Calcium/calmodulin inhibition of the Arabidopsis
BRASSINOSTEROID-INSENSITIVE 1 receptor kinase provides
a possible link between calcium and brassinosteroid signalling

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The receptor kinase BRI1 (BRASSINOSTEROID-INSENSITIVE 1) is a key component in BR (brassinosteroid) perception and signal transduction, and has a broad impact on plant growth and development. In the present study, we demonstrate that Arabidopsis CaM (calmodulin) binds to the recombinant cytoplasmic domain of BRI1 in a Ca^{2+}-dependent manner in vitro. In silico analysis predicted binding to Helix E of the BRI1 kinase subdomain VIa and a synthetic peptide based on this sequence interacted with Ca^{2+}/CaM. Co-expression of CaM with the cytoplasmic domain of BRI1 in Escherichia coli strongly reduced autophosphorylation of BRI1, in particular on tyrosine residues, and also reduced the BRI1-mediated transphosphorylation of E. coli proteins on tyrosine, threonine and presumably serine residues. Several isoforms of CaM and CMLs (CaM-like proteins) were more effective (AtCaM6, AtCaM7 and AtCML8, where At is Arabidopsis thaliana) than others (AtCaM2, AtCaM4 and AtCML11) when co-expressed with BRI1 in E. coli. These results establish a novel assay for recombinant BRI1 transphosphorylation activity and collectively uncover a possible new link between Ca^{2+} and BR signalling.

Key words: brassinosteroid, BRASSINOSTEROID-INSENSITIVE 1 receptor kinase, calcium signalling, signal transduction, transphosphorylation, tyrosine autophosphorylation.

INTRODUCTION

Plants contain a large family of RLKs (receptor-like kinases) that regulate various growth and developmental processes, phytohormone perception, and biotic and abiotic stress responses [1–3]. BRs (brassinosteroids) are essential plant steroid hormones that regulate multiple aspects of plant growth and development through a complex signal transduction pathway [4]. BR signalling is mediated by the BR receptor, BRI1 (BRASSINOSTEROID-INSENSITIVE 1) [5], and its co-receptor BAK1 (BRI1-ASSOCIATED KINASE 1) [6], both of which belong to the LRR (leucine-rich repeat) RLK family [7]. These plant RLKs contain an LRR extracellular domain, a single-pass transmembrane domain and a cytoplasmic domain that consists of a protein kinase domain flanked by a juxtamembrane and a C-terminal domain. BR signal transduction is initiated by the perception of the hormone in the extracellular domain of the BRI1 receptor kinase [8,9], which then binds to BAK1, with the result that the kinase domains activate by auto- and trans-phosphorylation of residues in the RLK cytoplasmic domains [10–14]. The signal is then transduced via intracellular kinases that phosphorylate downstream transcription factors that alter the expression of genes involved in cell elongation, division and differentiation [6,15,16]. Although the plant RLKs are classified as serine/threonine protein kinases, previous results show that BRI1 and BAK1 also autophosphorylate on tyrosine residues and are thus dual-specificity kinases [11,17]. Site-directed mutagenesis of specific tyrosine residues in BRI1 [11,18] and BAK1 [17] followed by in vitro biochemical analysis and an in vivo test demonstrated that tyrosine autophosphorylation plays an important role in plant receptor kinase function and is an important component in BR signalling.

Ca^{2+} is a universal second messenger that acts as a mediator of stimulus–response coupling in eukaryotes. In plants, various abiotic and biotic stimuli, including phytohormones, mechanical and oxidative stresses, and microbial elicitors, trigger changes in intracellular Ca^{2+} concentration [19,20]. CaM (calmodulin) is one of the primary Ca^{2+}-sensor proteins that can transduce cytosolic Ca^{2+} changes into a cellular response. CaM has no enzymatic activity, but functions by changing its conformation in the presence of Ca^{2+} and concomitantly binding and altering the activities of a wide array of target proteins [21,22]. Identification and characterization of the physiologically relevant protein targets of CaM is particularly important to understand how these proteins have a role in translating Ca^{2+} signals into cellular responses. In terms of BR signalling, previous studies have identified one enzyme involved in BR biosynthesis as a Ca^{2+}/CaM-regulated enzyme [23]. Interestingly, whereas several RLKs have also been reported as binding targets of CaM [24,25], BRI1 was reported to not interact with CaM in vitro when expressed as the recombinant cytoplasmic domain [25]. Curiously, even when binding of CaM to the recombinant receptor kinase cytoplasmic domains was shown to occur, no changes in kinase activities were found, and thus the functional significance of CaM–RLK interactions remains unclear.

In the present study, we show that the cytoplasmic domain of BRI1 interacts with CaM in vitro in a Ca^{2+}-dependent manner. A high-probability CaM-binding site in BRI1 was predicted to...
occur in subdomain VIa of the kinase domain using an algorithm based on known binding proteins (http://calcium.ahnres.utoronto.ca/cdthb/cdthb/home.html), and a synthetic peptide corresponding to the predicted CaM-binding domain was found to interact with CaM in vitro. We also report the novel result that co-expression of CaM with BRI1 in *Escherichia coli* specifically inhibited the kinase activity of BRI1. Both autophosphorylation of BRI1 and BRI1-mediated transphosphorylation of *E. coli* proteins (which is established as a new assay for BRI1 kinase activity) were inhibited. Differences between CaM isoforms were observed, suggesting specificity in the response. Our results suggest that Ca$^{2+}$/CaM may attenuate the kinase activity of BRI1 and thereby uncover a possible new link between Ca$^{2+}$ and BR signalling.

**EXPERIMENTAL**

**Materials**

> Synthetic peptides with an N-terminal biotin group were produced by GenScript and were >90% pure: W980, LNWSTRRKI-AIGSARGLAF; W1099, LVGWVKQHAKLRISDVFD.

**Pull-down assays**

For pull-down experiments, His$_6$-AtCaM7 (At is *Arabidopsis thaliana*) proteins (1 μg) immobilized on 150 μL of Ni-NTA (Ni$^{2+}$/nitrilotriacetate)-agarose beads (Qiagen) were incubated with FLAG-tagged recombinant BRI1 protein (1 μg) in 20 mM Mops (pH 7.5), 1 mM DTT (dithiothreitol) and protease inhibitor mixture (4 mM NaF, 0.2 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], 0.2 mM benzamidine, 0.2 mM capric acid and 0.4 μM E-64) for 1 h at 4°C with or without the addition of 100 μM CaCl$_2$. The agarose beads were washed twice with a buffer of 5 mM sodium phosphate and 150 mM NaCl (pH 7.4), and then twice with the binding buffer. Proteins were eluted using SDS/PAGE loading buffer by heating for 5 min at 95°C. The initial supernatant and the eluted proteins were loaded in equal volumes on to SDS/12% polyacrylamide gels, and immunoblots were performed with the FLAG-tag-specific antibodies. Pull-down assays using neutravidin-coated agarose beads (Pierce) and bionitlated synthetic peptides were conducted using the same buffer and reaction conditions as indicated. Briefly, the bionitlated synthetic peptides (BRI1 W980) containing the putative CaM-binding site of BRI1 were immobilized on neutravidin–agarose beads, and then incubated with the His$_6$–AtCaM7 protein. FLAG–BRI1 protein bound to the beads was eluted using sample buffer and detected by immunoblotting with anti-His$_6$ antibodies.

**Homology modelling of BRI1 kinase domain**

The homology structure of the BRI1 kinase domain (residues 883–1158) was obtained with MODELLER (http://www.salilab.org/modeller/), based on templates of tomato protein kinase Pto (PDB code 2QKW, ~38% identity) and human IRAK-4 (interleukin-1-receptor-associated kinase 4) (PDB code 2NRU, ~34% identity). Structural alignment and visualization were performed using visual molecular dynamics [26].

**Protein-interaction studies**

CaM binding to FLAG–BRII was measured using SPR (surface plasmon resonance) with a Biacore™ 3000 system (GE Healthcare). A total of 300 RU (response units) of the His$_6$–AtCaM7 protein was immobilized on to an Ni-NTA sensorchip. Six concentrations of recombinant BR1 protein were individually passed over the sensorchip in the running buffer [20 mM Mops (pH 7.5), 50 mM NaCl, 0.005% P20 surfactant (Biacore) and 5 mM CaCl$_2$] at a flow rate of 20 μl·min$^{-1}$ for 2 min, and the disassociation was monitored for 10 min immediately afterwards. The sensorchip was regenerated with running buffer plus 300 mM EDTA. All binding curves were normalized with a blank flow cell and a control curve of the empty analyte injection. The $K_d$ values were calculated assuming a 1:1 or 1:2 (CaM/BRI1) ratio with the Langmuir model. For the 1:2 ratio calculation, the molecular mass of analyte FLAG–BRI1 was taken as 88 kDa corresponding to the putative dimeric protein. The global fitting procedure of the software BLAevaluation 3.0 was used to calculate the $K_d$ value using data from the six binding curves.

**LC (liquid chromatography)-MS/MS (tandem MS) analysis**

Denatured *E. coli* soluble proteins were digested with trypsin (Promega) and sequentially enriched for phosphopeptides using TiO$_2$ (Pierce TiO$_2$, Phosphopeptide Enrichment and Clean-Up Kit) followed by IMAC (immobilized metal-affinity chromatography) (PHOS-Select Iron Affinity gel, Sigma–Aldrich). The insoluble protein fraction (referred to as ‘P-bodies’ in Supplementary Table S1 at http://www.BiochemJ.org/bj/443/bj4430515add.htm) was treated similarly. LC–MS/MS analysis was performed on a Waters Q-ToF API-US quadrupole–time-of-flight mass spectrometer with a nanoAcquity UPLC (ultra-performance liquid chromatography) system, with a linear gradient of 1–60% acetonitrile in 0.1% formic acid over 60 min. MS/MS data were collected using the Data Directed Analysis method in MassLynx to fragment the top four ions in each survey scan. Spectra were searched in MASCOT (http://www.matrixscience.com/search_form_select.html) against the NCBI non-redundant database limited to *E. coli*.

**Fluorescence spectroscopy**

Fluorescence spectra were acquired at room temperature (21 ± 1°C) with a PTI QuantaMaster4 spectrofluorimeter (Photon Technology) using excitation and emission bandwidths of 5 nm. The tryptophan residues of BRI1 W980 peptide were excited at 285 nm, and fluorescence emission was measured from 300 to 450 nm. Peptide concentrations were either 1 or 2 μM. Measurements were made in buffer consisting of 20 mM Hepes/KOH (pH 7.2), 100 mM KCl and 1 mM CaCl$_2$ or 5 mM EDTA. Purified recombinant CaM was prepared as described previously [27] and added as described in the Figure legends. For each concentration of CaM tested, the fluorescence of a blank consisting of identical reagents minus peptide was recorded and subtracted from the corresponding fluorescence spectrum containing peptide using the instrument software to account for the small contribution of fluorescence from the phenylalanine and tyrosine residues of CaM. Spectra were exported from the instrument software to Origin v8.0 for graphic presentation.

Dansylated CaM was prepared as described in [28]. Fluorescence of the modified protein was excited at 335 nm and emission was recorded from 400 to 600 nm. Fluorescence was measured in 20 mM Hepes/KOH (pH 7.2) and 100 mM KCl containing 150 mM dansylated CaM and recorded after sequential additions of 1 mM CaCl$_2$, 300 μM peptide and 5 mM EDTA. Peptides representing the CaM-binding domains of CaMKII (Ca$^{2+}$/CaM-dependent protein kinase II) and *Arabidopsis* CNGC2 (cyclic nucleotide-gated channel 2) were purchased from EMD Chemicals and GenScript respectively.
Co-expression of CaM and BRI1 in *E. coli*

*E. coli* cells co-expressing CaM and recombinant FLAG–BRI1 proteins were prepared by transformation of plasmids designed to express CaM proteins into *E. coli* BL21(DE3) pLYS cells (Novagen) harbouring a FLAG–BRI1 expression plasmid. The recombinant BRI1 cytoplasmic domain was expressed from a high-copy-number plasmid under the control of an inducible tac promoter. AtCaM proteins were constitutively expressed from a modified version of pACYC184, a low-copy-number plasmid, containing a p15A origin which allows it to coexist in cells with plasmids of the CoE1 compatibility group such as pBR322 [29]. Constitutive CaM expression driven by this plasmid in *E. coli* was confirmed by extracting soluble proteins and CaM proteins as described previously [27] (Supplementary Figure S1 at http://www.BiochemJ.org/bj/443/bj4430515add.htm). CaM proteins were expressed by this construct in *E. coli* at approximately 1 μg/mg of soluble protein, which is comparable with CaM concentrations in extracts from plants [30].

Recombinant protein purification and immunoblot analysis

Purification of FLAG–BRI1 protein was performed as described in [11] using anti-FLAG M2 affinity gel. Autophosphorylation of the recombinant BRI1 proteins purified from co-expressing *E. coli* cells was analysed by immunoblotting after transfer on to PVDF membranes using Pro-Q Diamond phosphoprotein stain (Invitrogen), anti-phosphotyrosine or anti-phosphothreonine antibodies with the detection of immune complexes using fluorescent secondary antibodies (Invitrogen) as described in [11]. Immunoblotting was carried out as described in [31], and densitometry was performed using an Odyssey Infrared Imaging System (LI-COR Bioscience) to quantify immunoblot results. Relative autophosphorylation levels of BRI1 co-expressed with CaM were calculated as the ratio of the specific phosphorylation signal to the anti-FLAG or Coomassie Brilliant Blue-stained protein signal and the values obtained with the control FLAG–BRI1 cytoplasmic domain expressed alone in *E. coli* were set as 1.

RESULTS AND DISCUSSION

Ca$^{2+}$-dependent binding of CaM to the BRI1 kinase domain

The interaction of CaM with the BRI1 cytoplasmic domain and its Ca$^{2+}$-dependence were tested by pull-down assays (Figure 1). When His$_{6}$–AtCaM7 was immobilized on Ni-NTA–agarose beads, recombinant FLAG–BRI1 protein bound to the beads in the presence of Ca$^{2+}$ (Figure 1A). No binding was observed in the absence of Ca$^{2+}$ or to the Ni-NTA–agarose beads in the absence of bound AtCaM7 (bead control, Figure 1A). Interestingly, CaM binding occurred primarily to the hypophosphorylated form of BRI1 (Figure 1A). In the supernatant fraction, the characteristic upper and lower bands of the FLAG–BRI1 protein (corresponding to hyper- and hypo-phosphorylated forms respectively) were clearly apparent as visualized by an approximate 1:2.9 ratio in densitometry between hyper- and hypo-phosphorylated forms. These results suggest that phosphorylation, perhaps of specific sites, of BRI1 inhibits CaM binding. In order to determine which portion of the BRI1 cytoplasmic domain contained the CaM-binding site, a pull-down experiment was performed with truncated BRI1 proteins lacking either or both of the domains flanking the kinase domain. As shown in Figure 1(B), all of the truncated proteins bound to immobilized AtCaM7, suggesting that the CaM-binding site was located within the kinase domain of BRI1.

We confirmed the binding of BRI1 and CaM using real-time measurements of protein–protein interaction. As shown in Figure 2, immobilized His$_{6}$–CaM7 bound FLAG–BRI1 cytoplasmic domain protein in the presence of Ca$^{2+}$. The calculated $K_d$ value was $\sim$58 nM, which is similar to the binding constant for other CaM-interacting proteins, and, when calculated at a 1:2 (CaM/BRI1) binding ratio (see Figure 5), the $K_d$ value was reduced to 13 nM.

CaM binds to BRI1 kinase subdomain VIa

Using algorithms for predicting CaM-binding domains [32], we identified a potential CaM-binding region in the BRI1 kinase domain (Supplementary Figure S2 at http://www.BiochemJ.org/bj/443/bj4430515add.htm). The sequence contains a cryptophan residue, which is often, but not always, found in CaM-binding sites, and structural modelling of the BRI1 kinase domain suggested that this region resides in surface-localized α-helix E of BRI1 kinase subdomain VIa (Supplementary Figure S3 at http://www.BiochemJ.org/bj/443/bj4430515add.htm). A basic amphiphilic α-helix structure is one of the common features found in most CaM-binding proteins characterized [33]. To test whether the predicted sequence could bind CaM, a synthetic peptide (designated W980 from Ala$^{99}$ to Phe$^{998}$ of BRI1)
spanning the potential CaM-binding domain was immobilized and tested for CaM binding in a pull-down assay (Figure 3A). As predicted, the W980 peptide interacted with His6–AtCaM7 and the binding was strongly Ca2+-stimulated; densitometry of the retained CaM7 in the presence of Ca2+ was 2.7-fold greater than the CaM7 retained in the beads without Ca2+. As an independent approach, the binding of the W980 peptide to dansylated AtCAM2 was assessed by monitoring fluorescence emission of the dansyl fluor, which has the advantage that both molecules are free in solution. As shown in Figure 3(C), the W980 peptide increased the dansyl-CaM fluorescence emission and blue-shifted the fluorescence maximum in the presence of Ca2+. Addition of EDTA to the peptide–Ca2+/-CaM mixture reduced the fluorescence emission, suggesting that the interaction between CaM and the W980 peptide was Ca2+-dependent. This fluorescence enhancement and Ca2+-sensitivity of dansylated CaM exerted by the W980 peptide was similar to that elicited from peptides derived from previously established CaM-regulated proteins, mammalian CaMKII [34] (Figure 3D) and Arabidopsis CNGC2 [35] (Figure 3E). As an additional control, another kinase domain sequence that contains a tryptophan residue (designated W1099 from Leu1096 to Glu1115 of BRI1) was produced and tested for binding to dansylated CaM2. As shown in Figure 3(B), there were some shifts in the fluorescence emission spectrum in the complete reaction, indicating some interaction in vitro, but, overall, the changes were substantially reduced compared with the W980 peptide or the positive control peptides (Figures 3D and 3E).

As a second independent approach to assess the interaction of the W980 peptide with CaM in vitro, we monitored the intrinsic fluorescence of the tryptophan residue in the W980 peptide. This assay can be used because CaM itself does not contain tryptophan residues. Fluorescence emission of the indole ring of tryptophan is a sensitive indicator of its surroundings: when tryptophan is in an aqueous environment, it fluoresces maximally around 355 nm, whereas when it is buried in a hydrophobic region, its fluorescence is enhanced and also blue-shifted [36]. Figure 4A shows that when recombinant AtCaM2 was mixed with the W980 peptide in the presence of Ca2+, fluorescence emission was enhanced and its maximum was blue-shifted to 327 nm. Conversely, EDTA addition reduced fluorescence and red-shifted the maximum to 350 nm. Similar results were obtained with AtCaM4, AtCaM6 and AtCaM7 (Figures 4B–4D). This confirms that binding occurs and suggests further that the indole ring of Trp980 in the peptide is buried in a hydrophobic pocket of CaM when the two interact. The lack of a shift in the fluorescence emission spectrum of the W980 peptide in the presence of CaM in vitro suggests that these CML (CaM-like) proteins do not bind to the peptide or that the binding pocket does not position the tryptophan residue in a hydrophobic environment. Nevertheless, two independent in-solution fluorescence-based assays along with demonstration of binding to an immobilized peptide are consistent with the computational prediction of the CaM-binding site as helix E of BRI1 kinase subdomain VIa.
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Calmodulin regulation of Arabidopsis BRI1

Figure 4 Steady-state tryptophan fluorescence of the BRI1 W980 peptide in the presence of various CaM and CML isoforms

Intrinsic tryptophan fluorescence of the W980 peptide was measured in 20 mM Hepes/KOH (pH 7.2), 100 mM KCl and 1 mM CaCl$_2$ in the presence of (A) AtCaM2 (Z12023), (B) AtCaM4 (Z12022), (C) AtCaM6 (Z12024), (D) AtCaM7 (UB2119), (E) ACML8 (U84678) or (F) ACML9 (U12490) (GenBank accessions are given within parentheses). In each experiment, emission of 1 μM W980 peptide was measured in the absence of CaM or CML proteins (continuous lines, a) and after sequential addition of 600 nM protein (dashed lines, b) and 5 mM EDTA (dotted lines, c).

Steady-state fluorescence titrations of AtCaM6 with the W980 peptide in the presence of Ca$^{2+}$ were measured because the AtCaM6–W980 peptide combination gave the most robust changes in fluorescence (Figure 4C). The results of this experiment, shown in Figure 5, suggest that the stoichiometry of the AtCaM6–W980 complex is 2:1. To confirm this result, we carried out gel-shift assays [37] on mixtures of AtCaM6 and W980 peptide. As shown in Supplementary Figure S4 (http://www.BiochemJ.org/bj/443/bj4430515add.htm), the mobility of AtCaM6 in a non-denaturing gel containing Ca$^{2+}$ was reduced in the presence of the W980 peptide; the mobility shift saturated at 2 mol equivalents of W980 peptide per mol of AtCaM6. In contrast, the mobility of AtCaM6 was quantitatively shifted in the presence of 1 mol equivalent of a peptide derived from the CaM-binding domain of mammalian CaMKII, a result that is consistent with the known atomic level structure of the CaM–CaMKII complex [34]. The contrast in sharpness between the AtCaM6 complexes with W980 and CaMKII peptides probably reflects their differences in affinity for AtCaM6. Nevertheless, the result is consistent with the idea that the AtCaM6–W980 binding stoichiometry is 2:1. Although somewhat atypical, there are other examples where CaM binds to two polypeptides rather than just one [38]. This could indicate that Ca$^{2+}$–CaM-mediated signalling may function in some capacity to regulate BRI1 dimerization, but further experiments will be required to test this notion.

AtCaM6 binding does not affect BRI1 kinase activity in vitro

We tested the effect of Ca$^{2+}/$CaM on BRI1 peptide kinase activity using the SP11 synthetic peptide as substrate [10]. As shown in Supplementary Figure S5 (http://www.BiochemJ.org/bj/443/bj4430515add.htm), addition of increasing concentrations of AtCaM6 in the presence or absence of Ca$^{2+}$ had no significant effect on transphosphorylation of the synthetic peptide. Likewise, previous studies found no effect of CaM binding to BoSRK (Brassica oleracea S locus receptor kinase) [25] or AtCaMRLK (At CaM-binding RLK) [24].

Co-expression of CaM and BRI1 in E. coli inhibits autophosphorylation of BRI1

Another assay of BRI1 kinase activity is autophosphorylation; however, analysis of autophosphorylation in short reactions in vitro [10] does not completely reflect the process that occurs in planta or when recombinant protein is expressed in E. coli [11]. Accordingly, to assess whether autophosphorylation of BRI1 is affected by CaM, we co-expressed the two proteins in E. coli and then examined BRI1 autophosphorylation by immunoblotting. As a control, we co-expressed 14-3-3ω with BRI1. As shown in Figure 6, co-expression of CaM with BRI1 altered the pattern of BRI1 autophosphorylation, and, in general, tyrosine autophosphorylation (pTyr in the Figure) of BRI1 was inhibited more strongly than threonine autophosphorylation or overall autophosphorylation as assessed by Pro-Q Diamond phosphoprotein staining. All CaM isoforms tested had some effect, but CaM6, CaM7 and CML8 were more effective than were CaM2, CaM4, CML11 (GenBank accession number U14842) and 14-3-3ω. The inhibition of BRI1 autophosphorylation is evident from the immunoblot and stain results shown in Figure 6(A), and as quantified in Figure 6(B), where values were normalized for BRI1 protein (Coomassie Brilliant Blue staining). Importantly, the expression of the different CaM isoforms did not vary appreciably as shown in the bottom panel of Figure 6(A), indicating that differences in isoform
effectiveness cannot be ascribed to variation in expression level. It is also worth noting that the production of recombinant BRI1 protein was not appreciably altered by co-expression of the second protein (results not shown). These results suggest that interaction with certain CaM isoforms inhibits BRI1 autophosphorylation and that phosphorylation of tyrosine residues may be affected more than serine and threonine residues.

**Co-expression of CaM and BRI1 in E. coli inhibits transphosphorylation of bacterial proteins**

During the production of recombinant BRI1 protein in E. coli, bacterial proteins are phosphorylated, which provides a novel assay for the transphosphorylation activity of the BRI1 cytoplasmic domain. The assay has been characterized (details are available from S.C.H. on request), but, in the present paper, we document the basic occurrence of this activity only. After 16 h of BRI1 induction, numerous E. coli proteins were phosphorylated as was evident by immunoblotting and Pro-Q Diamond phosphoprotein staining (Figure 7). The phosphorylation of bacterial proteins was dependent on the kinase activity of BRI1, because when the kinase-inactive BRI1(K911E) mutant was expressed, essentially no phosphorylation of E. coli proteins was observed (Supplementary Figure S6 at http://www.BiochemJ.org/bj/443/bj4430515add.htm). The samples analysed in Figure 6 had the majority of the FLAG–BRI1 protein removed by affinity-purification, but some of the BRI1 protein was still apparent at the position expected for a ∼50 kDa protein (Figure 7). The most important result is that co-expression of the CaM isoforms inhibited the transphosphorylation of E. coli proteins. This was most apparent with AtCaM6, AtCaM7 and AtCML8, which almost completely inhibited transphosphorylation on threonine and tyrosine residues. The strong reduction in Pro-Q Diamond staining also suggests that serine phosphorylation was also inhibited. Importantly, co-expression of 14-3-3ω had no apparent effect on the transphosphorylation activity of BRI1, indicating that the effects observed with CaM co-expression were specific. The CaM isoform specificity for inhibition of autophosphorylation (Figure 6) was generally similar to that for transphosphorylation (Figure 7), confirming the notion that CaM binding to BRI1 in situ attenuated kinase activity. Interestingly, these results also suggest that the lack of apparent binding of CML8 to the W980 peptide *in vitro* (Figure 4E) probably means that the binding pocket of CML8 does not position the tryptophan residue in a hydrophobic environment and therefore the fluorescence emission spectrum is unaltered.

To firmly establish the BRI1-mediated transphosphorylation of *E. coli* proteins, samples such as those used in the immunoblot analysis presented in Figure 7 were digested with trypsin and phosphopeptides were enriched with TiO2 or IMAC before analysis by LC–MS/MS. The two enrichment protocols are considered to be complementary [39–41]. Combining all of the results obtained in the present study, a total of 77 unique phosphopeptides consisting of 104 individual phosphorylation sites were identified. The sites were distributed among 41 different *E. coli* proteins that included many enzymes involved in gene expression, translation, protein folding and metabolism. One example is illustrated in Figure 8(A), but a complete list of the phosphopeptides identified is provided in Supplementary Table S1. As shown in Figure 8(A), the DNA-binding protein hupA was phosphorylated on seven distinct residues that were observed in six different phosphopeptides. Interestingly, the seven phosphorylation sites were located either in the N-terminal region of the protein. The results with hupA also illustrate that our analysis identified phosphopeptides that include single, doubly and triply phosphorylated peptides, with a predominance of doubly phosphorylated species (Figure 8B). From analysis of the multiply phosphorylated peptides, the number of residues between phosphorylation sites ranged from zero (i.e. adjacent phosphosites) to nine, with the largest number having four intervening residues (Figure 8C). These results are important because they firmly establish this new assay for BRI1-mediated transphosphorylation and perhaps will give insights into the nature of the sites targeted by BRI1, and, in this regard, the predominance of multiply phosphorylated peptides was certainly unexpected. It is important to note that bacterial cells do have endogenous protein phosphorylation on serine, threonine and tyrosine residues, although at much lower levels than in eukaryotes. In an intensive survey [42], 81 phosphorylation sites on 79 *E. coli* proteins were identified, and two of these endogenous phosphosites were detected in our study: Ser19 of rpmB and Ser45 of dnaK. It is possible that these sites were also targets of BRI1, but it is clear that the vast majority of the sites reported in Supplementary Table S1 were phosphorylated by BRI1 rather than endogenous bacterial protein kinases.

**Concluding remarks**

We have shown that Ca2+/CaM binds to the recombinant BRI1 cytoplasmic domain protein *in vitro* and have identified a likely binding site in subdomain Vla. Interestingly, a previous study found that CaM binds to BoSRK [25] at the equivalent site
Figure 7  Inhibition of BRI1-mediated transphosphorylation of *E. coli* proteins by co-expression of CaM proteins

Following affinity-purification of FLAG–BRI1 protein from cultures co-expressing CaM or CML isoforms, aliquots (containing 15 μg of predominantly *E. coli* proteins) of the remaining protein fraction were separated by SDS/PAGE and analysed for phosphorylation as described in Figure 6. w, 14-3-3ω; CBB, Coomassie Brilliant Blue; IB, Immunoblotting. Molecular masses are indicated in kDa.

In subdomain VIα and because of general conservation of this region, these authors suggested that CaM may interact with other receptor kinases. Indeed, binding of CaM to RLK4 and CLV1 (CLAVATA1) was demonstrated, but BRI1 was reported to not bind [25]; the basis for the discrepancy in results with BRI1 is not clear. However, what is emerging is that CaM appears to interact broadly with plant receptor kinases, including BoSRK, AtRLK4 and AtCLV1 [25], AtCaMRLK [24] and, in the present study, BRI1. Previous studies reported that CaM had no effect on the activity of these kinases, and thus our demonstration that CaM inhibits BRI1 kinase activity when co-expressed in *E. coli* provides the first information about a possible functional impact of CaM binding. The inhibition of BRI1 autophosphorylation and transphosphorylation activity by co-expression of certain CaM isoforms provides compelling evidence for the notion that CaM binding affects BRI1 kinase activity. Moreover, the results uncover a possible link between Ca2+ and BR signalling that will provide the foundation for future studies.

In the present study, we also established a new assay for monitoring transphosphorylation activity of recombinant receptor kinase cytoplasmic domains. This assay is based on the phosphorylation of *E. coli* proteins *in situ* as a result of expression of the recombinant protein kinase and is documented in the present study for BRI1. The assay will almost certainly will have relevance to studies with other receptor kinases as well. In the present study, we have established by immunoblotting that BRI1 phosphorylates numerous *E. coli* proteins on serine, threonine and tyrosine residues (Figure 7). Many specific serine and threonine phosphosites on *E. coli* proteins were identified by LC–MS/MS (Figure 8 and Supplementary Table S1). It is not clear why we were unable to identify any tyrosine phosphosites, but this perhaps reflects their lower relative abundance compared with serine/threonine phosphosites. Of particular relevance to the present study, we used the new assay to demonstrate the inhibitory effect of CaM on BRI1-mediated transphosphorylation activity (Figure 7). Isoforms of CaM and CML proteins differed in their effectiveness and those that inhibited transphosphorylation also inhibited BRI1 autophosphorylation. We observed that AtCaM6 and AtCaM7 were more effective than were AtCaM2 and AtCaM4, and, indeed, CaM6 and CaM7 are most similar to one another in sequence [43]. Interestingly, CML8 was as effective as CaM6 and CaM7 (Figures 6 and 7), suggesting that non-canonical CaM-like proteins may play a role in BRI1 regulation as well. Although the CML family has 50 members, CML8 and its parologue CML11 are most closely related to the CaM proteins, but differed strikingly in their ability to affect BRI1 kinase activity, with CML11 being inactive. Thus subtle sequence differences can have dramatic effects, as has been observed previously for interactions of CaM isoforms with their target proteins [44,45].

The results of the present study raise many important questions that remain to be addressed. First, do BRI1 and CaM/CML proteins interact *in vivo*, and, if so, under what conditions?
A Phosphorylation sites on *E. coli* hupA

| Source | Site | M(expt) | M(calc) | Delta | MC | Score | Expect | Peptide                  |
|--------|------|---------|---------|-------|----|-------|--------|--------------------------|
| TiO    | T4   | 1581.3848 | 1581.7837 | -0.3989 | 1  | 101   | 1.40E-08 | -MNkP10TLV/IAEK.A        |
| IMAC   | T19  | 1054.2634 | 1054.5806 | -0.2425 | 1  | 30    | 0.018  | KAELEkTQA1.A            |
| IMAC   | S17719 | 1134.2084 | 1134.4723 | -0.2638 | 1  | 65    | 0.00029 | KAELEkTQA1.A            |
| TiO    | 527733 | 1676.3594 | 1676.7674 | -0.4080 | 0  | 53    | 2.40E-05 | KAALSPSTLAIgTSKLKE       |
| TiO    | 728733 | 1676.3608 | 1676.7674 | -0.4066 | 0  | 57    | 8.90E-06 | KAELEPSSTLAIgTSKLKE      |
| TiO    | S81  | 1323.3224 | 1323.6588 | -0.3368 | 0  | 79    | 2.00E-07 | KIAANVPAPg8gkA           |

Figure 8 Identification of BRI1-mediated transphosphorylation sites on *E. coli* proteins

(A) Representative LC–MS/MS results identifying BRI1-mediated phosphorylation sites on the DNA-binding protein hupA, obtained by enrichment of phosphopeptides using TiO2 (TiO) or Fe³⁺-IMAC (IMAC) as indicated. The complete list of phosphosites identified is presented in Supplementary Table S1 at http://www.BiochemJ.org/bj/443/bj4430515add.htm. M(expt), expected molecular mass; M(calc), calculated molecular mass; MC, the number of missed cleavages; Score, Mascot score, which is a probability-based implementation of the Mowse algorithm. The total score is the absolute probability that the observed match is a random event and is calculated as $-10 \cdot \log_{10}(P)$, where $P$ is the absolute probability. Expect, expectation value, which is directly equivalent to the $E$-value in a BLAST search result. The lower the expectation value, the more significant the score. (B) Number of residues between adjacent phosphosites in multiply phosphorylated peptides. (C) Number of singly, doubly or triply phosphorylated peptides identified.

Presumably the interaction is regulated by increased cytosolic Ca²⁺ concentrations, which could occur under stress conditions [46,47] or in response to BR signalling [48]. Another important question is whether the inhibition of transphosphorylation activity by Ca²⁺/CaM binding to BRI1 occurs as a result of the inhibition of autophosphorylation. This is our current working model (Supplementary Figure S7), which we believe explains why addition of CaM in vitro had no effect on BRI1 peptide kinase activity (Supplementary Figure S5). If correct, then a rise in cytosolic Ca²⁺ concentration that occurs after the receptor kinase has fully activated would have no immediate consequence on kinase activity, but rather would inhibit the subsequent activation of additional BRI1 molecules. Another crucial area for future research will be to identify all of the downstream cytoplasmic targets (e.g. other protein kinases) that are transphosphorylated directly by BRI1 in vivo in *Arabidopsis* cells, the influence of phosphorylation on their biological functions, and roles that Ca²⁺ and CaM or CML proteins may have in this process, particularly in plants acclimating to biotic or abiotic stress. Although many questions remain, the new insights obtained provide a working model and foundation for continued studies. Moreover, the co-expression system in *E. coli* may be useful for studies of CaM and other proteins as potential interactors with a variety of receptor kinases.

**AUTHOR CONTRIBUTION**

Man-Ho Oh, Hyoung Kim, Xia Wu and Ray Zielinski conducted experiments, contributed to data analysis and preparation of the paper. Steven Clouse contributed to data discussion and preparation of the paper. Ray Zielinski and Steven Huber designed experiments, contributed to data analysis and discussion and assembled the paper.

**ACKNOWLEDGEMENTS**

We thank Dr Zaide Lusthey-Schulten, Dr John Earle and Dr Damien Mathew (all at School of Chemical Sciences, University of Illinois) for sharing their expertise in protein modelling, and Dr Peter Yau and Dr Brian Imai (Protein Sciences Facility, Roy J. Carver Biotechnology Center, University of Illinois) for help with the LC–MS/MS analysis.

**FUNDING**

This work was supported in part by the US Department of Agriculture (USDA)-Agricultural Research Service (ARS) and the National Science Foundation (NSF) [grant numbers IOS-1022177 and MCB-1021363]. Mention of a trademark or proprietary product does not constitute a guarantee or warranty by the USDA-ARS and does not imply its approval to the exclusion of other products that may also be suitable. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the NSF or USDA-ARS.

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SUPPLEMENTARY ONLINE DATA

Calcium/calmodulin inhibition of the Arabidopsis BRASSINOSTEROID-INSENSITIVE 1 receptor kinase provides a possible link between calcium and brassinosteroid signalling

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Figure S1 Constitutive expression of CaM in E. coli driven by prZ528 (CaM7) and S29 (CML8)

Total soluble proteins were extracted from late-exponential-phase bacteria, clarified by centrifugation and subjected to Ca2+ -dependent HIC (hydrophobic interaction chromatography) on phenyl-Sepharose. Samples of the crude extract, the fraction that did not bind to phenyl-Sepharose (unbound), and the fraction that eluted from the resin in the presence of EDTA (bound) were separated by SDS/PAGE and stained with Coomassie Brilliant Blue. Purified AtCaM and recombinant CaM (rCaM) were fractionated in parallel as positive controls together with molecular mass standards (Stds, sizes given in kDa).

Figure S2 BRI1 cytoplasmic domain sequence showing predicted probabilities of CaM binding

The numbers below the sequence indicate the probability of CaM binding (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html) with 9 being the highest score. The kinase domain of BRI1 is shown in red, and the sequences used to generate the W980 and W1099 peptides are underlined.

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Figure S3  Model of the BRI1 kinase domain structure
The BRI1 kinase domain was modelled as described in the Experimental section of the main text. The location of the predicted CaM-binding site (Leu978–Arg992) is shown in yellow and the Try980 side chain is shown in red. (A) Front view. (B) Side view.

Figure S4  Gel mobility-shift analysis of AtCaM6 interaction with W980 peptide
AtCaM6 (300 pmol/reaction) was incubated with 0 (lane 1), 150 (lane 2), 300 (lane 3), 600 (lane 4) or 1200 (lane 5) pmol of W980 peptide or 300 pmol of a CaM-binding peptide from CaMKII (lane 6) then fractionated in a non-denaturing gel containing Ca2+. Coomassie Brilliant Blue staining revealed the migration of free (F) and peptide-bound (B) AtCaM6. The larger size of the CaMKII peptide resulted in a slower mobility of the peptide–AtCaM6 complex compared with the W980 peptide complex.

Figure S5  AtCaM6 has no effect on BRI1 peptide kinase activity in vitro
BRI1 peptide kinase activity was assayed using [γ-32P]ATP and the SP11 synthetic peptide as substrate as described in [1] with the addition of 0.1 mM CaCl2 or 1 mM EGTA as indicated. Each reaction contained 0.5 μg of FLAG–BRI1 protein, and reactions were run for 5 min.
Calmodulin regulation of Arabidopsis BRI1

Figure S6  Kinase-inactive mBRI1 does not transphosphorylate E. coli proteins

BRI1 or the kinase-inactive mBRI1 (mutant BRI1) were expressed in E. coli and following purification of the FLAG-tagged proteins, the remaining E. coli proteins were analysed for phosphorylation by Pro-Q Diamond phosphoprotein staining or immunoblotting (IB) with generic anti-phosphotyrosine or anti-phosphothreonine antibodies. Molecular masses are indicated in kDa.

![Pro-Q Stain](image1)
![IB: anti-pY](image2)
![IB: anti-pThr](image3)
![CBB stain](image4)

**Figure S7  Working model for effects of Ca²⁺/CaM on BRI1**

We propose that Ca²⁺/CaM inhibits BRI1 autophosphorylation, which then affects transphosphorylation activity. Thus certain CaM and CML isoforms inhibit BRI1 autophosphorylation when co-expressed in E. coli and, as a result, also inhibit the transphosphorylation of E. coli proteins. This provides a plausible explanation for why Ca²⁺/CaM inhibits transphosphorylation in the *in situ* system, but does not affect transphosphorylation activity *in vitro* (peptide kinase activity). The model predicts that Ca²⁺ signalling during early stages of BRI1 activation will attenuate downstream signal transduction.

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LC–MS/MS results identifying BRIT1-mediated phosphorylation sites on *E. coli* proteins, obtained by enrichment of phosphopeptides using TiO<sub>2</sub>, Fe<sub>3</sub>–IMAC (IMAC) or processing bodies (P-bodies) as indicated. *M*(exp), expected molecular mass; *M*(calc), calculated molecular mass, MC, number of missed cleavages; Score, Mascot score, which is a probability-based implementation of the Mowse algorithm. The total score is the absolute probability that the observed match is a random event and is calculated as −10 log<sub>10</sub>(*P*), where *P* is the absolute probability. Expect, expectation value, which is directly equivalent to the *E*-value in a BLAST search result. The lower the expectation value, the more significant the score.

The values are expressed as Mr(expt) and Mr(calc) in daltons. The table includes peptide sequences corresponding to the phosphorylation sites identified.

| Source | Site | Name | Mr(expt) | Mr(calc) | Delta | MC | Score | Expect | Peptide |
|--------|------|------|----------|----------|-------|----|-------|--------|---------|
| TiO<sub>2</sub> | Ser<sup>93</sup>/Ser<sup>97</sup> | lacI | 1719.7304 | 1719.3072 | 0.423 | 53 | 7.9 × 10<sup>−8</sup> | 0.0016 | K.AANVPAPVPGSKA |
| TiO<sub>2</sub> | Ser<sup>27</sup>/Thr<sup>33</sup> | hupA | 1676.3608 | 1676.7674 | 0.408 | 0 | 0.89 × 10<sup>−6</sup> | 0.053 | 3.40 K.RALDKGSKAI |
| TiO<sub>2</sub> | Ser<sup>54</sup>/Thr<sup>60</sup> | infB | 1695.7674 | 1695.3088 | -0.46 | 1 | 4.00 × 10<sup>−9</sup> | 0.0001 | K.GGGPSGSGAQARI |
| IMAC | Ser<sup>30</sup> | rplO | 1486.3286 | 1486.5690 | 0.241 | 1 | 0.24 × 10<sup>−6</sup> | 0.067 | 0.018 K.AELSK |
| P-bodies | Ser<sup>47</sup> | rplM | 1122.2354 | 1122.5183 | 0.282 | 0 | 0.10 × 10<sup>−6</sup> | 0.010 | 7.00 K.LK |

**Table S1 Identification of BRIT1-mediated phosphorylation sites on *E. coli* proteins**
### Table S1 Continued

| Source | Site | Name | M (expt) | M (calc) | Delta | MC | Score | Expect | Peptide |
|--------|------|------|----------|----------|-------|----|-------|--------|---------|
| TiO₂   | Thr³ | gatZ | 911.2322 | 911.4663 | −0.2341 | 1  | 46    | 0.0025 | −MKpTLIAR.H |
| TiO₂   | Ser⁴⁷⁶| aspA | 1106.1322 | 1106.3917 | −0.2595 | 1  | 45    | 0.0017 | K.RYTDpSEQ |
| TiO₂   | Thr⁵⁴⁸/Thr⁵⁴⁰| pnp     | 1082.1788 | 1082.4563 | −0.2774 | 1  | 36    | 0.0098 | R.VIpTDpTR.I |
| TiO₂   | Ser⁶⁷/Thr⁶⁹ | gatD   | 1592.2680 | 1592.6671 | −0.3990 | 1  | 41    | 0.00036 | −MKpSVIpTDpTDGVNR.V |
| TiO₂   | Thr⁸⁷⁷/Thr⁹⁴⁷ | sucB | 1876.3088 | 1876.7761 | −0.4673 | 2  | 40    | 0.0014 | K.EIpTSASKEKApTpASQp |
| TiO₂   | Ser⁷⁵ | frr   | 927.1428 | 927.3773 | −0.2344 | 0  | 39    | 0.0078 | R.SMgpSPAVEnK.A |
| TiO₂   | Ser² | lipA | 1038.2284 | 1038.4933 | −0.2648 | 0  | 30    | 0.019 | M.pSKKpVEpVMER.G |
| IMAC   | Thr³ | yqjD | 1316.2666 | 1316.5874 | −0.3208 | 1  | 54    | 5.00 × 10⁻¹ | M.SKIpTIEpTHLR.A |
| IMAC   | Thr³ | yqjD | 1316.2684 | 1316.5874 | −0.3190 | 1  | 33    | 0.0047 | M.SKIpTIEpTHLR.A |
| IMAC   | Thr³/Thr⁷ | yqjD | 1396.2338 | 1396.5537 | −0.3199 | 1  | 67    | 5.80 × 10⁻⁴ | M.SKIpTIEpTHLR.A |
| IMAC   | Thr⁰⁶ | espF | 921.2758 | 921.4089 | −0.1311 | 0  | 29    | 0.026 | R.HIpTSASQp |
| IMAC   | Thr⁰⁶ | grpE | 1238.3146 | 1238.6020 | −0.2073 | 2  | 28    | 0.039 | R.RIpTEpTDIEK.A |
| IMAC   | Thr⁰⁴ | tulA | 2736.8350 | 2737.2639 | −0.4289 | 0  | 25    | 0.024 | K.NMpTGAGMDGAILVAATDGPMPQp |
| P-bodies | Thr¹⁷⁵/Ser¹⁹⁰ | ahpC | 1658.2694 | 1658.7205 | −0.4511 | 0  | 35    | 0.0025 | K.EEpApTLAfpSpSLpDVGKI |

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Received 19 October 2011; accepted 7 February 2012
Published as BJ Immediate Publication 7 February 2012, doi:10.1042/BJ20111871

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