-dependent receptor co-receptor heteromerisation in the cytoplasm has been provided in vitro (Li et al., 2002a; Nam and Li, 2002; Oh et al., 2010) and in vivo (Wang et al., 2008), but also ligand-independent heterooligomers have been reported in intact cells ( Bücherl et al., 2013). Recently, it has been demonstrated that both the extracellular and intracellular segments of BRI1 and SERK3 are critical for the interaction and for brassinosteroid signaling (Jaillais et al., 2011a). It is even possible to swap the kinase domains of FLS2 and SERK3 and still obtain a fully functional receptor-co-receptor pair, strongly supporting the heteromerisation model (Albert et al., 2013).

However, there is also substantial in vivo evidence for BRI1 (Wang et al., 2005b) and FLS2 (Sun et al., 2012) homomerisation. In the case of FLS2 the kinase domain and the membrane helix appear to be critically involved in the formation of homooligomers (Sun et al., 2012). Interestingly, BRI1 or FLS2 homomers appear to form independent of ligand stimulus, suggesting that this configuration could represent a resting state for these receptors (Hink et al., 2008; Sun et al., 2012). We speculate based on our biochemical assays, that BRI1 and SERK kinase domains are able to homodimerise using their JM and CT segments (Figure 6a-f), although we cannot exclude that the full-length proteins may behave differently in intact membranes ( Bücherl et al., 2013). Association of the receptor and co-receptor extracellular domains could affect this resting state, by bringing the kinase domains of BRI1 and SERKs into close proximity. Further mechanistic studies in vitro and in planta will be required to fully dissect the relative contributions of BRI1 homomers and heteromers to brassinosteroid signaling.

EXPERIMENTAL PROCEDURES

Protein expression and purification

Either wild-type, kinase-dead (Asp1027-Asn) or hyperphosphorylated (Thr872-Ala) BRI1 receptor kinase domain fragments BRI1865–1166, BRI1814–1160, BRI1865–1150 or BRI1814–1150, and SERK3250–615, SERK3272–566 or SERK2628–626 were recombinantly expressed in E. coli as previously described (Jaillais et al., 2011b). For protein purification, cells were thawed in lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, 4 mM MgCl2, 1 mM ATP and 2 mM β-mercaptoethanol (β-ME)) and lysed with an EmulsiFlex-C3 (Avestin, www.avestin.com). The lysate was centrifuged at 7000 g for 60 min. The supernatant was loaded onto a 5 ml HisTrap HP Ni2+ affinity column (GE Healthcare, www.gelifesciences.com/), washed with 10 column volumes of washing buffer (20 mM Tris pH 8.0, 500 mM NaCl, 4 mM MgCl2, 1 mM ATP, 10 mM imidazole pH 8.0, 2 mM β-ME) and eluted with lysis buffer supplemented with 200 mM imidazole pH 8.0. The 6 × His tag was removed with recombinant tobacco etch virus protease (TEV) at 1:100 molar ratio.

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Monomeric peak fractions were concentrated to about 10 mg ml\(^{-1}\) and dialysed to 5 mg ml\(^{-1}\). Reductive protein methylation of BRI1865 at Thr872 was performed by using 15% (v/v) ethylene glycol and snap-frozen in liquid nitrogen. Crystals of the BRI1865 Thr872-Ala apo complex were obtained when expressed in \(\varepsilon\). coli. These hexagonal crystals initially diffracted to approximately 3.5 \(\AA\) resolution. These crystals were cryoprotected by serial transfer into reservoir solution and using microseeding protocols. Crystals were cryo-cooled in a cryo-jar, and the solution was snap-frozen in liquid nitrogen. Crystals of the BRI1865 Thr872-Ala ATP and BRI1865 Thr872-Ala ADP are summarised in Table 1.

### HPLC-based activity assays

Analysis of ATP and GTP conversion by BRI1 was carried out in buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, 4 mM MgCl\(_2\), 0.5 mM TCEP at 25°C using 10 \(\mu\)M BRI1 and 5 mM ATP or GTP. Twenty microlitre aliquots of the reaction sample were injected onto a Vydac 218TP 5 \(\mu\)m C18 column (4.6 \(\times\) 250 mm) and product elution was analysed at 1 ml min\(^{-1}\) by monitoring the absorbance at 259 nm. The isotropic elution buffer contained 100 mM potassium phosphate (pH 6.5), 6% (v/v) acetonitrile and 10 mM tetrabutylammonium bromide.

### Analytical ultracentrifugation

The oligomeric state of the isolated BRI1 ectodomain and of a purified BRI1-brassinolide-SERK1 complex was investigated at 5 mg ml\(^{-1}\) in 25 mM citric acid/NaOH pH 5.0, 100 mM NaCl buffer at 4°C by monitoring its sedimentation properties at 280 nm using a Beckman Optima XL-A centrifuge fitted with a four-hole AN-60 rotor and double-sector Epon centerpieces. Molecular weight distributions were determined by the CsCl method (Schuch, 2000).

### Generation of antibodies

For generation of BRI1 and SERK3 specific antibodies, purified BRI1 and SERK3 were dialysed against phosphate-buffered saline (PBS) and injected into rabbits. The resulting sera were affinity-purified over BRI1- or SERK3-coupled Affigel 15 (BioRad, www.bio-rad.com) columns and eluted in 200 mM glycine pH 2.3, 150 mM NaCl.

### Size-exclusion chromatography and immunoblotting

To assess homomerisation of the BRI1, SERK3 and SERK2 cytoplasmic portions, gel filtration was performed using a Superdex 75 HR 10/30 column (GE Healthcare) pre-equilibrated in 20 mM HEPES pH 7.5, 100 mM NaCl buffer and 0.5 mM TCEP. The isolated wild-type BRI1, BRI1 brassinolide, and SERK3 were dialysed against phosphate-buffered saline (PBS) and injected into rabbits. The resulting sera were affinity-purified over BRI1- or SERK3-coupled Affigel 15 (BioRad, www.bio-rad.com) columns and eluted in 200 mM glycine pH 2.3, 150 mM NaCl.

### Structure solution and refinement

The structure of the BRI1-Thr872-Ala ADP was determined in space group \(P_6_2\) by the molecular replacement method as implemented in the program PHASER (McCoy et al., 2007), and using the recently reported SERK3 kinase domain structure as search model (Yan et al., 2012) (Protein Data Bank identifier 3uim, residues 272–573). The solution comprises one molecule per asymmetric unit with a solvent content of approximately 53%. The resulting electron density map was readily interpretable and the model was completed in alternating cycles of manual model building in COOT (Emsley and Cowtan, 2004) and restrained TLS refinement as implemented in PHENIX.REFINE (Adams et al., 2010). Analysis with MOLPROBITY (Davis et al., 2007) suggested excellent stereochemistry, with no outliers in the ramachandran plot. Refinement statistics for BRI1-Thr872-Ala ADP, BRI1-Thr872-Ala apo, BRI1-Thr872-Ala AppNH\(_2\), BRI1-Thr872-Ala ATP and BRI1-Thr872-Ala ADP are summarised in Table 1.
SDS-PAGE gel. Proteins were then transferred onto nitrocellulose membranes (GE Healthcare) and blocking was performed using TBS-0.1% Tween buffer and 5% powder milk. Immunoblotting was carried out first incubating the membranes with α-SRK3 or α-BRI1 antibodies (1 h incubation at RT), and then with an anti-rabbit peroxidase-conjugated (1 μg μl⁻¹) antibody (Calibiochem, www.emdbiosciences.com). Detection was performed using the ECL chemiluminescence blotting substrate (Roche, www.roche.com).

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SUPPORTING INFORMATION

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

Figure S1. Lattice arrangement in hexagonal BRI1(1995-1996) crystals.

Figure S2. Key sequence fingerprints in BRI1 kinase domain activation are conserved among other LRR receptor kinases.

Figure S3. Structural superposition of the BRI1 and SERK3 activation loop segments.

Figure S4. SDS-PAGE analysis of analytical size-exclusion chromatography experiments shown in Figure 6c,d.

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