

ABSTRACT

BAJWA, VIKRAMJIT SINGH. Comparative Analysis of Early Events in Tomato and Arabidopsis Brassinosteroid Signal Transduction. (Under the direction of Dr. Steven D. Clouse.)

Brassinosteroids (BRs) are essential plant hormones required for regulating numerous aspects of plant growth and development. BR-deficient and BR-insensitive mutants displaying a characteristic dwarf phenotype were instrumental in understanding the molecular mechanisms of BR biosynthesis and signaling in the experimental plant *Arabidopsis thaliana*. Isolation of BR-mutants from tomato, rice, barley and pea, expands the importance of these compounds from a model system to crops and the detailed genetic map, EST sequence database and relatively high transformation efficiency of tomato makes this plant an excellent model system for studying the molecular biology of horticultural crops. Our experiments examined the conservation in BR signaling mechanisms between Arabidopsis and tomato.

In Arabidopsis, BRs are perceived at the cell surface by a membrane bound leucine-rich repeat receptor-like kinase, BRASSINOSTEROID INSENSITIVE 1 (BRI1). A second LRR-RLK known as BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) interacts with BRI1 *in vivo*, suggesting an important role of receptor kinase heterodimerization and transphosphorylation in BR signaling. The cytoplasmic kinase domains (CDs) of tomato and Arabidopsis BRI1 and BAK1 show high sequence similarity with conservation of numerous Ser and Thr residues. Kinase assays of affinity purified CDs of tomato BRI1 (tBRI1-CD) and BAK1 (tBAK1-CD) revealed that both kinases autophosphorylate independently, and can also transphosphorylate each other. Tomato BRI-CD also phosphorylates peptide substrates with a similar sequence motif required for optimal phosphorylation by Arabidopsis BRI1.

We further determined specific phosphorylation sites of tBRI1-CD and tBAK1-CD using liquid chromatography-tandem mass spectrometry (LC/MS/MS and/or LC/MS^E) and were able to identify 12 *in vitro* phosphorylation sites in tBRI1 and seven in tBAK1. Interestingly, 10 of the tBRI1 sites and five of the tBAK1 sites were conserved in Arabidopsis, but two sites each of tBRI1 and tBAK1 were not, suggesting significant conservation but also possible differences in BR signaling events between these species.

Site-directed mutagenesis of identified and predicted tBRI1 phosphorylation sites revealed that phosphorylation of highly conserved residues in the kinase domain activation loop of tBRI1, was essential for kinase function *in vitro*. Furthermore, mutations in specific juxtamembrane residues to their unphosphorylated counterparts, increased tBRI1 kinase activity. Analysis of tomato transgenic lines expressing full-length 35S::tBRI1-Flag wild-type and mutated constructs in the weak tomato *bri1* allele, *curl3^{-abs1}*, showed that the highly conserved residue T-1054 is essential for normal BRI1 signaling *in planta*. We attempted to identify *in vivo* tBRI1 phosphorylation sites using transgenic tomato lines expressing full-length tBRI1-Flag, and while we did find peptides for tBRI1-FLAG and its *in vivo* interacting proteins by LC/MS^E analysis, no *in vivo* phosphorylation sites of tBRI1 have been found.

Finally, we cloned the tomato ortholog of Transforming Growth Factor- β Interacting Protein *TRIP1* (*tTRIP1*), which was previously shown to be a BRI1 interacting protein and kinase domain substrate in Arabidopsis. *In vitro* kinase assays demonstrated that tTRIP-1 is a substrate of tBRI1 and tBAK1 kinases and that tBRI1 can phosphorylate the same TRIP1 peptide substrates recognized by Arabidopsis BRI1. Tomato plants constitutively overexpressing tTRIP1 (containing N and C terminal epitope tags) were also generated for future studies of *in vivo* function of TRIP1 in tomato.

In summary, we examined early events in tomato BR signal transduction mechanisms using molecular genetics, proteomics, kinase biochemistry and mass spectrometry. The comparative functional analysis of tomato BRI1 and BAK1 kinase activity and phosphorylation sites with their Arabidopsis counterparts revealed significant inter-species conservation but also possible divergence in receptor kinase mechanisms involved in BR signaling regulating plant growth and development.

Comparative Analysis of Early Events in Tomato and Arabidopsis Brassinosteroid Signal
Transduction

by
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DEDICATION

My Guru, Baba Kashmira Singh Ji

And

My Parents

BIOGRAPHY

Vikramjit Singh Bajwa was born on November 20, 1979, in his ancestral village Basarpur, Punjab (India) to S. Beant Singh Bajwa and Mrs. Harbhajanjeet Kaur. He has an elder sister Navdeep Kaur, who is now married and blessed with two children. Vikramjit completed his middle school education from Saint Francis Convent School, Fatehgarh Churian, and moved to Amritsar with his parents where he completed his high school education from Sri Guru Harkrishan Public School, Amritsar. After finishing his high School, he joined Punjab Agricultural University (PAU) in Ludhiana, India as an undergraduate student. He successfully completed his undergraduate studies in Agriculture with a major in Horticulture. After completing his undergraduate degree he joined Post Graduate Diploma in Information Technology at PAU in 2001, which he completed in 2002. During his years in PAU, Vikramjit was part of a basketball team and represented his University at inter-varsity level competitions.

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CHAPTER One: Literature review: Brassinosteroids

1.1. History of Brassinosteroids

Brassinosteroids (BRs) are essential plant hormones that are ubiquitous throughout the plant kingdom (Takatsuto, 1994). The first report describing the role of what would later be identified as BRs was published in *Nature* by Mitchell et al. (1970), where they reported the growth-promoting activity of *Brassica napus L.* pollen extracts at a very low concentrations. They partially isolated the specific growth-promoting compound from rape pollen by chromatographic methods and termed it “Brassins”. The biological activity of Brassins was measured using a bean second-internode assay. Mandava and Mitchell (1971) detected brassin-like activity in extracts of 16 different plant pollens using the bean second internode assay to monitor activity. The application of brassins to field crops was shown in some cases to enhance plant growth, crop yield and seed viability (Mitchell and Gregory, 1972). Japanese scientists also isolated a compound from the leaves of *Distylium racemosum*, which they named Distylium factor, that had growth-promoting activities similar to Brassins in a rice lamina assay. Later it was found that Brassins and Distylium factor were very similar chemically as well as functionally, so both were subsequently classified as BRs (reviewed by Yokota, 1999).

1.2. Chemical Structure

Brassinolide (BL) was the first BR whose chemical structure was determined. Spectroscopic methods, including X-ray crystallography were used to identify the structure of BL isolated from *Brassica napus* pollen. BL was determined to be (22R,23R,24S)-

2 α ,3 α ,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5 α -cholestan-6-one (Grove et al., 1979).

Structurally, all BRs are composed of a steroid nucleus with four rings, A, B, C and D and a side chain at C-17 (Figure 1).

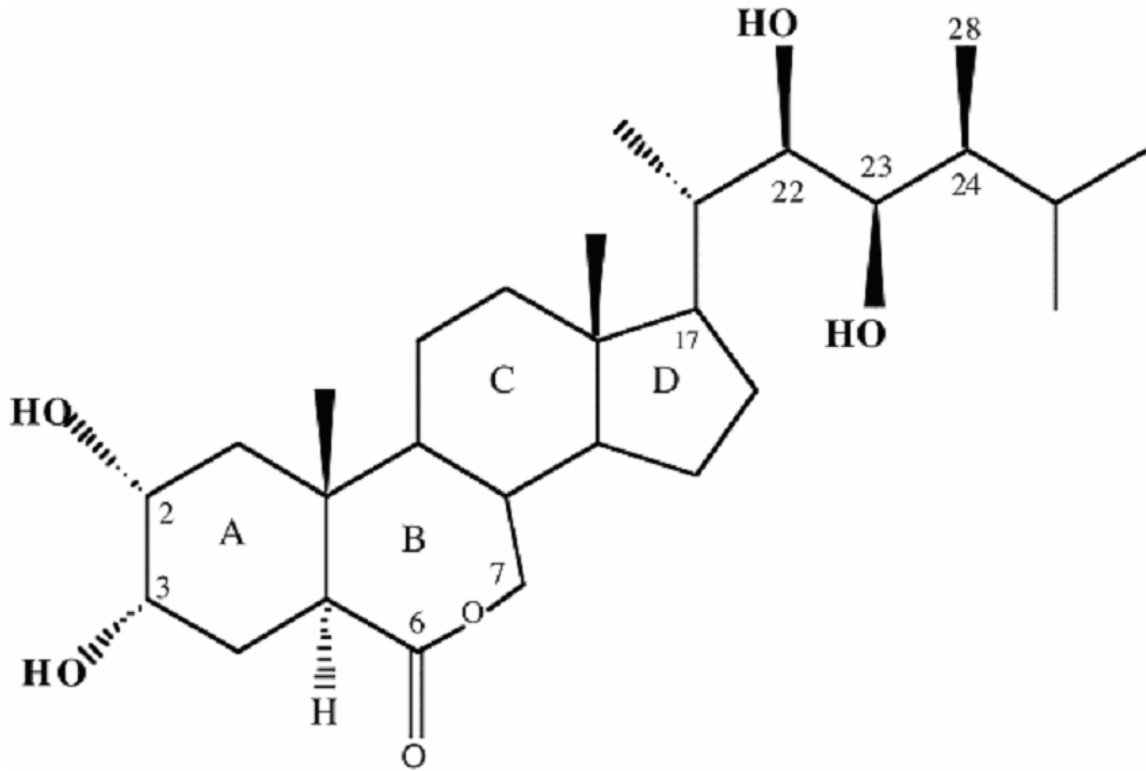


Figure 1. Chemical structure of plant steroid hormone, brassinolide, composed of a steroid nucleus with four rings, A, B, C and D (adapted from Grove et al., 1979). The structural requirements associated with optimal BR activity include: 1) presence of oxygen at C-6 in ring B; 2) presence of cis-glycols at C-2 and C-3 in ring A; 3) presence of hydroxyl groups at C-22 and C-23; 4) presence of a trans A/B ring junction; and 5) presence of a methyl or ethyl group at C-24 (reviewed by Mandava, 1988).

1.3. Natural Occurrence of Brassinosteroids in the Plant Kingdom

BRs have been detected throughout the plant kingdom in every species that has been examined. To date, 54 BRs and five of its conjugates have been isolated from 58 plant species. These BRs have been detected in angiosperms, gymnosperms, pteridophytes, bryophytes and a chlorophyte or alga. Pollen grains and immature seeds are the richest source of BRs with contents ranging from 1-100 ng/g of fresh weight, whereas the stems and shoots have the lowest BR content ranging from 0.01-0.1 ng/g of fresh weight (reviewed by Bajguz and Tretyn, 2003).

1.4. Biochemical Analysis of Brassinosteroids

Extraction and purification of BRs from vegetative tissue generally involves a solvent-based partitioning followed by a chromatographic separation. The active fractions containing BRs are tracked via one of the three bioassays described below. Further analysis of the purified fractions can be performed using analytical techniques such as gas chromatography-mass spectrometry (GC/MS), GC-MS selected ion monitoring (SIM), fast atom bombardment-mass spectrometry (FAB), high pressure liquid chromatography, and liquid chromatography-mass spectrometry (LC/MS) (Takasuto and Yokota, 1999).

The three main bioassays that are utilized to study structure-activity relationships of BRs are:

1) Bean second-internode bioassay: In this assay, elongation in the bean second internode length is measured four days after application of the test chemical. Application of BL not only increases the internode length but also causes swelling, curvature and splitting of the internodes (Thompson et al., 1981). This assay played a critical role in isolation of BL containing fractions for its structural analysis (Grove et al., 1979).

2) Rice-lamina inclination bioassay: This is the most commonly used bioassay to isolate BRs. Rice explants composed of the leaf blade, leaf sheath and the lamina joint are cut and floated on a solution of test chemical or distilled water (control). This is a very sensitive bioassay as BRs cause lamina bending even at very low concentrations. The rice lamina bioassay was used to isolate castasterone (CS) from chestnut insect galls (Yokota et al., 1982).

3) Wheat leaf unrolling test: This bioassay was developed by Wada et al. (1985). This is a relatively convenient bioassay, but is 10 times less sensitive than the rice-lamina inclination test (Takatsuto and Yokota, 1999).

1.5. Physiological Effects of Brassinosteroids

Similar to other endogenous plant hormones like auxins, gibberellins, ethylene, cytokinins, abscisic acid, jasmonic acid and salicylic acid, BRs affect multiple physiological processes in plants. A role for BRs is known in cell expansion and organ elongation, promotion of cell division, regulation of vascular differentiation, promotion of senescence, regulation of fertility, modulation of biotic and abiotic stresses, skotomorphogenesis and photomorphogenesis, timing of flowering, and regulation of gene expression (reviewed by Clouse and Sasse, 1998; Mandava, 1988; Sasse, 2003; Sasse, 1999).

The identification and characterization of BR-deficient and BR-insensitive mutants with pleiotropic phenotypic defects further confirmed the physiological importance of BRs (Clouse and Feldmann, 1999).

1.5.1. Cell elongation

The application of BRs at nM to μ M concentrations enhances cell elongation which leads to expansion of hypocotyls, epicotyls and peduncles in dicots, and coleoptiles and mesocotyls in monocots (Clouse, 1996; Mandava, 1988; Sasse, 1991). The elongation response caused by exogenous application of BRs is mostly observed in young tissue, whereas mature tissue shows little or no elongation (Sasse et al., 1992). The mechanism of BR-induced cell elongation is similar to that of auxins, which involves the extrusion of protons via ATPases leading to hyperpolarization of the cell membrane and acidification of the apoplast, leading to changes in the activity of wall-modifying enzymes (Bajguz and Czerpak, 1996; Cao and Chen, 1995). Some auxin-regulated genes including, *GHI* and *SAUR* that are involved in cell elongation, are also up-regulated by BRs, however, the kinetics of regulation is very different between auxins and BRs. In general, the auxin-mediated response is much quicker, whereas the BR-mediated response is delayed (Clouse and Sasse, 1998). The cross-talk between auxin and BR signaling is important for cell elongation (Nakamura et al., 2003).

BRs and auxins synergistically affect physiological processes such as cell elongation by regulating the expression of common target genes known as auxin-BR genes or A/B genes. At a molecular level, the synergism between auxins and BRs occurs at a transcriptional level, and is largely independent of their biosynthetic pathways (Hardtke, 2007). Recent studies also indicate that cross-talk between auxin and BRs also modulates photomorphogenesis. Transcription of *BASI* and *SOB7* genes, encoding enzymes that

inactivate BRs, is upregulated by indole acetic acid (IAA) (Papanov et al., 2008; Song et al., 2009). Both *BASI* and *SOB7* are involved in regulating photomorphogenesis (Turk et al., 2005). Genome wide analysis showed that BR deficiency upregulates, while BL treatment downregulates, the expression of light-responsive genes, confirming a negative role of BRs in photomorphogenesis (Song et al., 2009).

BRs regulate cell wall elongation by inducing a number of genes. Cell walls consist of a complex network of microfibrils tethered to xyloglucans. For cell expansion to occur, these tethers need to be broken or loosened. BRs regulate a number of cell wall modifying enzymes, including endo-1,4-beta-glucanases, xyloglucan *endo*-transglycosylase/hydrolases (XTHs, formerly known as xyloglucan endotransglycosylases or XETs), expansins, sucrose synthase and cellulose synthase, indicating that all of these proteins may play an important role in BR-mediated cell expansion (reviewed by Zurek et al., 1994; Sasse, 2003).

Several recent studies have contributed significantly to our understanding of the molecular basis of BR-regulated cell expansion. Brassinazole resistant 1 (BZR1) and BRI1-EMS suppressor (BES 1) belong to a family of transcription factors that regulate the expression of many BR-responsive genes. In rice, BZR1 positively regulates the expression of transcription factor ILI1 which is involved in cell elongation of the rice-lamina joint cells, and negatively regulates the expression of the ILI1-binding basic helix-loop-helix (bHLH) protein, IBH1 which inhibits cell elongation in rice (Zhang et al., 2009). This mechanism of BR mediated cell elongation was also found to be conserved in Arabidopsis (Zhang et al., 2009). In Arabidopsis, BR-mediated cell elongation is also developmentally regulated by

BES1, which is in turn developmentally regulated by the jumonji domain-containing proteins, early flowering 6 (ELF6) and relative of early flowering 6 (REF6) (Yu et al., 2008). In Arabidopsis, BES1 also regulates the expression of *Catharanthus roseus* receptor-like kinase (CrRLK1) family members, HERCULES1 (HERK1), HERCULES2 (HERK2), THESEUS1 (THE1) and FERONIA (FER) which are involved in BR-mediated cell elongation during vegetative growth (Guo et al., 2009a; Guo et al., 2009b)

Yamagami et al. (2009) used a chemical genetics approach to demonstrate the role of the transmembrane protein BRZ-insensitive-long hypocotyl 4 (BIL4) in BR-mediated cell expansion in Arabidopsis. In addition the rapid alkalization peptide growth factor (RALF) impairs hypocotyl elongation in seedlings. However, BL treatment down regulates AtRALF23 expression, thereby possibly relieving the growth-retarding effect of this peptide growth factor (Srivastava et al., 2009)

1.5.2. Cell division

The mechanism of BR-induced cell division is not clearly understood. The histological examination of the BR-deficient *cbb* mutant showed that dwarfism in the mutant was mainly a result of reduced cell size rather than number (Kauschmann et al., 1996). This is puzzling because BRs have been shown to enhance cell division in the presence of auxins and cytokinins in cell cultures and protoplasts (Clouse and Zurek, 1991; Oh and Clouse, 1998). The transcript level of the cell cycle regulatory enzyme, CYCD3, involved in regulation of G1/S transitions in the cell cycle, is up-regulated in Arabidopsis *det2* cell suspension cultures treated with 24-epibrassinolide (Hu et al., 2000), which indicates a positive role of BRs in controlling cell division. However, anti-proliferation activity of BR

analogues, 28-homocastasterone and 24-epibrassinolide in breast cancer and prostate cancer cells has been reported, indicating BRs may also have a role as cell division inhibitors (Malikova et al., 2008).

1.5.3. Vascular Differentiation

The role of BRs in cell differentiation has been studied in detail in two model systems, *Helianthus tuberosus* explants and isolated mesophyll cells of *Zinnia elegans*, where nM concentrations of BR promote xylem differentiation (Clouse and Zurek, 1991; Iwasaki and Shibaoka, 1991; Fukuda, 1997; Fukuda, 2004). Subsequently, identification and characterization of a BR-deficient mutant, *constitutive photomorphogenesis and dwarfism (cpd)* that had unequal cambium division established the importance of BRs in regulating vascular differentiation *in vivo* (Szekeres, et al., 1996). A putative role of other signaling sterols has been proposed in vascular differentiation as well (Clouse, 2002; Carland et al., 2002; Sasse, 2003). The interaction between BRs, auxins and cytokinin signaling pathways play a critical role in the regulation of vascular development (Dettmer et al., 2009).

1.5.4. Reproduction and Senescence

Male sterility in the BR-insensitive mutant, *brassinosteroid insensitive1 (bri1)* and BR-deficient mutants such as *dwarf 4 (dwf4)* and *cpd* demonstrate the importance of BRs in controlling fertility (Clouse et al., 1996; Choe et al., 1999; Szekeres, et al., 1996). A number of additional studies have furthered our understanding of BRs role in regulating fertility. Application of BL promoted pollen tube elongation *in vitro* in *Prunus avium* at nM concentrations (Hewitt et al., 1985). Treatment with BL induced parthenogenetic production of haploid seeds in *Arabidopsis* and *Brassica juncea* (Kitani, 1994). Although the

BR-deficient mutant *dwf5-1* had fertility comparable to that of WT plants, its seeds required exogenous BR treatment for full germination (Choe et al., 2000). BR signaling is also apparently involved in the induction of flowering. Two key proteins, ELF6 and REF6, involved in regulating time of flowering, directly interact with BES1, a transcription factor controlling BR-regulated gene expression (Yu et al., 2008). Ye et al. (2010) also showed that BES1 regulates a number of key genes involved in Arabidopsis anther and pollen development.

Several studies also suggest a role for BRs in controlling senescence. In one such study, application of BL enhanced senescence in Xanthum and Rumex explants (Mandava, 1981). In contrast, in Arabidopsis BR mutants, senescence is delayed by approximately 40 days (reviewed by Clouse, 2002). Delayed senescence is correlated with reduced fertility in BR mutants, e.g. the *bri1* mutant has very delayed senescence, whereas *dwf 5-1* has normal senescence compared to WT (Choe et al., 2000).

BRs also play an important role during tomato fruit development. Tomato extreme dwarf (d^x) is a BR-deficient mutant that lacks the *DWARF (D)* gene. This gene encodes a cytochrome P450 (CYP), CYP85A1, required for the catalysis of C-6 oxidation of 6-deoxo-castasterone (6-deoxo-CS) to CS, a rate limiting step in BR biosynthesis (Bishop et al., 1999). BR-deficiency in d^x mutants results in metabolic changes in the fruits of d^x mutants including, reduced dry matter content, reduced sugar levels and increased amino acid levels. Both flowering and fruiting are delayed in the d^x mutant compared to wild-type (WT) plants (Lisso et al., 2006). In WT tomato plants, high expression of the *D* gene was observed in fruits especially during early seed development. The higher expression of the *D* gene

correlated with higher levels of BRs, indicating that higher BR biosynthesis is occurring during early fruit development in tomato (Montoya et al., 2005). Analysis of gene and metabolite regulatory networks of early developing tomato fruit tissue identified the *Dwarf1* gene as one of the eight hub genes that is correlated with 10 or more regulatory transcripts, and that is embedded in a large regulatory network (Mounet et al., 2009). Application of 28-homo-BL and 24-epi-BL to tomato fruit pericarp discs resulted in elevated levels of lycopene and lowered chlorophyll levels. This accelerated ripening was associated with increased ethylene production, which is a known ripening hormone in climacteric fruits (Vardhini and Rao, 2002)

1.5.5. Environmental Stress

Exogenous application of BRs increases resistance of some field plants to environmental stresses, resulting in higher crop yield in specific cases (Hamada, 1986). BRs can enhance host-plant resistance to various abiotic and biotic stresses such as temperature, salt, drought and fungal infections (reviewed by Krishna, 2003). BR treatment reduced chilling injury in rice, maize and cucumber by enhancing chlorophyll synthesis, osmoregulation and membrane stability in cells (He et al., 1991; Katsumi, 1991). The role of BRs in stabilizing the production of plant proteins at high temperature has been reported by Kulaeva et al. (1991). Treatment of wheat leaves with BRs increases the production of heat-shock protein aggregates known as heat shock granules. The BR-treated wheat leaves contained 2.5-fold higher protein levels compared to the untreated wheat leaves at 43 °C.

BR-treatment also has an ameliorative effect on rice and barley plants grown under high salt conditions (Hamada, 1986; Kulaeva et al., 1991). Treatment with 24-epi-BL

increased seed germination and growth of *Eucalyptus camaldulensis* seeds under high salt conditions (Sasse et al., 1995).

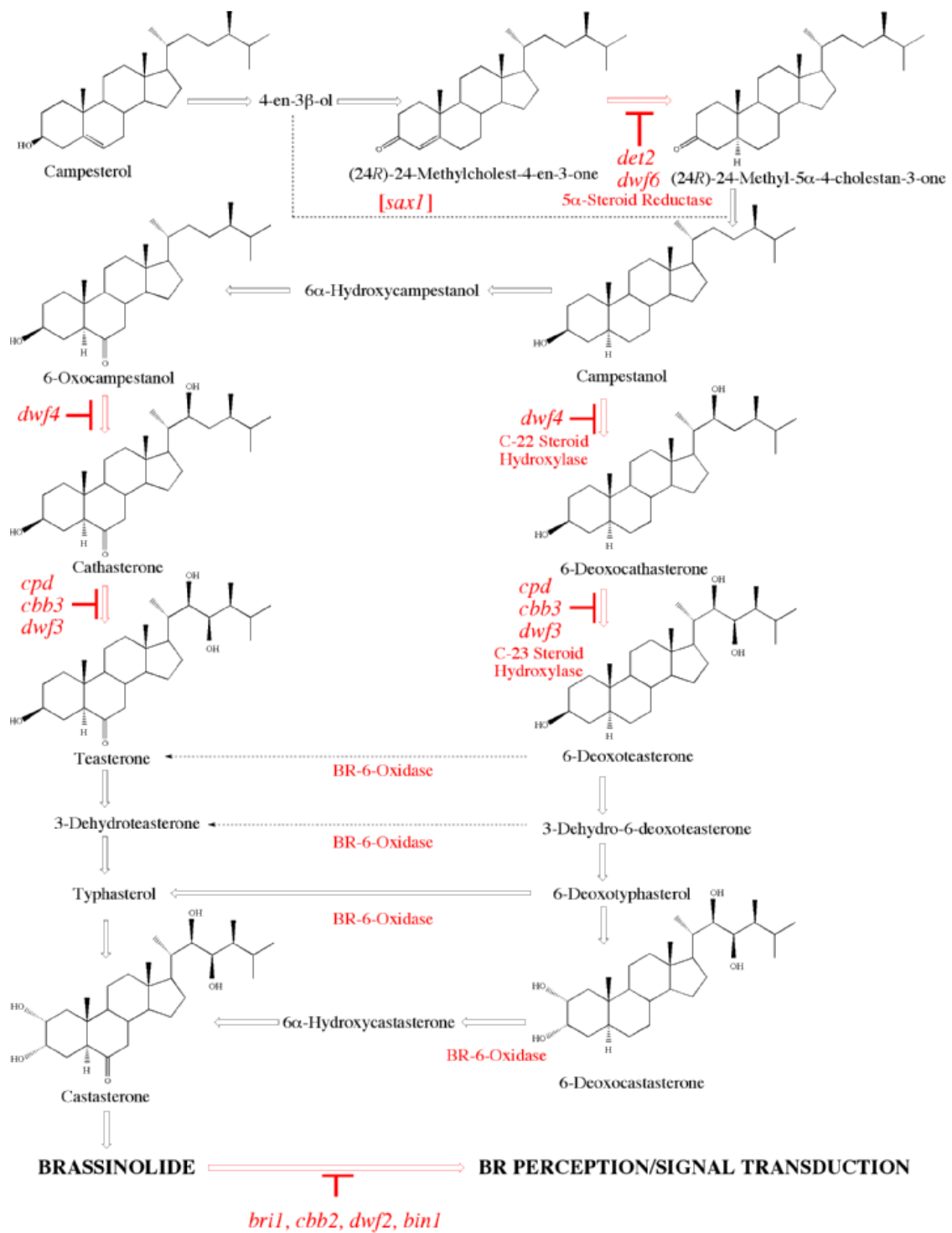
1.6. Brassinosteroid Biosynthetic Pathways

BR biosynthesis mainly has been studied using isotope labeled *Catharanthus roseus* cells and BR biosynthetic mutants in Arabidopsis and tomato. Naturally occurring C-27, C-28 and C-29 BRs are synthesized from plant sterols including campesterol, sitosterol and cholesterol. C-28 BRs, which include BL and its precursors CS, typhasterol (TY) and teasterone (TE), are derived from campesterol, whereas C-27 and C-29 BRs are derived from sitosterol (Takasuto and Yokota, 1999).

The classical scheme of biosynthesis of C-28 BRs involves two main branches, 1) an early C6 oxidation pathway and 2) a late C6 oxidation pathway, which are interconnected at various points (Fujioka and Yokota, 2003).

Biosynthesis of BL from campesterol via early C-6 oxidation involves these steps; campesterol → campestanol → 6-oxocampestanol → cathasterone → teasterone → typhasterol → castasterone → brassinolide, whereas BL biosynthesis from campesterol via late C-6 oxidation includes the following main steps: campesterol → campestanol → → 6-deoxocathasterone → 6-deoxoteasterone → 6-deoxotyphasterol → 6-deoxocastasterone → brassinolide (Figure. 2) (Fujioka and Yokota, 2003).

Figure 2. Overview of the Classical brassinosteroid biosynthetic pathways. Locations of the brassinosteroid-deficient and -insensitive mutants are highlighted in red. Enzymes involved in the conversion of intermediary steps in the pathway are indicated. Used with permission from S.D. Clouse, "Brassinosteroids", *The Arabidopsis Book*, American Society of Plant Biologists, 2002.



More recently Ohnishi et al. (2006) reported a new preferred route for BR biosynthesis. The role of two C-23 hydroxylases, CYP90C1 and CYP90D1, involved in the conversion of various substrates to their respective 23-hydroxylated forms during BR biosynthesis was clarified. This new preferred pathway circumvents the need to synthesize certain intermediates in the BR biosynthesis pathway such as 6-deoxocathasterone and 6-deoxoteasterone (Ohnishi et al., 2006; Bishop, 2007).

The fact that many biochemical conversions during BR biosynthesis are catalyzed by P 450 enzymes (P450s or CYPs) is not surprising, because BR biosynthesis requires multiple oxidative reactions, and this type of reaction is generally catalysed by P450s (Bishop, 2007).

1.7. Brassinosteroid Mutants

The study of BR mutants has played a major role in understanding the molecular mechanisms of BR biosynthesis and signaling. There are two main types of BR mutants 1) BR-deficient mutants, which contain lesions in genes involved in BR biosynthesis and, 2) BR-insensitive mutants, which contain mutations in genes involved in the signal transduction pathway that results either in the loss of hormone perception or disruption of downstream events in signaling. Exogenous application of BRs can rescue BR-deficient but not BR-insensitive mutants.

1.7.1. Brassinosteroid-deficient Mutants

BR-deficient mutants exhibit certain characteristic features: In the light, the mutants are severely dwarfed, have curled dark-green leaves, very short stems, delayed senescence and reduced male fertility. Additionally, in the dark Arabidopsis mutants have a de-etiolated dwarf phenotype with expanded cotyledons.

BR-deficient mutants that have been isolated from *Arabidopsis* include *de-etiolated2* (*det2*), *dwarf4* (*dwf4*) and *constitutive photomorphogenesis and dwarfism* (*cpd*) (Chory et al., 1991; Azpiroz et al., 1998; Szekeres et al., 1996). The *det2* mutant encodes a 5 α -reductase involved in the reduction steps converting (22*S*,24*R*)-22-hydroxyergost-4-en-3-one to (22*S*,24*R*)-22-hydroxy-5 α -ergostan-3-one during C-28 biosynthesis, and (22*S*)-22-hydroxycholest-4-en-3-one to (22*S*)-22-hydroxy-5 α -cholestan-3-one during C-27 biosynthesis (Fujioka et al., 2002; Noguchi et al., 1999). The *dwf4* and *cpd* mutants are deficient in hydroxylases required during C-22 and C-23 α -hydroxylation, although the role of CPD as a C-23 hydroxylase has recently been questioned (Szekeres et al., 1996; Choe et al., 1998; Choe, et al., 1999; Bishop, 2007).

BR-deficient mutants also have been isolated from crop species including pea *lk* and *lkb* mutants (equivalent to the *Arabidopsis det2* and *dwf1* mutants, respectively), and *brd* mutants, deficient in C-6 oxidation steps (Bishop, 2003). Several BR-biosynthetic mutants have been isolated from tomato including *d* and *d^x* mutants deficient in C-6 oxidation steps (Bishop et al., 1996; Bishop et al., 1999) and the *dpy* mutant, putatively defective in a C-23 hydroxylation step (Koka, et al., 2000).

1.7.2. Brassinosteroid-insensitive Mutants

Similar to BR-deficient mutants, BR-insensitive mutants also display multiple defects in growth and development. The first BR-insensitive mutant, *brassinosteroid-insensitive 1* (*bri1*) was isolated from *Arabidopsis* by Clouse et al. (1993) by a root growth inhibition assay. The *bri1* mutant was insensitive to BRs but responded to other plant hormones including auxins, cytokinins, ethylene, ABA and GAs in the root growth inhibition assay.

The *bri1* mutant exhibits multiple defects in growth and development. It is an extreme dwarf with very dark-green leaves, shows a de-etiolated phenotype, is male sterile, and exhibits much delayed senescence (Clouse et al., 1996). Several other alleles of *bri1* have been isolated from Arabidopsis including *cbb2* (*bri1-2*), *dwf 2-1/5* (*bri1-3/7*) and *bin1-18* (*bri1-101/118*) (Kauschmann et al., 1996; Li and Chory, 1997).

BR-insensitive mutants equivalent to Arabidopsis *bri1* have been identified in crop plants as well. These include the *curl3* mutant from tomato (Koka et al., 2000), *lka* from pea (Nomura et al., 2003) and *d61* from rice (Yamamuro et al., 2000).

Mutants that are defective in downstream components of BR signal transduction pathway have also been identified. The study of these mutants played a critical role in understanding BR signaling. These include *BRI1-associated receptor kinase 1* (*bak1*) (Li, et al., 2002; Nam and Li, 2002), *brassinosteroid insensitive 2* (*bin 2*) (Li et al., 2001) *brassinazole resistant 1* (*bzr1*) (Wang et al., 2002), and *bri1-EMS suppressor* (*bes1*) (Yin, et al., 2002).

1.8. Brassinosteroid Signal Transduction Pathway

1.8.1. Brassinosteroid Insensitive 1 (BRI1)

A recessive mutation in the single *BRI1* gene was linked to the DHS1 marker on the bottom of Arabidopsis chromosome IV (Clouse et al., 1996). Later, the *BRI1* gene was identified by positional cloning and found to encode a plasma membrane localized, leucine-rich repeat receptor-like kinase (LRR RLK) (Li and Chory 1997). The BRI1 protein is composed of an extracellular domain consisting of 24 LRRs, a transmembrane domain (TM), and a cytoplasmic domain (CD) consisting of, juxtamembrane (JM), kinase (KD) and

C-terminal (CT) subdomains (Li and Chory, 1997). Photoaffinity experiments using photolabelled CS identified a BR binding site in the 70 amino acid island domain (ID) present between LRR 20 and 21. These experiments also showed that the ID-LRR21 domain was sufficient for BR binding and identified a new steroid-binding motif in plants (Kinoshita et al., 2005).

Molecular and biochemical analyses of BRI1 have revealed the functional importance of the different BRI1-CD subdomains. The JM domain was shown to play a positive role in BRI1 function, as its deletion abolished BRI1 signaling, whereas the CT domain played an auto-inhibitory role as deletion of 49 amino acids of this domain enhanced BRI1 kinase activity *in vitro* and more completely rescued the *bri1* mutant phenotype *in vivo* (Wang et al., 2005b). The autophosphorylation activity of the BRI1 kinase has also been assessed using biochemical and molecular approaches (Oh et al., 2000). Using mass assisted laser desorption ionization mass spectroscopy (MALDI-MS) analyses they identified 12 (Ser/Thr) autophosphorylation sites in the BRI1-CD. The BRI1-CD was also found to transphosphorylate specific substrate peptides. Wang et al. (2005a) identified six specific *in vivo* phosphorylation sites using immunoprecipitation followed by liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis, and presented evidence for an additional five sites. Functional roles of the identified phosphorylation sites in BR signaling were examined in detail both *in vivo* and *in vitro* by mutational analysis. Mutation of the phosphorylation sites in the KD activation loop of BRI1 almost abolished its kinase activity, emphasizing the importance of these sites in generating a functional BRI1 kinase. Originally, BRI1 was classified as a Ser/Thr kinase. Recently, however, tyrosine phosphorylation of

BRI1 has been detected as well, and some of these tyrosine sites play an important role in BRI1 activity (Oh et al., 2009).

Orthologs of BRI1 have been cloned from a number of important crop species including tomato (*Curl3/tBRI1*) (Montoya et al., 2002), rice (*OsBRI1*) (Yamamuro et al., 2000), pea (*LKA/PsBRI1*) (Nomura et al., 2003), cotton (*GhBRI1*) (Sun et al., 2004), barley (*HvBRI1*) (Chono et al., 2003) and grape (*VvBRI1*) (Symons et al., 2006). The *bri1* null mutants of these crop plants show a close resemblance to the Arabidopsis *bri1* phenotype, however there is some species-specific divergence in the phenotype as well. For example, tomato BRI1/SR160 was also identified as a systemin receptor. Systemin is an 18-22 amino acid peptide involved in wound response and defense signaling (Scheer and Ryan, 2002). The *lka* mutant of pea does not show the same de-etiolation phenotype as Arabidopsis *bri1* (Nomura et al., 2003), and the rice orthologs, *OsBRL1*, 2 and 3, differ from *AtBRI1* in that their expression is specific to roots (reviewed by Morillo and Tax, 2006).

1.8.2. BRI1-Associated Receptor Kinase 1 (BAK1)

BAK1 was first isolated from Arabidopsis by Li et al. (2002) using an activation-tagging screen to identify dominant negative suppressors of the *bri1* mutant. The overexpression of kinase inactive *mBAK1* resulted in a dominant negative phenotype in the weak *bri1-5* mutant. Sequencing BAK1 revealed that it was an LRR-RLK with a short LRR sequence. They also discovered that BAK1 interacted with BRI1 both *in vivo* and *in vitro*. These results revealed BAK1 as an important component of the BR signal transduction pathway. Nam and Li (2002) also demonstrated BRI1/BAK1 interaction in a yeast two hybrid screen and found that the interaction between BRI1 and BAK1 increased their kinase

activities through transphosphorylation. Wang et al. (2005a) subsequently showed that BL treatment enhanced the *in vivo* interaction and autophosphorylation of the BRI1/BAK1 pair.

BAK1 is also involved in a range of BR-independent functions including light responses, cell death and plant innate immunity (Chinchilla et al., 2009). BAK1, also known as SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 3 (SERK3), belongs to a small family of LRR-RLKs known as SERKs, which include SERK1, known for its role in enhancing embryo competence in culture (Hecht et al., 2001). A critical role of BAK1 has recently been identified in pathogen induced innate immunity in plants. FLAGELLIN RECEPTOR 2 (FLS2), is an LRR-RLK that binds to flg22, a bacterial peptide. The binding of flg22 to FLS2 initiates a signaling pathway that enhances plant resistance against bacterial pathogens. The interaction between FLS2 and BAK1, in a flg22 dependent manner, was shown to be an important component in plant defense response (Chinchilla et al., 2007). Wang et al. (2008) proposed a sequential transphosphorylation model of the BRI1/BAK1 interaction, in which BRI1 kinase is first activated in a BL dependent manner and then transphosphorylates BAK1 on KD residues. Once BAK1 kinase is activated by BRI1, it transphosphorylates BRI1 JM and CT residues in a reciprocal manner. This quantitatively enhances BRI1 kinase activity towards its specific substrates. Wang et al. (2008) also identified and characterized various functionally important BRI1 and BAK1 transphosphorylation sites. This model of plant LRR-RLK function has similarities to, but also distinct differences from, mechanisms of action of animal receptor kinases.

1.8.3. Other components of BR signal transduction

Another important component of the BR signal transduction pathway is the negative regulator, BKI1, a plasma membrane-associated protein that interacts directly with BRI1-CD in the absence of BRs and inhibits its interaction with BAK1. Perception of BRs by BRI1, however, leads to the dissociation of BKI1 from the plasma membrane, allowing BRI1/BAK1 association to occur (Wang and Chory, 2006). The activated BRI1/BAK1 hetero-oligomer in turn inactivates BIN2, a glycogen synthase kinase 3 (GSK-3) (Li et al., 2001), that is also a negative regulator of BR signaling. BIN2 phosphorylates transcription factors BZR1/BZR2 or BES1 and inhibits their binding to DNA, BIN2 also promotes binding of the 14-3-3 protein to BZR1 which reduces the nuclear localization of BZR1 (Gampala et al., 2007). BRI1 suppressor 1 (BSU1), a Kelch-repeats-containing phosphatase (Mora-Garcia et al., 2004), inactivates BIN2 by dephosphorylating a conserved phospho-tyrosine residue in a BL dependent manner (Kim et al., 2009). Inactivation of BIN2 leads to dephosphorylation and nuclear accumulation of transcription factors BES1 (Yin et al., 2002) and BZR1 (Wang et al., 2002), which can then bind to the promoters of BR-regulated genes (reviewed by Belkhadir et al., 2006). A large number of genes are regulated by BRs; some examples are given in Table 1.

Another proposed component of downstream BR signaling is a putative cytoplasmic substrate of BRI1, the TGF-beta receptor interacting protein (TRIP-1). Arabidopsis *TRIP-1* gene expression is regulated by BR treatment and transgenic plants expressing antisense *TRIP-1* RNA had phenotypes that resembled BR-deficient and BR-insensitive mutants (Jiang and Clouse, 2001). Ehsan et al. (2005) showed that TRIP-1 interacted with the BRI1 kinase

domain *in vivo* and identified three sites on TRIP-1 that were phosphorylated by BRI1. TRIP-1 is a WD domain protein that also functions as a subunit of the eukaryotic translation initiation factor 3 (eIF3) in animals, yeast and plants (Jiang and Clouse, 2001). Another BRI1 interacting protein, identified by yeast two-hybrid screening, is the plasma membrane localized transthyretin-like (TTL) protein (Nam and Li, 2004). BRI1-CD was shown to phosphorylate TTL *in vitro* and overexpression of TTL in transgenic plants resulted in slight dwarfism, suggesting a negative role for TTL in BR signaling.

Finally, another recently identified group of BRI1-interacting proteins known as BR signaling kinases (BSKs), with BSK1, BSK2, BSK3 as three of their members, have been shown to be critical for downstream BR signaling (Tang et al., 2008). BSKs interact with BRI1 *in vivo* and are phosphorylated by BRI1 *in vitro*. The amount of BSK that coimmunoprecipitated with BRI1 was shown to be decreased by BR treatment, indicating that BSKs dissociate from BRI1 upon phosphorylation. Phosphorylation of BSK1 by BRI1, promotes its binding to BSU1 phosphatase, which leads to the activation of BSU1. The activated BSU1 in turn inactivates BIN2 kinase by dephosphorylating a conserved phospho-tyrosine residue of BIN2. The inactivation of BIN2 promotes the accumulation of the unphosphorylated form of BZR transcription factors in the nucleus resulting in BR-regulated gene expression (Kim et al., 2009)

Table 1. BR-Regulated Genes in Arabidopsis.

Gene/protein title	(Putative) Function	Fold change	Ref.
BAS1D	Cytochrome P450, CYP72B, steroid biosynthesis	3.4 ^a	Yin et al., 2005
SAUR-AC1	Auxin-induced protein	3.3 ^a	Yin et al., 2005
GASA3	Giberellin-regulated protein	2.2 ^a	Yin et al., 2005
TCH4	Xyloglucan transglycosylase/ hydrolases, cell elongation	6.0 ^a	Yin et al., 2005
AtEXPA8	Expansin, cell elongation	1.4 ^a	Yin et al., 2005
HERCULES1	Cell elongation	1.3 ^b (approx.)	Guo et al., 2009a
THESUS1	Cell elongation, cell wall integrity	2.5 ^b (approx.)	Guo et al., 2009a
FERONIA	Cell elongation, cell wall integrity	1.4 ^b (approx.)	Guo et al., 2009a
MS1	Male fertility (anther and pollen development)	2.0 ^c	Ye et al., 2009
MS2	Male fertility (anther and pollen development)	2.0 ^c	Ye et al., 2009
SPL/NZZ	Male fertility (anther and pollen development)	2.0 ^d	Ye et al., 2009
TDF1	Male fertility (anther and pollen development)	1.4 ^d	Ye et al., 2009
AAP4	Amino acid transporter	3.6 ^e	Mussig et al., 2002
AMT1	Ammonium transporter	3.6 ^e	Mussig et al., 2002
Hsc70-G8	Heat shock protein	2.0 ^e	Mussig et al., 2002
PRO1	Osmotic stress-induced Protein DH	1.0 ^e	Mussig et al., 2002
PRL	Cell division	-2.5 ^e	Mussig et al., 2002
ROT3	Steroid synthesis	-3.7 ^e	Mussig et al., 2002
DIM	Steroid synthesis	-6.6 ^e	Mussig et al., 2002
CPD	Steroid 23-hydroxylase	-1.0 ^a	Song et al., 2009
DWF4	Steroid 22-hydroxylase	-1.7 ^a	Song et al., 2009
LTP	Auxin stimulus	-3.8 ^a	Song et al., 2009
ERF subfamily	Ethylene-med signaling	2.0 ^a	Song et al., 2009
ARR5	Cytokinin-med signaling	-1.3 ^a	Song et al., 2009
Sedoheptulose-bisphosphatase	Starch biosynthesis	3.3 ^f	Song et al., 2009
C3HC4-type RING finger protein	Ubiquitin cycle	3.8 ^f	Song et al., 2009

a = WT plants treated with BL vs WT plants not treated with BL

b= WT plants treated with BL vs *bri1-5* mutant plants treated with BL

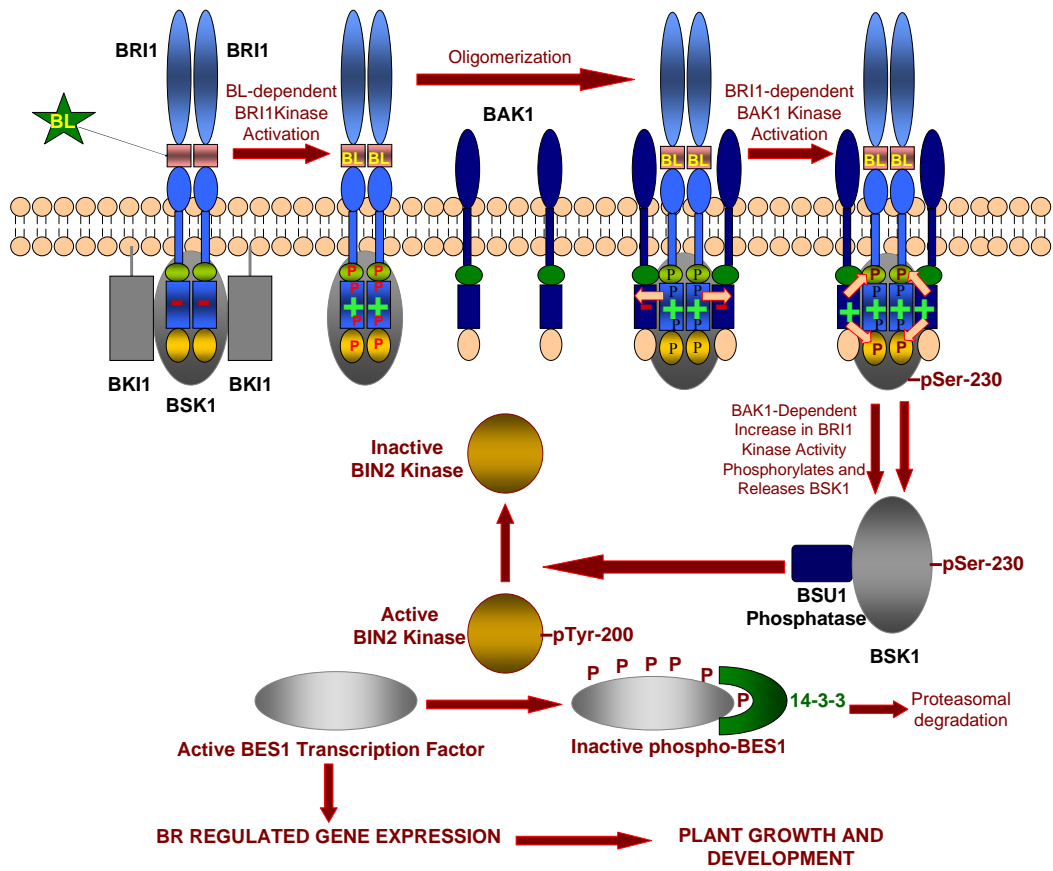
c= WT plants treated with BL vs *bri1-116* mutant plants treated with BL

d= The fold-change calculated based on the relative change in anti-BES1 compared with a non-specific antibody

e= WT plants treated with BL vs *dwf1-6* mutant plants treated with BL

f= WT plants treated with BL vs *det2* mutant plants treated with BL

Figure 3. Model of BR signal transduction in *Arabidopsis*. BL binds directly to inactive BRI1 in the island domain, resulting in the activation of the BRI1 kinase domain. This initial activation of BRI1 is independent of BAK1. The activated BRI1 then oligomerizes and transphosphorylates inactive BAK1 on its kinase domain residues. Once activated by BRI1, BAK1 quantitatively enhances the phosphorylation of BRI1 downstream substrates by transphosphorylating BRI1 on its juxtamembrane and c-terminal residues, therefore enhancing the BR dependent signaling output. Activated BRI1 phosphorylates BSK1, leading to its dissociation from the BRI1/BAK1 receptor complex. Dissociated BSK1 associates with and presumably activates BSU1. Activated BSU1 inactivates BIN2 by dephosphorylating it at the pTyr-200, allowing nuclear accumulation of unphosphorylated transcription factors, BZR1/BES1. BZR1/BES1 bind to the promoters of BR-target genes, leading to growth and developmental responses.



1.8.4. Tomato Brassinosteroid Signal Transduction Pathway

Tomato BRI1 (tBRI1) was cloned using degenerate primers from the altered brassinolide *sensitivity* (*abs1*) mutant, a weak allele at the *curl3* (*cu3*) locus (Montoya et al., 2002). Sequence analysis of *cu3* and *abs1* mutants revealed that *cu3* contained a nonsense mutation, whereas *curl3^{abs1}* contained a missense mutation in the tBRI1 sequence. Pairwise sequence comparison identified tomato and Arabidopsis BRI1-cytoplasmic domains as highly conserved (>80% homology) at the nucleotide level (Montoya et al., 2002). Tomato BRI1/ SR160 was also isolated earlier as a systemin receptor from the plasma membranes of tomato suspension cell cultures, using photoaffinity labeled ¹²⁵I-azido-Cys-3, Ala-15-systemin (Scheer and Ryan, 2002). Mass spectral fingerprinting of SR 160 revealed that it has high sequence similarity to Arabidopsis BRI1. These experiments suggested a putative role for tBRI1 as a dual receptor for BRs and systemin, a peptide involved in defense signaling (Ryan and Pearce, 2003).

In solanaceous plants, systemin and Hyp-rich systemin act as signaling intermediates in the JA-signaling pathway. Systemin homologs are present only in solanaceous plants belonging to subtribe solaneae which include tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), black nightshade (*Solanum nigrum*), and pepper (*Capsicum annuum*) (Constabel, et al., 1998), whereas hyp-rich systemins are present in both tomato and tobacco (*Nicotiana tabacum*) (Ryan and Pearce, 2003)

Holton et al. (2007) were able to restore the extremely dwarf phenotype of the *curl3* mutant by overexpressing *tBRI1* in the mutant background. Tritium labeled BL was not

detected in the microsomal fraction of *curl3* due to non-functional tBRI1. However, restoring the wild type phenotype by overexpressing tBRI1 in *curl3* restored the BL binding, confirming that tBRI1 is involved in BR perception and signaling. Normal response to wound and systemin signaling was observed in the *curl3* mutant, indicating that systemin signaling can occur via a non-BRI1 mechanism. The binding of the BL, but not systemin, to tBRI1 in tBRI1 over expressing suspension-cultured tobacco cells led to phosphorylation of tBRI1-FLAG (Malinowski et al., 2009). These experiments indicate that tBRI1, even though it binds systemin, may not be a part of systemin signaling.

Other downstream components of the tomato BR signal transduction pathway have not been studied in detail.

1.9. Practical implications of studying BR biosynthesis and Signaling for Agriculture and Horticulture

Since BRs affect multiple aspects of growth and development and increase plant resistance against environmental stress, there is potential to use BRs to increase crop yield and quality. A number of field studies from various countries, including Japan, China, Russia, Belarus, Poland and the US have reported the use of BRs in promoting plant yield (reviewed by Khripach et. al, 2000). In recent years, a number of studies have implicated BRs as a biotechnological target for enhancing crop yield and stress tolerance in plants (reviewed by Divi and Krishna, 2009).

One of the major factors in the success of the green revolution was the introduction of high yielding, semi-dwarf varieties of wheat and rice. Because of their small size, these

varieties were tolerant to lodging damage by wind and rain, and had a higher harvest index (grain/ grain plus straw) (Khush, 1999). In recent years, genetic manipulation of BR-biosynthetic and signaling genes has had a positive impact on increasing grain yield in rice. For instance, the erect leaf phenotype of the BR-deficient mutant *osdwarf4-1* was linked to enhanced grain yield under dense planting conditions without any extra fertilizer. This increase in yield was attributed to the fact that erect leaves allow more penetration of light to the lower leaves. The resulting increase in photosynthetic rates results in an increase in the number of panicles. The *osdwarf4* was found to be defective in two functionally redundant cytochrome P450's required in C-22 hydroxylation, a rate limiting step in BR biosynthesis (Sakamoto et al., 2006). Morinaka et al. (2006) demonstrated that a weak allele of the rice *osBR11* mutant, *d61-7*, has an erect phenotype and has up to 35% higher biomass production than the wild-type (WT) under dense planting conditions. However, this mutant had smaller grains which resulted in reduction in total grain yield compared to WT. To overcome this problem, Morinaka et al. (2006) made transgenic plants with partial suppression of the endogenous *OsBR11*. These new transgenic plants showed erect leaf phenotype without significant change in grain size, which resulted in about 30% higher grain yield compared to WT rice plants. Modulation of *OsBAK1* levels in rice also has potential for improving agriculturally important traits such as plant height, leaf erectness, grain morphology and disease resistance (Li et al., 2009). Constitutive overexpression of *GhDET2* in cotton seed-coat increased fiber number and length by 22.6% and 10.7%, respectively. Unfortunately, there were also undesirable effects including severe sterility and boll abortion (Luo et al., 2007). These studies together suggest that there is potential to improve

agriculturally important traits by regulating genes involved in BR-biosynthesis and signaling. Since BRs regulate many physiological processes in plants, it is critical to modify the levels of BRs in such a way that desirable traits can be achieved with limited negative effects on plant phenotype.

Application of BRs can reduce toxicity of pesticides in plants by promoting pesticide metabolism. Several pesticide detoxification genes including P450 monooxygenases, glutathione-S-transferases and UDP-glycosyltransferases are regulated by BRs (Mussig et al., 2002; Goda et al., 2002). Xia et al. (2009b) found that application of 24-epi-BL on cucumber enhanced degradation of many fungicides and insecticides. They linked this degradation to the increased expression of detoxification genes. This study suggests the role of BRs as safeners, suitable for reducing the risk of pesticide toxicity to humans and the environment.

BRs induce a wide range of stress tolerances in many crop species. For instance, treatment of cucumber with BRs increased its tolerance to both photo-oxidative and cold stress, and increased resistance to cucumber mosaic virus. In these studies, Xia et al., (2009a) further showed that reactive-oxygen species played a central role in BR-induced stress tolerance. Many other studies document role of BRs in enhancing the plant's ability to cope with various abiotic and biotic stresses, such as water stress, salt stress and diseases (reviewed in Krishna, 2003).

Water stress is one of the major environmental stresses that negatively impacts plant growth and development. During water stress various changes happen in plants, including stomatal closure, leaf abscission, and changes in cell wall and plasma membrane composition

that cause a decrease in photosynthesis and turgor pressure which ultimately reduces the plant's growth (Jarger et al., 2008). A study with cucumber showed that plants treated with 24-epi-BL maintained growth during drought conditions by retaining more water than untreated control plants (Pustovoitova et al., 2001). BR treatment of sugar beet (*Beta vulgaris*) plants compensated for the reduction in taproot mass normally caused by water stress (Schilling et al., 1991). In wheat, BR application increased relative water content (RWC), nitrate reductase activity, chlorophyll content and photosynthesis during water stress, resulting in higher leaf area, biomass, and grain yield (Sairam, 1994). BR-treated sorghum (*Sorghum vulgare*) plants showed higher germination rates compared to non-treated control plants under osmotic stress (Vardhini and Rao, 2003), and 24-epi-BL treatment of *Arabidopsis* and *B. napus* seedlings increased their survival rate under drought conditions (Kagale et al., 2007). These studies also indicate that BR levels increased under drought conditions (Catala et al., 2007).

BRs increase plants' resistance against a broad range of viral, fungal and bacterial diseases (Khripach et al., 2000). BRs enhance disease resistance in crop plants including potato (Khripach et al., 2000), cucumber (Churikova et al., 2003), tomato (Krishna, 2003), rice and tobacco (Nakashita et al., 2003). Nakashita et al (2003) found that BL-treated, WT tobacco plants exhibited enhanced resistance to the viral pathogen tobacco mosaic virus, the bacterial pathogen *Pseudomonas syringae pv. tabaci* (Pst), and the fungal pathogen *Oidium* sp., and that treatment of rice plants with BL increased their resistance towards rice blast and bacterial blight diseases caused by *Magnaporthe grisea* and

Xanthomonas oryzae pv. *oryzae*, respectively. This BL-induced disease resistance (BDR) was found to induce innate immunity of higher plants, this is distinct from systemic acquired resistance (SAR) and wound-inducible disease resistance, which require salicylic acid (SA) biosynthesis and PR gene expression, respectively (Nakashita et al., 2003).

Research Objectives

Research in our laboratory is mainly directed towards understanding the molecular mechanism of BR signal transduction. During the past 15 years, much has been done to elucidate BR signaling in Arabidopsis. However, much less detail on this important pathway is known in tomato, an important model crop plant. Understanding the molecular mechanism of BR signal transduction in tomato can have practical implications in generating transgenic crop plants with altered growth properties.

The objectives of my doctoral research were to understand the early events of BR signaling in tomato, and compare them with what occurs in Arabidopsis, and to determine the degree of conservation in the BR signaling pathway between these two plant species. In order to perform this comparative analysis we first introduced tBRI1-CD containing N-terminal FLAG or MBP epitope tags into bacterial expression vectors. Immunoprecipitation was used to purify *E. coli*-expressed recombinant tBRI1-CD and tBAK1-CD. Kinase assays were used to detect autophosphorylation of tBRI1-CD and tBAK1-CD, and transphosphorylation of kinase inactive mtBAK1 by tBRI1-CD and vice versa. Peptide substrate phosphorylation analysis was used to see if tBRI1 can phosphorylate Arabidopsis BRI1-CD substrate peptides, and if substrate phosphorylation is enhanced in the presence of tBAK1-CD.

Experiments were also performed to assess the kinetics of tBRI1 phosphorylation of substrate peptides.

Second, LC/MS/MS and LC/MS^E analysis was used to map *in vitro* autophosphorylation and transphosphorylation sites on tBRI1-CD and tBAK1-CD proteins. Site-directed mutagenesis was then performed on the tomato tBRI1-CD identified sites to change S/T sites to A, and Y sites to F, thus preventing phosphorylation at those sites. The effect of these mutations was observed regarding autophosphorylation, peptide substrate transphosphorylation, and activity of tomato BRI1-CD. Transgenic tomato plants overexpressing the tomato *BRI1-Flag* gene were generated and tBRI1-FLAG protein was isolated from the total membrane fraction of these transgenic tomatoes using immunoprecipitation with anti-Flag antibody-linked beads. A gel-based LC/MS^E approach was used to identify *in vivo* tBRI-interacting proteins. Attempts were also made to identify the *in vivo* phosphorylation sites using an LC/MS/MS approach in these transgenic plants. Functional effects of mutating specific phosphorylation sites on the phenotype of the weak *curl3^{-abs1}* mutant were also determined. Transgenic plants overexpressing, tomato BRI1-FLAG, T-1054-A and K-916-E (kinase inactive) in the *curl3^{-abs1}* background, were also generated.

Finally, the tomato *TRIP1 (tTRIP1)* gene was cloned and recombinant tTRIP1 protein was overexpressed in *E.coli*, and *in vitro* and kinase assays were performed to show that tTRIP-1 is a tBRI1 kinase substrate. Peptide substrate assays were also performed to determine which TRIP1 peptides are transphosphorylated by tBRI1-CD. Tomato plants

constitutively overexpressing tTRIP1 (containing N and C terminal FLAG epitope tags) were also generated.

BRs are a comparatively new group of plant steroidal hormones with structural similarity to animal and insect steroids (Grove et al., 1979; Clouse and Sasse, 1998). BRs regulate multiple physiological responses in plants including, cell elongation, xylem differentiation, fertility, photomorphogenesis, seed germination and stress responses (reviewed by Clouse and Sasse, 1998; Mandava, 1988; Sasse, 2003; Sasse, 1999; Krishna, 2003). BR-deficient and BR-insensitive mutants display pleiotropic phenotypic defects, such as dwarfism, small dark-green leaves, a compact rosette structure, delayed flowering and reduced fertility and senescence (Clouse et al., 1996; Szekeres et al., 1996). These mutants were instrumental in understanding the molecular mechanism of BR biosynthesis and signaling in plants. Understanding the molecular details of BR signaling and biosynthesis in crop plants such as tomato has practical implications in generating transgenic crop plants with altered growth properties and improved crop yield and stress tolerance.

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Chapter Two: Cloning and Biochemical Properties of Tomato BRI1 and BAK1

Introduction

Brassinosteroids (BRs) are essential plant steroid hormones that regulate many processes in plants including cell expansion, cell division and differentiation, senescence, male fertility, seed germination and responses to the environment (reviewed by Mandava, 1988; Clouse and Sasse, 1998; Krishna, 2003). Mutants in BR biosynthesis or signaling have a characteristic dwarf phenotype (Clouse et al., 1993; Clouse et al., 1996; Szekeres et al., 1996; Bishop, 2003). Studies of molecular mechanisms of BR signal transduction were initiated in the early 1990's with identification of the *Arabidopsis brassinosteroid insensitive 1 (bri1)* mutant that showed multiple defects in growth and development (Clouse et al., 1996). The *BRI1* gene encodes a leucine-rich repeat receptor-like kinase (LRR-RLK) (Li and Chory, 1997). BRI1 is a member of a large family of LRR-RLKs with more than 220 members in *Arabidopsis* and nearly 400 members in rice (Shiu and Bleecker, 2001; Shiu et al., 2004). Only a small number of LRR RLKs have been studied in detail, but those with known functions play indispensable roles in plant growth, development and biotic defense (Torii, 2004). Other important LRR-RLKs include, *CLAVATA1*, involved in meristem proliferation (Clark et al., 1997), *ERECTA*; regulating plant structure (Torii et al., 1996); *HAESA*, involved in regulating abscission (Jinn et al., 2000); *FLAGELLIN SENSITIVE 2 (FLS2)* (Gomez-Gomez and Boller, 2000); and *XA21* (Song et al., 1995), both involved in pathogen defense. LRR-RLKs are also involved in establishing symbiotic relationships with microbes (Endre et al., 2002; Stracke et al., 2002) and in the signaling pathways for the plant peptide hormones phytosulfokine (Matsubayashi et al., 2002) and systemin (Scheer and Ryan, 2002).

BRI1-associated receptor kinase 1 (BAK1), also known as SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK 3), belongs to a subfamily of SERK LRR-RLKs with five closely related members in Arabidopsis. BAK1 interacts with BRI1 both *in vitro* and *in vivo* to modulate BR signal transduction (Li et al., 2002; Nam and Li, 2002). Interaction and transphosphorylation activity between BRI1 and BAK1 quantitatively enhance BRI1 kinase activity (Wang et al., 2005; Wang et al., 2008). The overexpression of a kinase inactive mutant of *BAK1* in the *bri1-5* mutant background (a weak allele of *bri1*) led to a severe dwarf phenotype similar to the *bri1* null mutant, suggesting a dominant negative effect due to disruption of a heterodimeric complex between BRI1 and BAK1 (Li et al., 2002). BAK1 supports many BR independent roles including a positive regulatory role in cell death pathways (He et al., 2007). Moreover, BAK1 interacts with another LRR-RLK, FLS2, and promotes the function of FLS2 in plant defense against pathogens (Chinchilla et al., 2007).

During the past decade, various biochemical and genetic details of BRI1 receptor action in Arabidopsis have been described, and a number of additional BR signaling components have been identified and their role studied. These include BRI1 interacting plasma membrane-associated proteins, BAK1, BRI1 KINASE INHIBITOR 1 and SERK1 as well as putative cytoplasmic substrates of BRI1, TGF-beta Receptor Interacting Protein 1 and the recently identified BR SIGNALING KINASES (reviewed by Belkhadir et al., 2006; Clouse, 2007; Kim and Wang, 2010). Other downstream components identified include the cytoplasmic kinase, BRASSINOSTEROID-INSENSITIVE 2, a negative regulator of BR

signaling, and the transcription factors, *bri1*-EMS SUPPRESSOR 1 and BRASSINAZOLE RESISTANT 1 (reviewed in Kim and Wang, 2010).

In vitro study of the biochemical properties of *E. coli* recombinant Arabidopsis BRI1-CD revealed that it is a S/T kinase (Oh, et al., 2000). Further studies of substrate phosphorylation activity of BRI1 determined an optimum consensus sequence for substrate recognition and phosphorylation by BRI1 (Oh, et al., 2000). These *in vitro* biochemical studies of BRI1 accurately predicted its *in vivo* function determined later by Wang et al. (2005). The identification of BRI1 substrate peptides was also useful in studying transphosphorylation between BRI1 and BAK1, which led to the proposal of the sequential transphosphorylation model of BRI1 and BAK1 interaction (Wang et al., 2008).

Orthologs of BRI1 are found in several plant species including tomato. Tomato is an emerging model crop plant that is widely used for genetic and biochemical research. The study of molecular genetics in tomato has advantages over other model plants such as Arabidopsis: 1) transgenic tomato plants with agriculturally important traits can be developed 2) fruit growth and development can be easily studied. In tomato, the *curl3* mutant was the first to be characterized as BR-insensitive (Koka et al., 2000). A weak allele of the *curl3* mutant, *curl3^{abs1}*, facilitated cloning of the tomato *BRI1* (*tBRI1*) gene which was identified as an ortholog of Arabidopsis *BRI1*, sharing >80% sequence similarity between kinase domains of the two species (Montoya et al., 2002). Constitutive overexpression of *tBRI1* in the *curl3* mutant restores a WT phenotype, confirming the role of tBRI1 as a BL receptor in tomato (Holton et al., 2007). However, little is currently known about the detailed function of

tBRI1 or tomato BAK1 (tBAK1), recently cloned by Dr. Gerard Bishop, Imperial College, London, UK (unpublished). The objective of our current study was to analyze the biochemical properties of recombinant tBRI1 and tBAK1 *in vitro*. We report here that both tBRI1 and tBAK1 are active kinases that can autophosphorylate their own cytoplasmic kinase domains as well as transphosphorylate each other. Tomato BRI1-cytoplasmic kinase domain (tBRI1-CD) can also phosphorylate the peptides BR13 and SP11, which contain the consensus sequence required for Arabidopsis BRI1 substrate phosphorylation. Kinetic analysis confirmed both BR13 and SP11 peptides were substrates of tBRI1. The substrate phosphorylation activity of tBRI1 was significantly enhanced with the addition of tBAK1-CD, suggesting that the sequential transphosphorylation model originally proposed in Arabidopsis (Wang et al., 2008) might also function in tomato.

Materials and Methods

Cloning the Tomato BRI1 Cytoplasmic Domain for Expression

An *Escherichia coli* expression construct was generated in the pFLAG-MAC expression vector (Sigma, St. Louis, MO). The tomato BRI1 cytoplasmic domain (CD) was amplified from tomato genomic DNA isolated from tomato leaves using a Qiagen DNAeasy Kit (Qiagen, Valencia, CA), with sense (5'-cgatctcgaggagacgaagaagaggaggagg-3') and antisense (5'cgatggtacctcaaggtgttgctcagctc-3') primers. The 20- μ L PCR reaction contained buffer [4- μ L 10X pfx amplification buffer (2X final conc.)], 1 mM MgSO₄, 300 μ M dNTPs, 50 ng tomato genomic DNA, 5 μ M of each primer, and 5 units cloned *pfx* DNA polymerase (Invitrogen, Carlsbad, CA). After pre-incubation at 94°C for 2 min, 31 cycles (of 94°C, 20 s;

58°C, 40 s; 68°C, 75 s) were performed followed by an extension step of 7 min at 68°C. PCR products were gel purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR product was digested with *XhoI/KpnI* (New England Biolabs, Ipswich, MA), gel-purified and ligated with *XhoI/KpnI* digested, gel-purified pFLAG-MAC vector to yield FLAG-tBRI-CD, consisting of an 11-amino acid N-terminal Flag tag (DYKDDDDKVKL) followed by amino acids 824 through 1,208 of tBRI1. The FLAG-tBRI-CD plasmid was sequenced completely to verify the construct. Transformation of FLAG-tBRI-CD plasmid into Chaperone Competent Cells, pG-Tf2/BL21, containing GroES-GroEL-tig chaperones was performed as described in manufacturer's instruction (Takara Bio Inc., Otsu, Shiga, Japan). Overexpression of FLAG-tBRI-CD in pG-Tf2/BL21 cells was conducted as per manufacturer's instructions, (Takara Bio Inc) except that cells were incubated at 25°C overnight after adding 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Tetracycline (5ng/ml) was used for the induction of the chaperone-containing (pG-Tf2) plasmid for 2 hours at 37 °C, and IPTG was added to induce expression of FLAG-tBRI-CD at 25 °C overnight. Induced and uninduced bacterial cells were collected, ultrasonicated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

A second *E. coli* expression construct was generated in the pMAL-c4X expression vector (New England Biolabs). The tomato BRI1 cytoplasmic domain (CD) was amplified from tomato genomic DNA with sense (5'-gagacgaagaagaggaggaggaggag-3') and antisense (5'cgatggatctcaaaggtgtttgctcagct-3') primers. The PCR reaction and PCR product gel purification was performed as described above. The purified PCR product was digested with

*Bam*HI (Promega, Madison, WI), gel-purified and ligated with *Xmn*I/*Bam*HI digested, gel-purified pMAL-c4X vector to yield MBP-tBRI-CD, containing an N-terminal MBP tag. The MBP-tBRI-CD plasmid was sequenced completely to verify the construct. Overexpression and analysis of induced MBP-tBRI-CD in pG-Tf2/BL21 cells was conducted as described above.

To generate mutant FLAG-tBRI1-K916E, the conserved Lys at position 916 in subdomain II was substituted with Glu, which is predicted to eliminate kinase activity (Hanks, et al., 1988). The QuickChange site directed mutagenesis kit (Stratagene, La Jolla, CA) was used for *in vitro* mutagenesis with sense (5'-gagtgtgtagctattgagaaattgatacacg-3') and antisense (5'-cgtgtatcaatttctcaatagctacaacactc-3') primers as per manufacturer's instructions. The resulting FLAG-tBRI1-K916E construct was sequenced to verify the correct mutation.

Constructs containing MBP-tBAK1-CD and mutant MBP-tBAK1-K317E with the conserved Lys at 317 mutated to Glu, were a gift from Dr. Gerard Bishop, Imperial College, London, UK.

Purification of Recombinant Proteins

The purification of FLAG tagged fusion constructs using M2 agarose beads was conducted essentially as per manufacturer's instructions (Sigma-Aldrich). The cell pellet was frozen at 20°C overnight, and lysis buffer [20mM Tris-HCl, pH 7.4; 200mM NaCl; 1mM EDTA, 0.25mg/ml lysozyme] was added to the frozen pellet. The pellet was solubilized in lysis buffer using a paint brush and incubated at room temperature for 5 min followed by sonication for 2.5 min. The sonicated samples were centrifuged at 9000×g for 20 min, and

the supernatant was collected in new tubes. The soluble total protein extract containing FLAG M₂ agarose beads was incubated at 4°C, overnight on a rotator to bind the FLAG protein. The FLAG elution buffer [20 mM Tris-HCl, pH 7.4; 200 mM NaCl; 1 mM EDTA, 0.25 mg/ml FLAG peptides] was used to elute the FLAG-tBRI1-CD protein bound to the agarose beads.

Purification of the MBP tagged proteins using amylose resins was carried out as per manufacturer's instructions (New England Biolabs) except that the concentration of maltose in the elution buffer was 20 mM rather than 10 mM.

Western Blotting Analysis of FLAG-tBRI1-CD

Proteins (0.5µg/lane) were separated by 10% (w/v) SDS-PAGE (Gallagher and Smith, 1994) and transferred to PVDF membrane by the wet transfer method using Tris-glycine transfer buffer [25 mM Tris, 192 mM glycine, 20% methanol, pH8.3]. Transfer was carried out overnight at 20 V at 4 °C. After protein transfer the PVDF membrane was incubated in blocking solution [TBST (150 mM NaCl, 10 mM Tris pH 8.0, Tween 20 (0.1% v/v) + 5% Milk Powder] for 1 hr with shaking. After blocking was complete, the membrane was incubated in an anti-Flag M₂ primary antibody (Sigma-Aldrich) at 1:5000 dilution. Following incubation with the primary antibody, the PVDF membrane was washed 3 times with TBST buffer, 5 min per wash and then incubated in horseradish peroxidase-linked secondary anti-mouse antibody (Amersham Biosciences, Piscataway, NJ) at 1:4000 dilutions for 1 hr. The incubation with secondary antibody was followed by 4 washes with TBST, 5 min each wash, and detection with the ECL chemiluminescence detection system (Amersham Biosciences).

Autophosphorylation Assay

For the autophosphorylation assay a 40 μ l reaction containing 1 μ g of affinity purified FLAG-tBRI1-CD or MBP-tBRI1-CD or MBP-tBAK1-CD was incubated with 20 μ Ci of [γ -³²P] ATP in kinase buffer (50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-KOH, pH 7.9, 10 mM MnCl₂, 1.0 mM dithiothreitol, and 0.2 mM unlabeled ATP) at room temperature for 1 hr. Reactions were terminated by adding 20 μ L of 2X Laemmli loading buffer (Laemmli, 1970), heating the sample at 95°C for 5 min followed by 10% (w/v) SDS-PAGE and autoradiography.

Peptide Substrate Assay

Peptide substrate assays were performed as described (Oh et al., 2000) with some changes in protein and peptide amounts. A 20 μ L reaction contained 0.10 mg/mL synthetic peptide, 0.5 μ g FLAG-tBRI1-CD or MBP-tBRI1-CD or MBP-tBAK1-CD, 0.8 μ Ci [γ -³²P] ATP and 0.1mM unlabelled ATP in a kinase buffer consisting of 50 mM MOPS [3-(*N*-morpholino) propanesulfonic acid], pH 7.4; 10 mM MgCl₂, and 0.2 mM CaCl₂. Following 22 min incubation at room temperature, 20 μ L of the reaction solution was spotted on a 2 \times 2-cm piece of P81 phosphocellulose paper. The paper was washed four times in 75 mM H₃PO₄ (4 min per wash) and the amount of ³²P incorporation into the peptide incubated with FLAG-tBRI1-CD or MBP-tBRI1-CD or MBP-tBAK1-CD was determined by liquid scintillation counting of the washed squares. Each reaction point was determined in triplicate, and the results are presented as mean \pm SE. The sequence of peptides used is as follows, with J representing norleucine, a methionine amino acid substitute:

BR9 – GRJRRIASVEJAKK

BR10 – GAJRRIASVEJAKK

BR11 – RRAASAAAAKA

BR12 – ARJRRASAAAJKA

BR13 – GRJKKIASVEJJKK

BR14 – GRJHHIASVEJJKK

BR15 – GRJRRIASVEJJKK

SP11 – GRJRRIASVEJJKK

Results

Both tBRI1-CD and tBAK1-CD Autophosphorylate and Transphosphorylate Each

Other

To see if tBRI1 and tBAK1 are active kinases, the *tBRI1* cytoplasmic kinase domain was cloned into the *E. coli* expression vectors pFLAG-MAC and pMAL-c4X to obtain *tBRI1-CD* fused with an N-terminal FLAG (*FLAG-tBRI1-CD*) or an N-terminal maltose binding protein (*MBP-tBRI1-CD*) (Figures 1 and 2).

Both *FLAG-tBRI1-CD* and *MBP-tBRI1-CD* containing constructs were verified for the presence of tBRI1-CD by restriction enzyme digestion (Figures 1A and 2A) and by sequencing. Kinase inactive forms of tBRI1 were also generated by mutating a conserved lysine to glutamic acid (K 916 E) in subdomain II of tBRI1-CD (*FLAG-mtBRI1-CD*) and were verified for the correct mutation by sequencing. This substitution was predicted to abolish protein kinase activity (Hanks, et al., 1988; Horn and Walker, 1994). *FLAG-tBRI1-CD*, *FLAG-mtBRI1-CD (K916E)* and *MBP-tBRI1-CD* constructs were transferred into BL-21

cells containing pGTf-2 plasmid with GroES-GroEL and tig chaperones for proper folding of the overexpressed proteins. Overexpressing recombinant FLAG-tBRI1-CD and FLAG-mtBRI1-CD (K916E) were verified by western blotting and gel-Coomassie Brilliant Blue (CBB) staining (Figure 1B), and purified using FLAG M₂-agarose beads (Figure 1C). Recombinant MBP-tBRI1-CD overexpression was verified by gel-CBB staining (Figure 2B), and the protein was purified using amylose resin (Figure 2C). The MBP-tBAK1-CD and kinase inactive MBP-mtBAK1-CD (K317E) constructs were gifts of Dr. Gerard Bishop. Both, MBP-tBAK1-CD and MBP-mtBAK1-CD constructs were transformed into *E. coli* BL-21 cells containing a pGTf-2 plasmid, overexpressed, and purified using amylose resin beads (Figures 3A and 3B).

Purified recombinant MBP-tBRI1-CD and MBP-tBAK1-CD were tested for autophosphorylation activity (Figure 4, lanes 1 and 2) and both were shown to be active kinases. Mutated forms MBP-mtBAK1-CD (K 317 E) and FLAG-mtBRI1-CD (K 916 E) produced no detectable kinase activity (Figure 4, lanes 3 and 5).

To determine if tBRI and tBAK1 can transphosphorylate each other, mutant MBP-mtBAK1-CD was incubated with FLAG-tBRI1-CD and mutant FLAG-mtBRI1-CD with MBP-tBAK1-CD. The results show that both tBAK1 and tBRI1 can transphosphorylate each other (Figure 4, lanes 4 and 6).

Tomato BRI1 can Phosphorylate Substrate Peptides

Recombinant Arabidopsis BRI1 cytoplasmic kinase domain (BRI1-CD) undergoes autophosphorylation and phosphorylates synthetic peptides with a conserved sequence motif. This conserved motif is, [RK]-[RK]-X(2)-[ST]-X(3)-[LMVIFY]-[RK], where residues in

brackets are the amino acid(s) that can occur at that position (Oh et al., 2000). To see if tBRI1-CD can recognize a similar substrate sequence and have structural requirements similar to Arabidopsis BRI1, the peptide substrate phosphorylation assays were performed with 0.5 µg of tBRI1/reaction. Modifications of the SP11 peptide, designed around the regulatory phosphorylation site (Ser-158) of spinach SPS (McMichael et al., 1995) were used to determine the structural requirement of Arabidopsis BRI1-CD (Oh et al., 2000). The synthetic peptides, BR9-BR15 and SP11, were used to see if the same structural elements are required to be a good substrate for tomato BRI1-CD. Tomato BRI1-CD was able to strongly phosphorylate synthetic peptides BR15, BR13, BR 10, BR 9, SP11 and BR 14, indicating these peptides are good tBRI1-CD substrates (Figure 5). However, the peptides BR12 and BR11 showed poor phosphorylation indicating these peptides are poor substrates for tBRI1-CD. This indicates there are similar structural requirements for phosphorylation by tBRI1 and Arabidopsis BRI1. When ability of these SP 11 variant peptides to be phosphorylated by tBRI1-CD, were compared , it was found that a) changing the Lys at P+5, relative to the phosphorylated Ser (P=0), in SP11 to an Arg (BR 15) increases kinase activity by about 40%; b) Substituting Arg of SP11 at P-3 and P-4 with Lys (BR13), enhanced the kinase activity by about 35%, whereas replacement with His residues (BR 14) decreased the activity by about 34%; c) Exchanging the Arg residue at P-6 with Ala (BR10) increased the activity by 30%; d) Replacing the hydrophobic residue at P+4 of SP11 with Ala (BR 9) resulted in 29% increase in the activity; and e) Replacing Val, Glu, nor-Leu, Lysine and Glycine at P+1, P+2, P+3, P+6 and P-7 respectively of SP11 with Ala (BR 12) reduced the kinase activity by about 70%.

Overall, it was determined that the structural requirements for efficient substrate peptide phosphorylation by tBRI1-CD are similar to those of Arabidopsis BRI1, although some variations were evident. In the case of Arabidopsis BRI1-CD the substrate peptides, BR 9, BR 10 and BR 15 showed phosphorylation levels lower than the SP11 peptide (Oh et al., 2000), whereas in case of tBRI1-CD, phosphorylation levels of BR 9, BR 10 and BR 15 peptides were higher than SP11. To compare the level of peptide substrate phosphorylation between Arabidopsis and tomato BRI1, three highly phosphorylated synthetic peptides, BR 15, BR 13 and SP 11 were tested. This comparative analysis showed that Arabidopsis BRI1 has significantly higher kinase activity with respect to these peptide substrates compared to tomato BRI1 (Figure 6). Arabidopsis BRI1 phosphorylation of BR 13 and BR 15 was 62% and 63% higher than tomato BRI1, respectively. With the SP11 substrate peptide, Arabidopsis BRI1 showed 72% higher kinase activity than tomato BRI1 (Figure 6). These results show that although tBRI1 can recognize the same substrate peptides as Arabidopsis BRI1, it has lower kinase activity than its Arabidopsis counterpart.

Kinetics of tBRI1-CD Kinase Activity in vitro

To see if tBRI1-CD phosphorylated peptides SP11 and BR13 were realistic substrates comparable to other known peptide substrates of plant kinases, we calculated K_m and V_{max} for both peptides using Lineweaver-Burk plot (double reciprocal plot) analyses with seven substrate concentrations (3mM, 6mM, 12mM, 18mM, 24mM, 30mM and 36mM). The K_m values of SP11 (109 μ M) and BR13 (193 μ M) fall within the range of K_m s for soluble plant kinases using peptide substrates that do reflect true physiological substrates, e.g. the

cauliflower HMR kinase has a K_m of 95 μM for the SAMS peptide (Weekes et al., 1993). These results indicate that both SP11 and BR13 are reasonable substrates of tBRI1-CD.

Tomato BRI1-CD showed higher affinity for BR 13 than for SP 11 (K_m of 109 versus 193 μM , respectively) and the V_{max}/K_m ratio was 1.1 times higher for BR13 than for SP 11 (Figure 7). Arabidopsis BRI1-CD also had a much greater affinity for BR 13 than for SP 11 (K_m of 81.7 versus 427 μM , respectively) and the V_{max}/K_m ratio for BR 13 was 2.5 times higher than for SP 11 (Oh et al., 2000). Overall these data suggest that both tomato and Arabidopsis BRI1-CD, preferred BR 13 as a substrate over SP11. However, comparison of K_m values for tomato and Arabidopsis, suggest that Arabidopsis BRI1 has a higher affinity for BR 13 than does tomato BRI1 (K_m of 81.7 versus 109 μM , respectively).

Transphosphorylation of tBRI1 by tBAK1 Increases tBRI1 Kinase Activity in Peptide Substrate Phosphorylation Assays

To determine if the transphosphorylation between tBRI1 and tBAK1 leads to increased kinase activity of either receptor, MBP-tBAK1-CD was incubated with FLAG-tBRI1-CD and their kinase activities monitored by assessing phosphorylation levels of the synthetic peptide SP11. FLAG-tBRI1-CD alone phosphorylated SP11 peptide to a greater degree than did MBP-tBAK1-CD alone, although it appears MBP-tBAK1 does have some ability to phosphorylate SP11. However, the mixture of equal amounts of MBP-tBAK1-CD and FLAG-tBRI1-CD increased the level of SP11 phosphorylation more than four-fold compared to tBRI1-CD alone (Figure 8). This enhancement in phosphorylation was dependent on MBP-tBAK1-CD kinase activity, because substitution of a kinase inactive form MBP-mtBAK1-CD resulted in greatly reduced SP11 phosphorylation (Figure 8).

The kinase inactive forms FLAG-mtBRI1-CD (K 916 E) and MBP-mtBAK1 (K 317 E) were unable to phosphorylate the SP11 peptide either alone or in combination (Figure 8).

Discussion

Rapid progress in BR signal transduction research over the past several years has made it one of the best-understood signaling cascades in plants. However, most of our mechanistic understanding is based on research done in the model plant *Arabidopsis*, and not as much detail is known in crop plants such as tomato. Understanding the molecular mechanism of BR signal transduction in tomato can have practical implications in generating transgenic plants with valuable agronomic traits such as altered growth architecture.

In *Arabidopsis*, the BR-signaling pathway involves two main transmembrane receptor kinases BRI1 and BAK1. The signaling cascade is initiated by BR binding to the extracellular domain of BRI1, which leads to the activation of BRI1's cytoplasmic kinase domain (He et al., 2000; Wang et al., 2001; Kinoshita et al., 2005). Activated BRI1 interacts with BAK1 both *in vitro* and *in vivo* (Li et al., 2002; Nam and Li, 2002). The association and phosphorylation of BRI1 and BAK1 is enhanced by Brassinolide (BL), a biologically active BR (Wang, et al., 2005).

In vitro biochemical properties of plant kinases, especially those of *Arabidopsis* BRI1 and BAK1, are highly predictive of their *in vivo* function (Oh et al., 2000; Wang, et al., 2005). In order to understand the *in vitro* biochemical properties of tBRI1 and tBAK1 and to determine the extent of conservation in the early events of BR signal transduction between *Arabidopsis* and tomato, epitope tagged recombinant tomato BRI1 and BAK1 cytoplasmic kinase domains (CD) were constructed. Kinase assays using [γ -³²P] ATP verified that both

tBRI and tBAK1 are active kinases that can autophosphorylate and transphosphorylate each other *in vitro* (Figure 4). This indicates that similar to Arabidopsis BRI1 and BAK1, tomato BRI1 and BAK1 have *in vitro* autophosphorylation and transphosphorylation activities. The association and transphosphorylation between Arabidopsis BRI1 and BAK1 are essential for the phosphorylation of downstream signaling components in the BR signal transduction pathway (Wang et al., 2008; Li et al., 2002). Our results suggest that BRI1/BAK1 interaction and transphosphorylation may also be important in tomato BR signaling. *In vivo* interaction studies, such as those conducted in Arabidopsis (Wang et al. 2005; Wang et al. 2008) will be required to verify this hypothesis. Similar to the kinase inactive forms of Arabidopsis BRI1 and BAK1, mutation of the conserved lysines at position 916 and 317 to glutamic acid in kinase subdomain II of tBRI1 and tBAK1, respectively, resulted in kinase inactive forms of tBRI1 and tBAK1, which can neither autophosphorylate nor transphosphorylate each other.

In order to identify the structural elements required for optimal substrate phosphorylation by Arabidopsis BRI1, various peptide substrates having specific variation in their sequence were previously used for *in vitro* kinase assays (Oh et al., 2000). The optimal peptide identified (BR13) was further used to test the *in vitro* biochemical, functional importance of BRI1 autophosphorylation sites (Wang et al., 2005) and also to understand the details of Arabidopsis BRI1 and BAK1 transphosphorylation and substrate phosphorylation (Wang et al., 2008). Similarly, we tested the ability of tBRI1 to phosphorylate eight different substrate peptides that had variations introduced in their sequence around the serine that is phosphorylated by BRI1-CD. It was determined that while tBRI1 and Arabidopsis BRI1 have similar sequence preferences for peptide substrate phosphorylation (Figure 5), tBRI1-CD had

lower kinase activity than Arabidopsis BRI1-CD for the three peptides tested under the same experimental conditions (Figure 6). This difference in phosphorylation activity between Arabidopsis and Tomato BRI 1 indicate that there may be some variation in affinity for downstream substrates between Arabidopsis and tomato BRI1. This may be due a divergence in the sequence of BRI1 downstream substrates between these two species. The kinetic data for BR 13 and SP 11 peptides indicate that both peptides behave as reasonable substrates, i.e. the K_m values of these peptides lie in the range of those found for soluble plant kinases with peptide substrates known to reflect true physiological substrates. The tBRI1-CD showed slightly higher affinity for BR 13 peptide than for SP 11, with a K_m of 109 μM for BR 13 versus 193 μM for SP 11 peptide. The V_{max}/K_m ratio for BR13 is also about 1.1 times higher than SP11. However, in the case of Arabidopsis BRI1-CD, the V_{max}/K_m ratio for BR13 is about 2.5 times higher than SP11 and the K_m for BR13 is 81.7 μM compared to 427 μM for SP 11. These data indicate that although tBRI1-CD shows some preference for BR 13 peptide compared to SP 11, the difference in its affinity for substrate peptides is not as much as shown by Arabidopsis BRI1-CD (Oh et al., 2000). These kinetic data further support the hypothesis that there is divergence between the substrates of Arabidopsis and tomato BRI1-CD.

In Arabidopsis, BRI1 and BAK1 can transphosphorylate each other in a sequential manner. First, the BRI1 kinase domain is activated in response to BR binding in its extracellular domain. Once activated, BRI1 associates itself with BAK1, positively regulating BAK1 by transphosphorylating it on the BAK1 kinase domain residues. The activated BAK1 can then transphosphorylate the BRI1 juxtamembrane (JM) and c-terminal

(CT) residues (JM and CT domains are often important for docking of receptor kinase substrates), thereby quantitatively enhancing BRI1 kinase activity towards its substrates (Wang et al., 2008). Using a peptide substrate assay, a greater than 4-fold increase in the level of phosphorylation of SP11 substrate peptide was observed when tBRI1-CD is used in combination with tBAK1-CD compared to substrate phosphorylation by BRI1 alone (Figure 8). This experiment suggests that the sequential transphorylation model of BRI1 and BAK1 that occurs in Arabidopsis may also be true in tomato. Tomato BAK1 also appears to have some activity towards BR13 whereas no such activity was observed for Arabidopsis BAK1-CD. This may be due to some difference in the BAK1 substrate sequences between these two species.

Overall, our initial advances in understanding the biochemical properties of tomato BRI1 and BAK1 *in vitro* suggest a significant conservation in the early events of BR signal transduction pathway between Arabidopsis and tomato. However, there appears to be some divergence between these two species in the preference of the downstream substrates of BRI1. Our understanding of the biochemical properties of tBRI1 and tBAK1 *in vitro* can prove useful in understanding their *in vivo* function.

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Figures

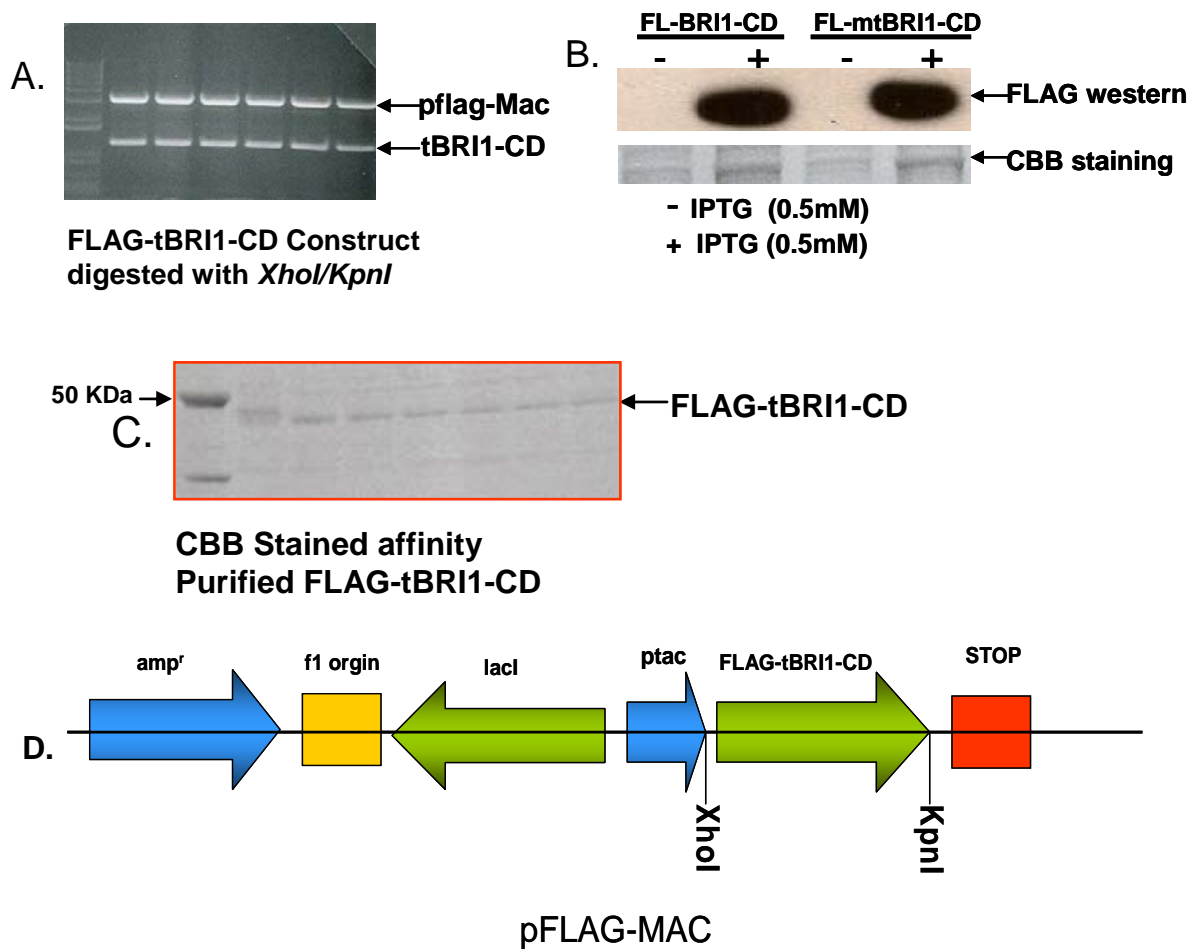


Figure 1. Cloning of tBRI1 cytoplasmic domain into pFLAG-MAC expression vector (**A**) Digestion of the construct with restriction enzymes (*XhoI/ KpnI*) to verify the presence of tBRI1-CD. (**B**) Western blot analysis and coomassie brilliant blue (CBB) staining to detect tBRI1-CD after induction with IPTG (0.5mM). (**C**) CBB stained gel containing purified FLAG-tBRI1-CD protein. (**D**) Map of the expression vector pFLAG-MAC with the restriction enzymes, *XhoI* and *KpnI* used for cloning.

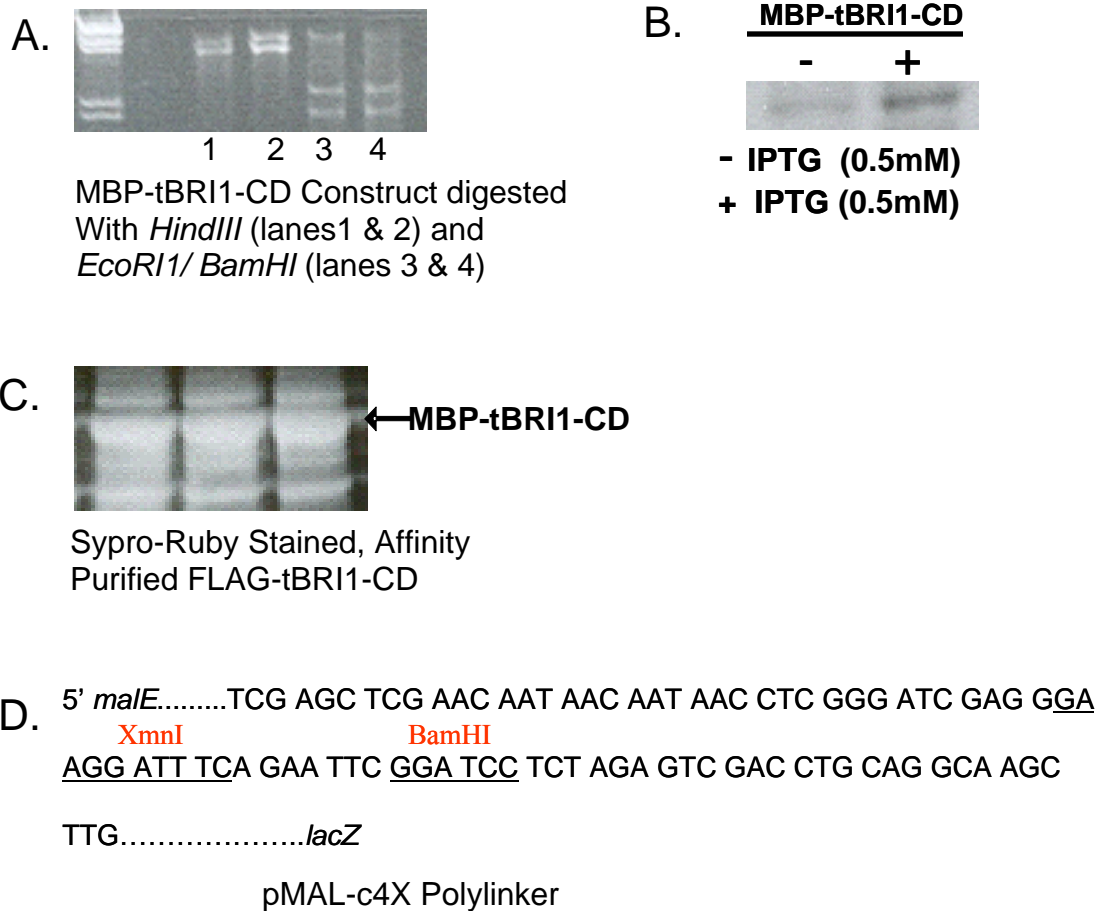


Figure 2. Cloning of tBRI1 cytoplasmic domain into pMAL-c4X expression vector (A) digestion of the construct with restriction enzymes, *HindIII* and *EcoRI*/*BamHI* to verify the presence of tBRI1-CD. (B) CBB staining to detect the presence of tBRI1-CD after induction with IPTG (0.5mM). (C) Gel picture of Sypro Ruby stained purified MBP-tBRI1-CD protein. (D) Multiple cloning site of the expression vector pMAL-c4X with the restriction enzymes, *XmnI* and *BamHI* used for cloning are underlined and highlighted in red.

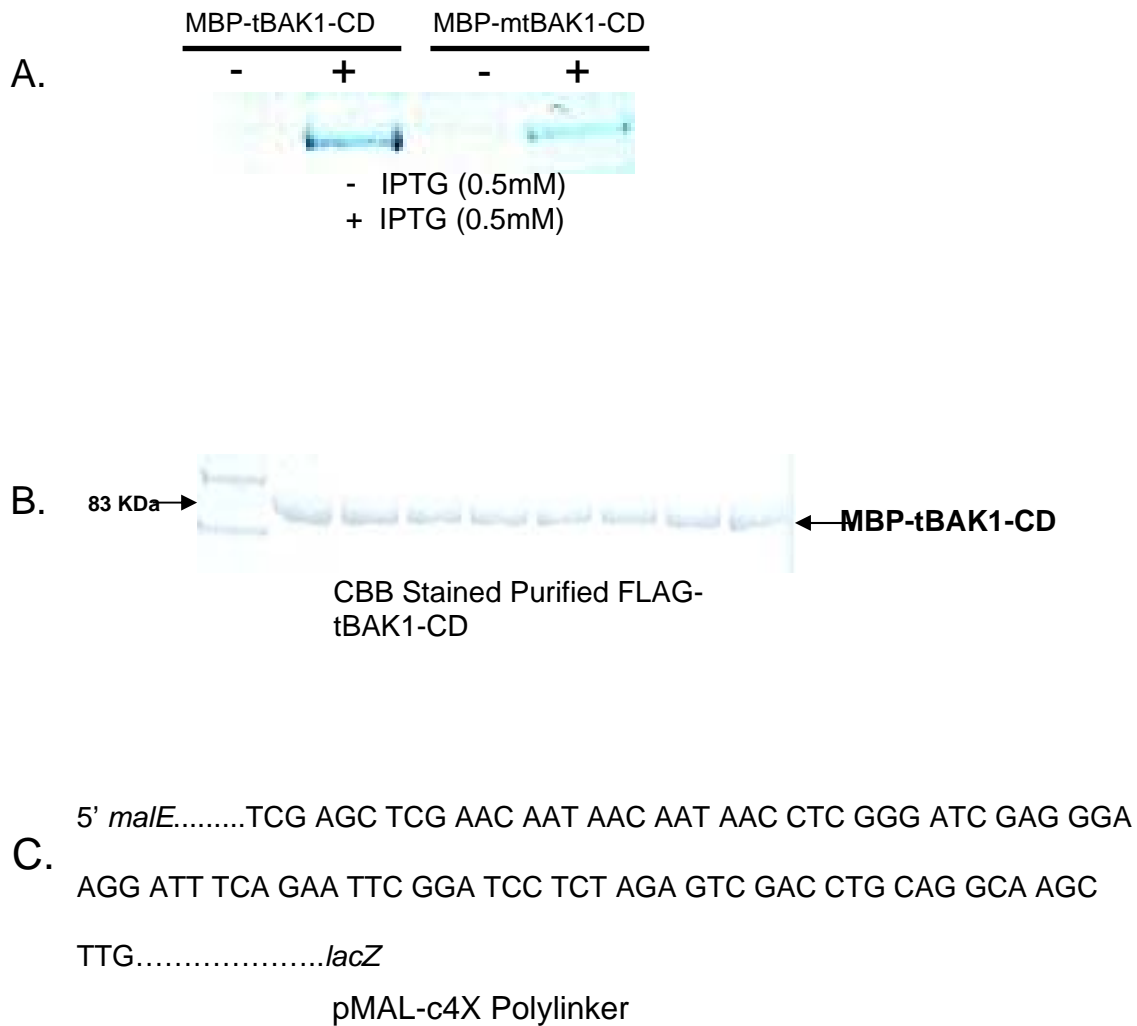


Figure 3. Overexpression and purification of tBAK1 cytoplasmic domain. **(A)** CBB staining to detect the presence of tBAK1-CD after induction with IPTG (0.5mM). **(B)** CBB stained gel purified MBP-tBAK1-CD protein. **(C)** Multiple cloning site of the expression vector pMAL-c4X used for cloning tBAK1-CD into pMAL-c4X.

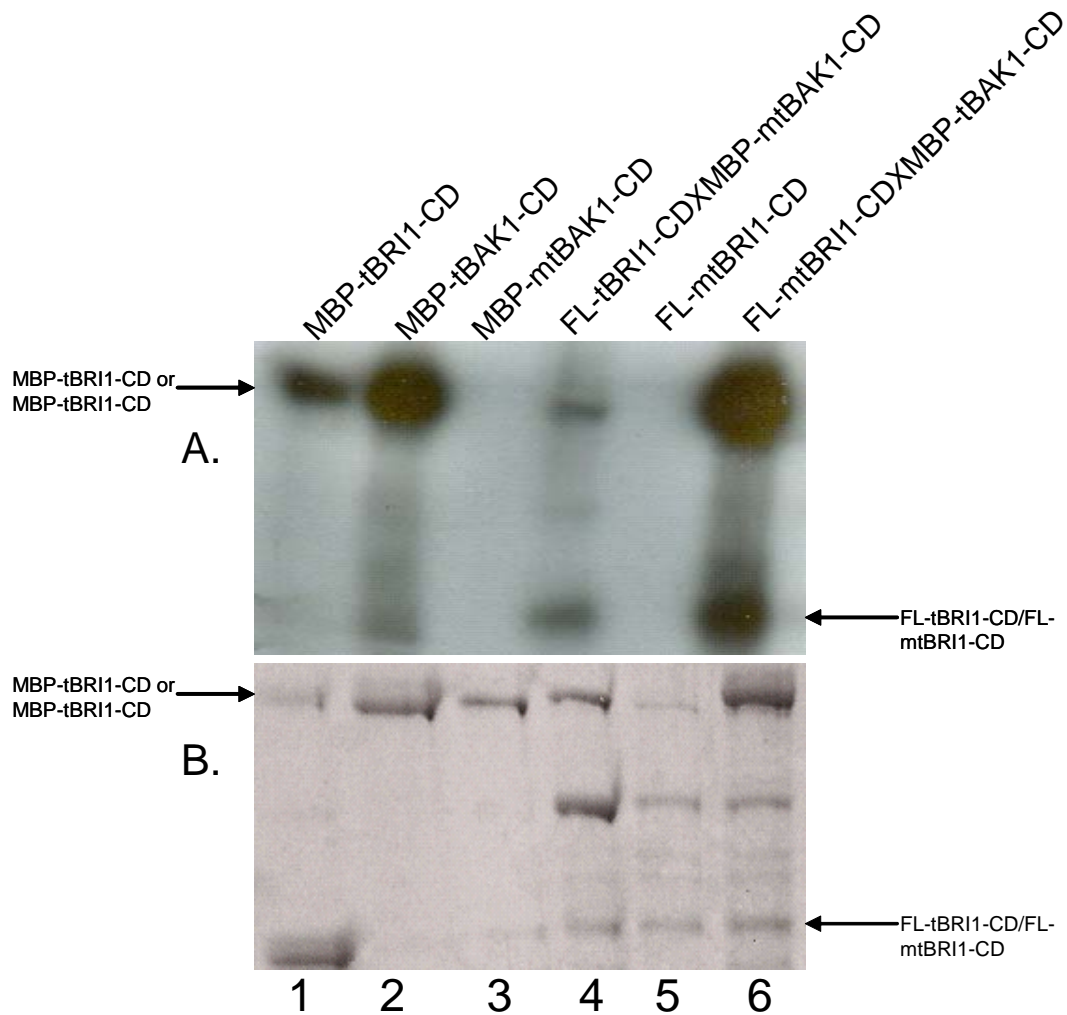


Figure 4. Both tomato BRI1-CD and BAK1-CD can autophosphorylate and transphosphorylate each other. **(A)** Autoradiograph of protein samples after exposing to X-ray film for 1 hr. Proteins were incubated with [γ - 32 P] ATP in kinase buffer for 1hr followed by separation by 10% SDS-PAGE. **(B)** CBB stained protein samples from the same gel.

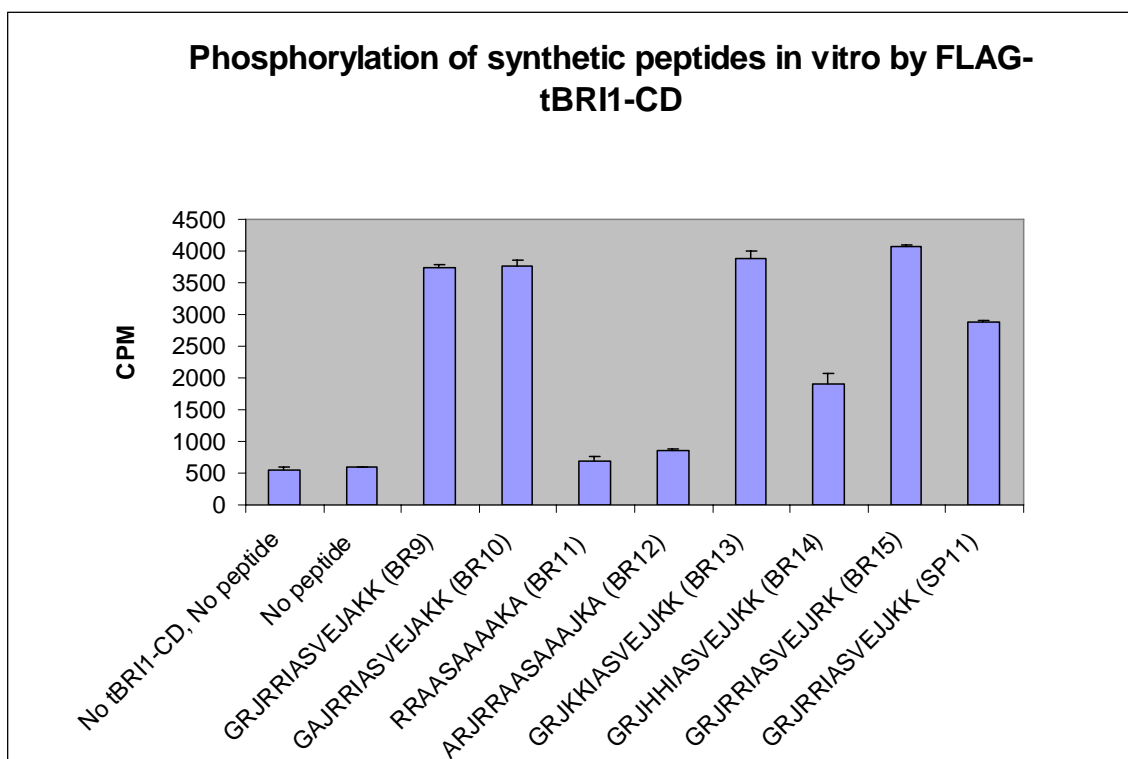


Figure 5. Phosphorylation of synthetic peptides *in vitro* by FLAG-tBRI1-CD. A typical 20- μ L reaction mixture contained 0.5 μ g of affinity-purified FLAG-tBRI1-CD, 0.8 μ Ci [γ - 32 P] ATP (888 cpm/pmol), and 0.10 mg/mL of synthetic peptide in kinase buffer. Reactions were incubated for 20 min at room temperature and incorporation of 32 P into the synthetic peptide was quantified by binding to P81 phosphocellulose paper, followed by liquid scintillation counting. All peptides are sequence variants of SP11 based on the regulatory phosphorylation site (Ser-158) of spinach Sucrose Phosphate Synthase. J indicates nor-Leu, a nonoxidizing functional equivalent of Met. Error bars are SE, $n = 3$.

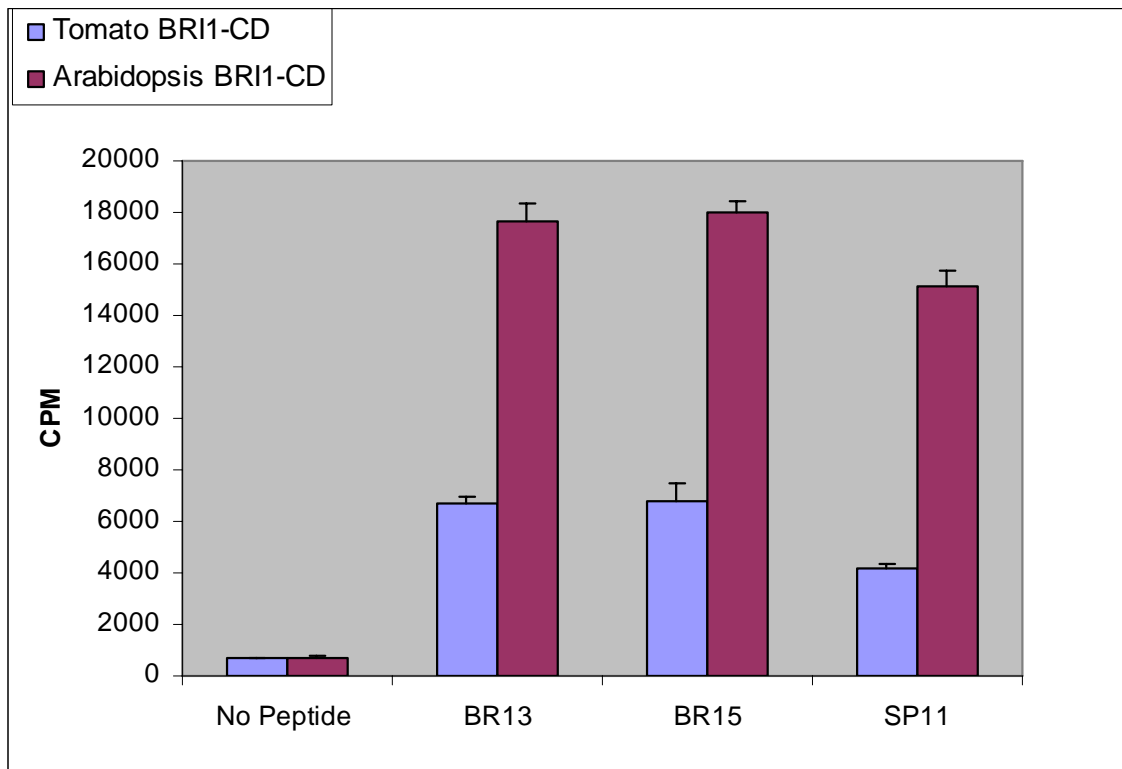
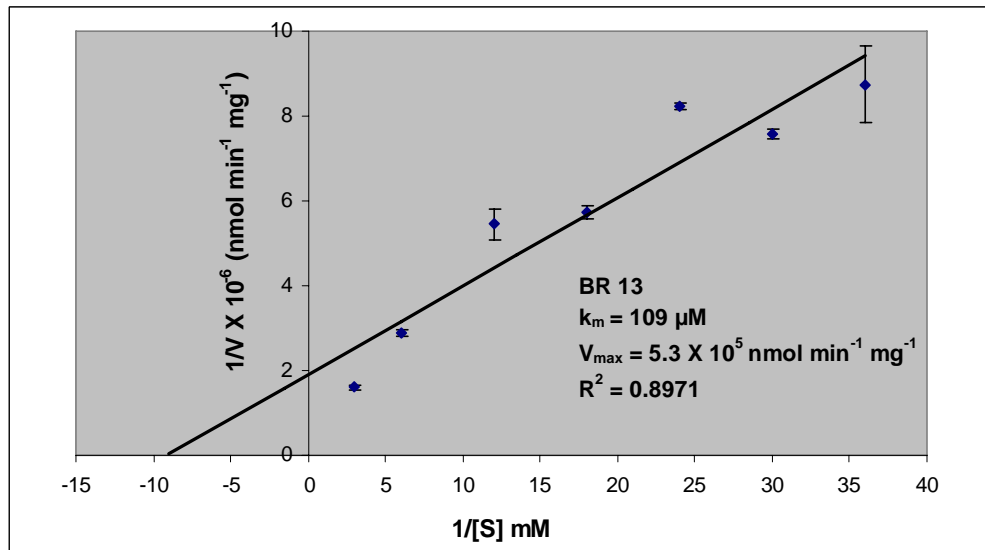


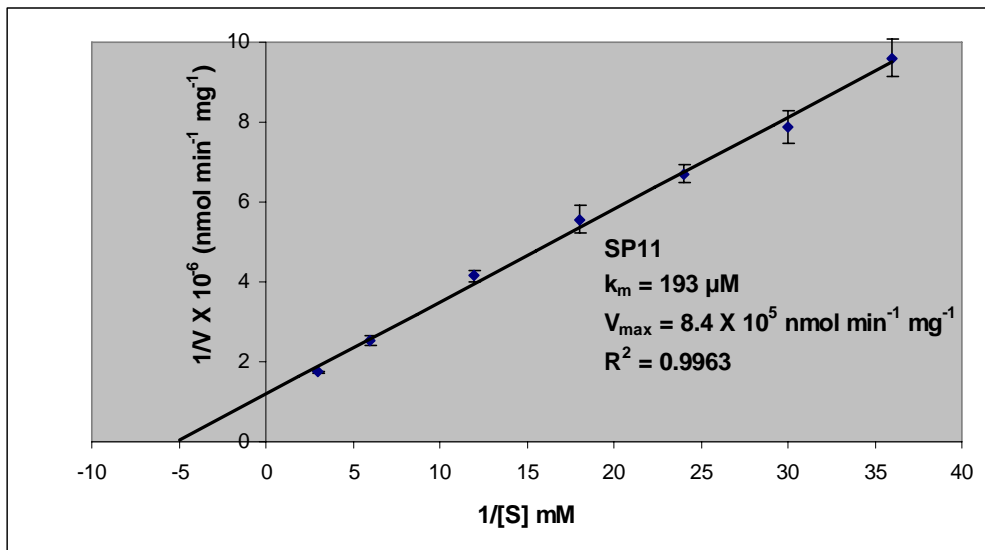
Figure 6. Comparative analysis of phosphorylation of three synthetic peptides *in vitro* by Arabidopsis or Tomato FLAG-BRI1-CD. A typical 20- μ L reaction mixture contained 0.5 μ g of affinity-purified Arabidopsis or Tomato FLAG-BRI1-CD, 0.8 μ Ci [γ - 32 P] ATP (888 cpm/pmol) and 0.10 mg/mL of synthetic peptide in kinase buffer. Reactions were incubated for 22 min at room temperature and incorporation of 32 P into the synthetic peptide was quantified by binding to P81 phosphocellulose paper, followed by liquid scintillation spectrometry. Error bars are SE, $n = 3$.

Figure 7. Peptide substrate kinetics of tBRI1-CD. Lineweaver-Burk double reciprocal plots for BR13 (A) and SP11 (B) were constructed using the indicated substrate concentrations, 0.5 μg of FLAG-tBRI1-CD and 0.1mM ATP (888 cpm/pmol), in kinase buffer. Reactions were incubated for 20 min at ambient temperature and processed as described in Figure 5. Linear regression lines, K_m and V_{max} were determined using Microsoft Excel software.

A.



B.



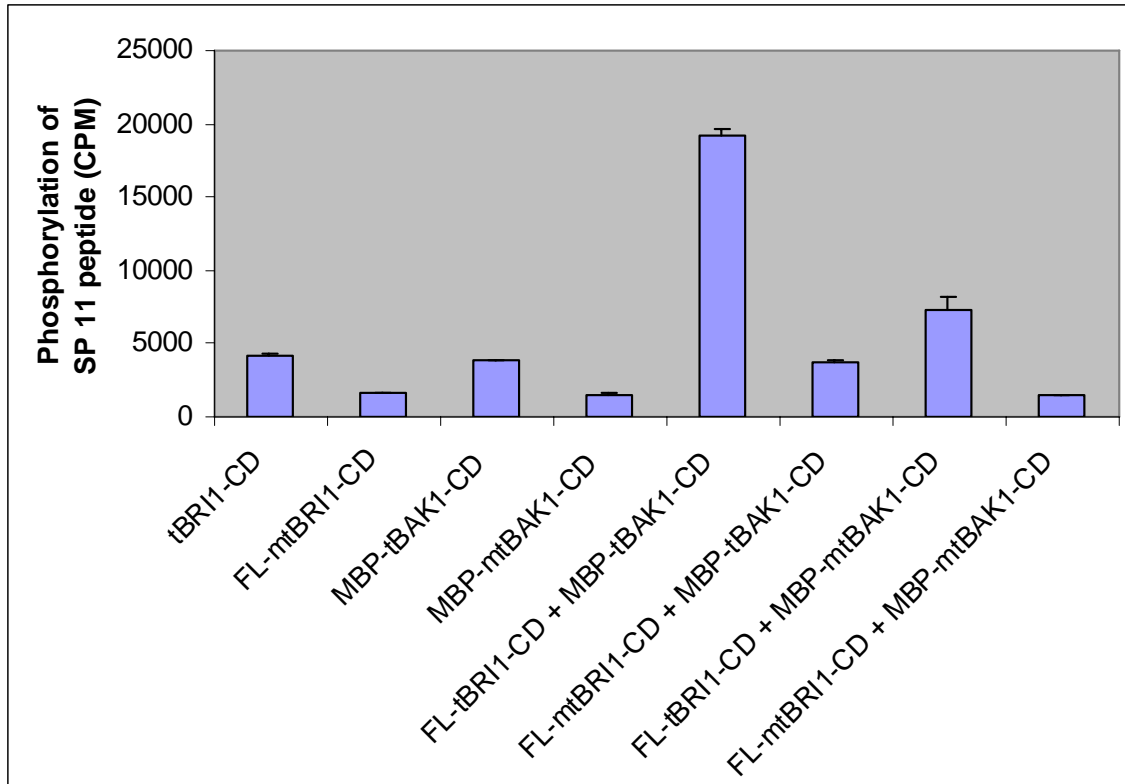


Figure 8. Phosphorylation of the peptide substrate SP 11 (GRJKKIASVEJJKK, J = norLeu) by FLAG-tBRI1-CD (1.0 μ M) is increased over four-fold by the addition of MBP-tBAK1-CD (1.0 μ M) and is dependent on an active tBAK1 kinase. A typical 20- μ L reaction mixture contained 0.5 μ g of affinity-purified FLAG-tBRI1-CD, 0.8 μ Ci [γ - 32 P] ATP (888 cpm/pmol) and 0.10 mg/mL of synthetic peptide in kinase buffer. Reactions were incubated for 20 min at room temperature and incorporation of 32 P into the synthetic peptide was quantified by binding to P81 phosphocellulose paper, followed by liquid scintillation spectrometry. Error bars are SE, $n = 3$.

Chapter Three: Phosphorylation Site Analysis of Tomato BRI1 and BAK1

Introduction

Brassinosteroids (BRs) are naturally occurring plant hormones that have structural similarity to animal and insect steroid hormones (Bishop and Koncz, 2002). A great deal of interest in elucidating the BR signal transduction pathway followed the identification and characterization of the *brassinosteroid-insensitive 1 (bri1)* mutant in Arabidopsis, which exhibited multiple defects in growth and development (Clouse et al., 1993; Clouse, et al., 1996). Cloning of the gene in Arabidopsis revealed that *BRI1* encoded a leucine-rich repeat receptor-like kinase (LRR-RLK) (Li and Chory, 1997). Further work confirmed the role of BRI1 as a plasma membrane associated BR receptor, which is structurally distinct from the animal nuclear steroid receptor family (Wang et al., 2001; Kinoshita et al., 2005). However, BR signaling does show mechanistic similarity to animal receptor kinases recognizing peptide ligands, such as mammalian epidermal growth factor receptor, insulin receptor, and transforming growth factor β (TGF- β) receptor kinases, all of which have multiple steps between ligand perception and gene activation, where phosphorylation status of the receptor and downstream signaling components play an indispensable role (Clouse, 2002). The Arabidopsis receptor kinases BRASSINOSTEROID-INSENSITIVE 1 (BRI1) and BRI1 ASSOCIATED RECEPTOR KINASE 1 (BAK1) share many conserved features with the molecular mechanism of animal receptor kinase action, including ligand-dependent autophosphorylation and oligomerization which ultimately leads to regulation of BR-responsive gene expression (reviewed by Clouse, 2007).

In animal receptor kinases, ligand dependent autophosphorylation of one to three residues in the activation loop is required for kinase activity (Adams, 2003), whereas phosphorylation of juxtamembrane (JM) and C-terminal (CT) domains provide the docking sites for substrate phosphorylation (Pawson, 2004).

Oh et al. (2000) used Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) to identify up to 12 phosphorylation sites on S/T residues in the cytoplasmic kinase domain (CD) of Arabidopsis BRI1. These *in vitro* phosphorylation sites were highly predictive of *in vivo* sites, later identified using liquid chromatography-tandem mass spectrometry (Wang, et al., 2005). The biochemical and genetic analysis of *in vivo* phosphorylation sites in Arabidopsis BRI1 showed that phosphorylation of two highly conserved residues, S1044 and T1049, in the activation loop of BRI1 is essential for its kinase activity, while phosphorylation of residues in the JM and CT domains are important for substrate phosphorylation. These results demonstrated that BRI1 shares general mechanistic features with animal kinase activation and function (Wang, et al., 2005). Wang et al. (2008) also found that at least three residues in the activation loop of BAK1, T-446, T-449 and T-455 (equivalent to the highly conserved T-1049 in BRI1) were critical for its function. They proposed a sequential transphosphorylation model, in which BRI1 phosphorylation precedes its association with BAK1. The association of activated BRI1 with BAK1 leads to BAK1 activation by transphosphorylation on its kinase domain residues. Once activated, BAK1 can in turn transphosphorylate BRI1 JM and CT residues, thereby quantitatively increasing the signaling output of BRI to its specific substrates.

Although BRI1 was previously classified as an S/T kinase, recent studies have shown that it is also phosphorylated on tyrosine residues and is therefore a multifunctional enzyme. Tyrosine phosphorylation of BRI1 was shown to play an important role in its kinase function since expression of BRI1 carrying a mutation in one of the phosphorylated tyrosine residues resulted in plants with larger leaves and early flowering (Oh, et al., 2009).

In order to compare BR signaling mechanisms in Arabidopsis and tomato, it is essential to identify and characterize various autophosphorylation and transphosphorylation sites of BRI1 and BAK1 to understand their role in BR signal transduction. The objective of this study was to identify and perform biochemical functional analysis of tomato BRI1 (tBRI1) and BAK1 (tBAK1) phosphorylation sites. We report here identification of up to 12 *in vitro* phosphorylation sites in tBRI1-CD (six of which were uniquely identified), and up to seven phosphorylation sites in tBAK1 (five of which were uniquely identified). Site-directed mutagenesis of phosphorylated sites showed that highly conserved residues in the activation loop of tBRI1 were essential for BRI1 kinase function, while mutation of phosphorylated residues in the juxtamembrane domain enhanced kinase activity.

Materials and Methods

Determination of *In Vitro* Autophosphorylation and Transphosphorylation Sites of tBRI1 and tBAK1 by Q-TOF LC/MS/MS and Q-TOF LC/MS^E

Five micrograms of affinity purified recombinant proteins (FLAG-tBRI1-CD, MBP-tBRI1-CD, MBP-tBAK1-CD) were autophosphorylated in kinase buffer (50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-KOH, pH 7.9, 10 mM MnCl₂,

1.0 mM dithiothreitol, and 2 mM unlabeled ATP and separated by 10% Bis-Tris NU-PAGE gel (Invitrogen, Carlsbad, CA).

The protein bands were excised with a blade. In-gel reduction, alkylation and trypsin digestion was performed on the excised bands according to a published protocol (Rowley et al., 2000). After digestion, peptides were extracted in 5% (v/v) formic acid; 50% (v/v) acetonitrile; 45% (v/v) 50mM NH_4HCO_3 . The peptide samples were then filtered and injected into a nanoACQUITY UltraPerformance LC (UPLC) coupled to a Premier Q-TOF mass spectrometer (Waters Corporation, Milford, MA) equipped with a nanolock ion source (Waters). The injected peptides were first desalted and concentrated on a 300 μm x 1 cm C18 trapping column at a flow rate of 10 $\mu\text{l}/\text{min}$. The trapped peptides were then separated by an in-line 25cm x 75 μm (Waters) C18 column packed with Bridged Ethyl Hybrid (BEH) C18 matrix (Waters) as the stationary phase and using a mobile phase gradient composed of A=0.1% formic acid in water and B=0.1% formic acid in acetonitrile for the gradient. The peptides were eluted at flow rate of 300 nl/min for 60 min with a gradient of 7% B to 40% B. After the sample run the C18 BEH columns were equilibrated at initial conditions (2% mobile phase B) for 22 min prior to injecting the next sample.

The peptide samples were analyzed using LC/MS/MS, run under data dependent acquisition (DDA) and LC/MS^E modes. In DDA mode the top eight most intense ions were selected in each survey scan and were subjected to collision induced dissociation (CID) to obtain MS/MS spectra. In the LC/MS^E mode LC/MS data is obtained by capturing alternating scans of 2s each between low (4V) and high (10-32V) collision energies (Blackburn et al., 2010). The low energy scans generated intact peptide and high energy

scans resulted in product ions, resulting in better overall coverage from low to high intensity ions. A predigested standard, rabbit phosphorylase B was run between the samples to verify the instruments sensitivity and performance.

Phosphopeptide Enrichment by Immobilized Metal Ion Chromatography (IMAC)

Peptides extracted after in-gel trypsin digestion were enriched for phosphopeptides using Phos-select Iron affinity gel (Sigma, St. Louis, MO). The peptides were dried completely by vacuum centrifugation and resuspended in 50 μ L of buffer [250 mM glacial acetic acid, 30% (v/v) acetonitrile]. Ten μ L of Phos-select resin prewashed with the same buffer was added to the peptide sample. The sample was then incubated at room temperature for 1h on a shaker. After incubation the resin was collected by centrifugation, washed extensively using the same buffer, and eluted with 50 μ L of the same buffer containing 400 mM NH_4OH .

Data Analysis for Phosphopeptide Identification

The raw data files obtained from LC/MS/MS analysis were searched against an *E. coli* database with the addition of tomato MBP-BRI1 and MBP-tBAK1 protein sequences, using Mascot 2.2 search algorithm (Matrix Science, London, UK) running on an in-house server. The dynamic mass modification of 80 Da for phosphorylation on S, T and Y residues were searched. The raw data obtained from LC/MS^E analysis was processed using ProteinLynx Global Server 2.4 (PLGS 2.4) software (Waters). Phosphorylation on S, T and Y residues were searched using PLGS 2.4.

Cloning of *tBRII-FLAG* gene into Plant Transformation Vector

Tomato *BRI1-FLAG* was amplified from tomato genomic DNA, isolated from tomato leaves using Qiagen DNAeasy Kit (Qiagen, Valencia, CA), with sense (5'-cgatcccgaggagttttattttaatttt ctttcaaatacttccatcatgaaagctcacaaaactgtg -3') and antisense (5'-gactgagctctcatttgcacgcgctccttgtagtcaagggtgttgctcagctccattg-3') primers. The sense primer contains 37 bp UTR sequence from alpha mosaic virus, upstream of the *tBRI1* start sequence. This UTR sequence has been previously shown to enhance the expression of transgenes in transformed tomato plants (Krasnyanski et al., 2001). The 20- μ L PCR reaction contained buffer [4- μ L 10X pfx amplification buffer (2X final conc.)], 1 mM MgSO₄, 300 μ M dNTPs, 50 ng of tomato genomic DNA, 5 μ M of each primer, and 5 units of cloned *pfx* DNA polymerase (Invitrogen). After pre-incubation at 94°C for 2 minutes, 31 cycles (of 94°C, 20 s; 58°C, 40 s; 68°C, 240 s) were performed followed by an extension step of 7 min at 68°C. PCR products were gel purified using the QIAquick Gel Extraction Kit (Qiagen). This PCR product was gel-purified, digested with *SmaI* and *SacI* and introduced into the binary vector pBI121 (Clontech, Palo Alto, CA) that had also been digested with *SmaI/SacI* and gel purified to remove the *uidA* gene. The resulting plasmid (35S:*tBRI1-FLAG*/pBI121) was transformed into *Agrobacterium* strain GV 3101.

Transformation of Tomato cv “Cobra” with *tBRII-FLAG*

Agrobacterium-mediated transformation was performed essentially as described by Krasnyanski et al. (2001) with modifications. Seeds of tomato cv. Cobra were obtained from Thompson and Morgan Seeds, Inc. (Buffalo, NY) and sterilized with 70 % ethanol for 1 min and 50% Clorox (commercial bleach) for 30 min. After sterilization, seeds were rinsed four

times with sterile distilled water (3-4 min per rinse), and then placed on tomato seedlings medium (1X MS salts + Gamborg's (B5) vitamins, 10 g/L Sucrose, 12 g/L Agar; pH 5.6) in glass jars, to germinate. Eight-day-old seedlings grown under the light (16 h light: 8 h dark) at 25 °C were used as a source of cotyledon explants.

Agrobacterium strain GV3101 containing 35S: tBRI1-FLAG/pBI121 plasmids was cultured in 25 ml of liquid YEP [10 g/L Yeast extract (Bacto), Bacto-peptone 10g/ L and NaCl 5 g/L] media containing [100 mg/l kanamycin, 50 mg/l rifampicin and 50 mg/l gentamicin] and grown overnight on a shaker at 220 rpm at 28°C. The overnight *Agrobacterium* culture was centrifuged at 1000×g to precipitate the cells. The cell pellet was resuspended in 25 ml of liquid resuspension (LR) medium [1× MS salts + MS vitamins, 30 g/L Sucrose, 2.0 g/L Glucose, 700 mg/L MES; pH 7.6] with 40 mg/L acetosyringone and grown for another 1-2 hours at 28°C. The suspension was then diluted to an OD₆₀₀ of 0.3-0.4 with LR medium containing 40 mg/L acetosyringone.

Cotyledons (well shaped but not curly) of eight-day-old seedlings were used for explants. The explants were cut and placed in Petri plates containing tomato co-cultivation (TCC) medium [1x MS salts + MS vitamins, 30 g/L Sucrose, 2.0 g/L Glucose, 0.5 mg/L IAA, 1.0 mg/L BA, 700 mg/L MES, 40 mg/L acetosyringone, 12 g/L Agar; pH 5.6] and wounded with multi-pronged needles. Wounded cotyledons were submerged in diluted liquid *Agrobacterium* suspension for 15 min with shaking at 50 rpm. Following inoculation, cotyledons were blotted dry on sterile paper towels and placed back on TCC medium for 48 h in the dark at 25 °C.

After co-cultivation, explants were washed 2-3 times with LR medium containing 300 mg/L timentin, blotted dry on sterile paper towels and were placed (15-20 explants per plate) on tomato shoot regeneration (TSR) medium [1x MS salts + MS vitamins, 30 g/L Sucrose, 0.1 mg/L IAA, 2.0 mg/L zeatin, 300 mg/L timentin, 100 mg/L kanamycin, 40mg/L acetosyringone, 8.0 g/L Agar; pH 5.6]. After one week on TSR medium, all explants were re-positioned to achieve full contact with regeneration medium. After two weeks on TSR medium, the explants were subcultured onto fresh TSR medium and further cultured under a 16 h photoperiod (provided by 20 watt cool-white fluorescent tubes yielding a light intensity of 30 mmol/m²/sec) at 25 C.

Five to six weeks later, one to two cm long shoots were excised and placed on a Tomato Rooting medium (TR) [1x MS salts + MS vitamins, 30 g/L Sucrose, 300 mg/L timentin, 50 mg/L kanamycin, 8.0 g/L agar; pH 5.6] containing 50 mg/L kanamycin for secondary selection. Tomato plants with well developed roots were transferred into small plastic pots with sterile soil and covered with clear plastic covers for a week of acclimatization in a growth chamber. Following acclimatization, plants were transferred to the greenhouse for further growth and fruiting.

PCR to Verify tBRI1-FLAG Transgene Expression

Total RNA was isolated from 100 mg of leaves harvested from transgenic tomato plants followed by grinding in liquid N₂ and isolation of RNA using the RNAeasy kit (Qiagen). One µg of total RNA was used for synthesis of first strand cDNA with the ImProm-II Reverse Transcription system using oligodT primer (Promega, Madison, WI). One µL of cDNA was used in a 20-µL PCR reaction (as described above) except that an

extension time of 2.5 min was used to amplify *tBRI1-FLAG* sequence. The PCR reaction was performed using a gene specific forward primer 5'-gttgaaggtagtatcccaaag-3' and reverse FLAG primer 5'-ttgtcatcgtcgtcctttagtc-3'.

Plant growth and Membrane Protein Isolation

Tomato plants overexpressing tomato BRI1-FLAG were grown in flats containing 'Farfard 4P' potting media in a greenhouse under natural light conditions. Twenty days after seeding, the seedlings were treated with foliar spray application of 1.0 μ M 24-epi-brassinolide (dissolved in water containing 0.001% ethanol; 0.1% Tween 20) to wet the leaf surface completely. Seedlings were harvested 120 min after the treatment.

Total membrane protein was isolated from the tomato seedlings as described by (Wang et. al, 2005) with minor modifications. Forty grams of tissue was ground in liquid nitrogen using a mortar and pestle followed by further homogenization in a blender in 100 ml of cold extraction buffer [20 mM Tris-HCl (pH 8.8), 150 mM NaCl, 1 mM EDTA (pH 8.0), 20% glycerol] containing 1% (v/v) Polyvinylpolypyrrolidone (PVPP), 1 mM PMSF, 20 mM NaF, 50 nM microcystin, and protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN). The extract was centrifuged at 6000 \times g for 15 min at 4°C, and the resultant supernatant was filtered through a cheese cloth and further centrifuged at 100,000 \times g for 2 h at 4°C to precipitate the microsomal fraction. The pellet containing the total membrane proteins was resuspended in 2 mL of solubilization buffer [10 mM Tris-HCl, pH 7.3, 150 mM NaCl, 1.0 mM EDTA, 10% glycerol, 1.0% Triton X-100, 1.0 mM PMSF, 20 mM NaF, 500 nM microcystin, and protease inhibitor cocktail (Roche)] and sonicated for 4 minutes.

Immunoprecipitation and Immunoblotting

Immunoprecipitation of tomato BRI1 was carried out as described by (Wang et. al, 2005) with modifications. Tomato BRI1-Flag was immunoprecipitated with prewashed anti-Flag M₂ agarose beads (Sigma-Aldrich) at 4°C overnight, washed extensively, and eluted in 50 µL of 2.0X SDS sample loading buffer (3% SDS, 94 mM Tris-HCl, pH 6.8, 15% glycerol, and 7.5% β-mercaptoethanol). After boiling for 7 min, 10 µL of supernatant was separated by electrophoresis on a 10% Bis-Tris NU-PAGE gel (Invitrogen). Immunoprecipitated proteins were detected by immunoblot analysis on PVDF membranes with anti-Flag M₂ primary monoclonal antibody (Sigma) at 1:1000 dilution. Blots were visualized with horseradish peroxidase–linked secondary antibody (sheep anti-mouse HRP-linked, GE healthcare, Piscatway, NJ) and the ECL chemiluminescence detection system (GE healthcare).

In Vitro Site-Directed Mutagenesis of FLAG-tBRI1-CD

A FLAG-tBRI1-CD construct was the template for site-directed mutagenesis of S, T and Y residues using the Quick Change II site-directed mutagenesis kit (Stratagene, La Jolla, CA). Fourteen *in vitro* mutant constructs containing substitutions in the tBRI1-FLAG amino acid residues, T-825-A, Y-839-F, S-846-A, T-848-A, S-863-A, T-877-A, S-968-A, S-1040-A, T-1044-A, T-1047-A, S-1049-A, T-1050-A, T-1054-A, or S-1073-A, were generated using the following primer pairs respectively:

(Forward) 5'-caagcttctcgaggagacgaagaagaggaggaggaag- 3'

(Reverse) 5'-cttcctcctcctctctctctcgtctcctcgagaagcttg- 3'

(Forward) 5'-ggctgctcttgaagcttatatggatggtcattcac- 3'

(Reverse) 5'-gtgaatgaccatccatataagcttcaagagcagcc- 3'

(Forward) 5'-ggatggcattcacattctgcaactgccaacagtgc- 3'
 (Reverse) 5'-gcactgttggcagttgcagaatgtgaatgaccatcc- 3'

(Forward) 5'-gtcattcacattctgcaactgccaacagtgcctgg- 3'
 (Reverse) 5'-ccaggcactgttggcagttgcagaatgtgaatgac- 3'

(Forward) 5'-gtgctcgtgagggcgtaagcatcaacctgcagc- 3'
 (Reverse) 5'-gctgcaagggtgatgcttaacgcctcacgagcac- 3'

(Forward) 5'-cctctcaggaagctcacatttgctgatcttctcg- 3'
 (Reverse) 5'-cgagaagatcagcaaatgtgagcttctgagagg- 3'

(Forward) 5'-gaatacatgaagtatggaagtcttgaagatgtcctgc - 3'
 (Reverse) 5'-gcaggacatctcaagacttccatacttcatgtattc- 3'

(Forward) 5'-gaatggcaaggtaatgagtgcctatggacactcatttg- 3'
 (Reverse) 5'-caaatgagtgtccatagcactcattaaccttgcattc- 3'

(Forward) 5'-gttaatgagtgcctatggacactcatttgagtgtcagcac - 3'
 (Reverse) 5'-gtgctgacactcaaatgagtgtccatagcactcattaac- 3'

(Forward) 5'-ctatggacactcatttgagtgtcagcactcttgccgg- 3'
 (Reverse) 5'-ccggcaagagtgtgacactcaaatgagtgtccatag- 3'

(Forward) 5'-gacactcatttgagtgtcagcactcttgccggcactc- 3'
 (Reverse) 5'-gagtgccggcaagagtgtgacactcaaatgagtgtc- 3'

(Forward) 5'-ctcatttgagtgtcagcactcttgccggcactccagg- 3'
 (Reverse) 5'-cctggagtgccggcaagagtgtgacactcaaatgag- 3'

(Forward) 5'-gtcagcactcttgccggcactccaggatacgtacctcc- 3'
 (Reverse) 5'-ggaggtacgtatcctggagtgccggcaagagtgtgac- 3'

(Forward) 5'-ggcatggattcgacatcgacaatcggagctgatg- 3'
 (Reverse) 5'-catcagctccgattgtcgtatgtcgaatccatgcc- 3'

A 20- μ L PCR reaction was performed for each mutation according to the manufacturer's instructions (Stratagene). After pre-incubation at 95°C for 3 minutes, 18 cycles (of 95°C, 30 s; 55°C, 60 s; 68°C, 390 s) were performed followed by an extension

of 7 min at 68°C. All constructs were sequenced completely to verify the presence of correct mutations and absence of other unexpected mutations.

Autophosphorylation Assay

For autophosphorylation assays a 40µl reaction containing 1.0 µg of affinity purified FLAG-tBRI1-CD carrying mutations in specific S/T residues was incubated with 20 µCi of [γ -³²P]ATP in kinase buffer (50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-KOH, pH 7.9, 10 mM MnCl₂, 1.0 mM dithiothreitol, and 0.2 mM unlabeled ATP) at room temperature for 1 h. Reactions were terminated by adding 20 µL of 2X Laemmli loading buffer, followed by 10% (w/v) SDS-PAGE and autoradiography.

Peptide Substrate Assay

Peptide substrate assays were performed as described by (Oh, et al., 2000) with some changes in protein and peptide amounts. A 20-µL reaction contained 0.10 mg/mL of synthetic peptide (BR 13), 0.5 µg FLAG-tBRI1-CD carrying mutations in specific S/T residues and 0.1mM unlabelled ATP in a kinase buffer consisting of 50 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], pH 7.4; 10 mM MgCl₂, and 0.2 mM CaCl₂. Following 22-min incubation at room temperature, 20 µL of the reaction solution was spotted on a 2- × 2-cm piece of P81 phosphocellulose paper [Whatman, Piscataway, NJ]. The paper was washed four times in 75 mM H₃PO₄ (4 min per wash) and ³²P incorporation into the BR 13 peptide incubated with mutant FLAG-tBRI1-CD was determined by liquid scintillation counting of the washed squares. Each reaction point was performed in triplicate, and the results are

presented as means \pm SE. The sequence of BR 13 peptide used is as follows, with J representing norleucine, a non-oxidizing methionine amino acid substitute:

BR13 – GRJKKIASVEJJKK

Cloning the Mutated *tBRII-FLAG* Constructs for Plant Transformation

The first 2,484 base pairs of *tBRI1* were PCR amplified from 35S:*tBRI1-FLAG/pBI121* construct using the sense primer 5'-cgatcccgggagttttattttaattttctttcaataacttccatcatgaaagctcacaaaactgtg-3' and antisense primer 5'-gactgagctcagtcaggcctcttctctctctatgg-3'. The resulting PCR product was gel purified and cloned into the pGemT-easy Vector (Promega) using a T/A cloning strategy. The *tBRI1-CD* gene carrying mutations was amplified from the mutant *FLAG-tBRI1-CD* constructs using the PCR primer pair 5'-aggaggaagaaggagctgc-3' (Forward) and 5'-gactgagctctcattgtcatcgtcgtcctttagtcaaggtgttgctcagctccattg-3' (Reverse). To get the full length mutant *tBRI1-FLAG* constructs into the pGemT-easy vector the amplified PCR products were gel purified, digested with *StuI/SacI* (Promega), gel purified and ligated with a *StuI/SacI* digested, and gel purified pGemT-easy vector containing the first 2,484 bps of *tBRI1* to obtain full length mutant *tBRI1-FLAG* constructs in the T-easy vector. All constructs were sequenced completely to confirm mutations.

The plant transformation vector pGPTV-35S-KAN, a vector with the NPT II gene next to left T-DNA border, was used for all mutant *tBRI1-FLAG* constructs. The *EcoRII/HindIII nosp-uidA* section of pGPTV-KAN plasmid was replaced with the 35S-GUS containing *EcoRII/HindIII* fragment of the plasmid pBI121 to obtain pGPTV-35S-KAN.

Full length *tBRI1-FLAG* genes carrying our mutations were released from pGemT-easy using *SmaI/SacI* restriction enzymes, gel purified and ligated into *SmaI/SacI* digested and gel purified pGPTV-35S-KAN vector to obtain full-length mutant versions of tBRI1-FLAG in the pGPTV-35S-KAN vector. Plant transformation of the full-length mutant constructs was performed as described above.

Results

Tomato BRI1 Autophosphorylates on Multiple Ser, Thr and Tyr Residues *In Vitro*

To identify specific pS, pT or pY phosphorylation sites in the tBRI1-cytoplasmic domain, MBP-tBRI1-CD was subjected to SDS-PAGE, followed by gel extraction, alkylation and trypsin digestion. The trypsin digested MBP-tBRI1-CD peptides were extracted and subjected to Q-TOF LC-MS/MS analysis, with or without IMAC to enrich for phospho-peptides. The MS/MS spectra of identified phosphorylated peptides are shown in Figure 1 and phosphorylated peptides are listed in Table 1. The identification of phosphorylated sites was based on the product ion scores of spectra generated by Mascot 2.0 (Perkins, et al., 1999) and by manual inspection of the data. The sequence coverage of the tBRI1-cytoplasmic domain was more than 85%. The six unambiguously assigned phosphorylation sites include Y-839, T-848, S-863 and T-877 in the juxtamembrane domain, S-963 in kinase subdomain VI and T-1054 in the kinase domain activation loop (Table 1 and Figure 1). All of these sites, except S-963, have been identified on the corresponding conserved (S/T/Y) residues in Arabidopsis BRI1 (Oh et al., 2000; Wang et al., 2005; Oh et al., 2009). The highly conserved residue corresponding to tomato BRI1 residue T-1054 (or T-1049 in Arabidopsis BRI1) has not been unambiguously assigned previously in Arabidopsis

BRI1 (Table 2) (Oh et al., 2000; Wang et al., 2005). Based on the Q-TOF-LC/MS/MS data, up to three of the five sites (T-1040, T-1044, S-1047, S-1049 and T-1050) present in the activation loop of subdomain VII/VIII, spanning residues 1032-1061 of tomato BRI1, were detected to be phosphorylated (Figure 1E, Tables 1 and 2). These residues were identified in the peptide 1039-LMSAMDTHLSVSTLAGTPGYVPPEYYQSFR-1066 which contains six T and S residues in total. The longer size and absence of any basic residue in this peptide makes it hard to fragment, leading to low fragment ion coverage with aberrantly low scores. However based on manual inspection of the MS/MS spectra, up to three sites were detected to be phosphorylated in this peptide but the specific location of these sites was ambiguous. In addition to these sites, T-825 in the JM domain of tBRI1-CD might also be phosphorylated based on the Mascot assignment, however due to poor quality of the fragment ion spectrum it is hard to manually verify the assignment of this site. No S/T residue is present in Arabidopsis at a location corresponding to tBRI1 residue T-825 (Table 2). Tomato BRI1 sites that were identified as phosphorylated or have a possibility of being phosphorylated are listed in Tables 1 and 2. Summary of the tBRI1-CD *in vitro* phosphorylation sites is given in Figure 2. Overall, up to 12 *in vitro* phosphorylation sites were identified in tomato BRI1 compared to the 11 sites previously reported in Arabidopsis (Oh et al., 2000; Wang et al., 2005). Interestingly, ten of the tomato BRI1 phosphorylated sites have been identified on the corresponding Arabidopsis residues, but two were not (Table 2).

Tomato BAK1 Autophosphorylate on Multiple Ser and Thr Residues *In Vitro*

Tryptic digested peptides from *in vitro* autophosphorylated MBP-tBAK1-CD were used to identify the tBAK1 phosphorylation sites by either Q-TOF-LC/MS/MS (DDA) or

Q-TOF LC/MS^E approach. Using these approaches, we uniquely identified tomato BAK1 phosphorylated residues corresponding to S-286, S-290, T-449, T-455, and S-465 in Arabidopsis BAK1 based on Mascot and PLGS data (Table 3 and Figure 3). The PLGS and/or Mascot data further suggested sites equivalent to Arabidopsis BAK1 T-324, S-370 (or S-373), T-446 (or T-449 or T-450) and S-465 (or S-466). The residues corresponding to Arabidopsis T-446, T-449, T-450 and T-455 are present in the activation loop of BAK1. Two of the activation loop residues, T-449 and T-455 are unambiguously assigned as tBAK1 phosphorylation sites, while the phosphorylation site assignment of the other two residues (T-446 and T-450) is ambiguous. The MS^E or MS/MS spectra of some identified phosphorylated peptides are shown in Figure 3 and listed in Table 3. Tomato BAK1 sites corresponding to S-286, T-446, T-449, and T-455 in Arabidopsis BAK1, were previously reported as Arabidopsis BAK1 phosphorylation sites (Wang et al., 2005; Wang et al., 2008). However, the tomato sites corresponding to T-324, S-370 (or S 373), T-465 (or T-466) in Arabidopsis BAK1 have not been reported previously (Figure 4). The activation loop sites of Arabidopsis BAK1 are essential for its kinase activity (Wang et al., 2008).

Tomato BAK1-CD is transphosphorylated by tBRI1-CD

We had detected the transphorylation activity between tBRI1 and tBAK1 using an *in vitro* kinase assay with [γ -³²P] ATP (see chapter 2). However, to understand the precise molecular mechanism of tBRI1 and tBAK1 interaction and activation, it was essential to identify the specific transphosphorylation sites. In order to identify the tBAK1 transphosphorylation sites, we incubated kinase inactive MBP-mtBAK1-CD (K317E) with FLAG-tBRI1-CD in kinase buffer containing unlabelled ATP for 1 hr. MBP-mtBAK1-CD

(K317E) tryptic peptides that were transphosphorylated by FLAG-tBRI1-CD were subjected to LC/MS/MS analysis. Using this approach we were able to identify a tBAK1 transphosphorylation site corresponding to Arabidopsis residue T-455 in the activation loop of tBAK1 (Figure 5). Interestingly, T-455 has also been previously reported as one of the Arabidopsis BAK1 transphosphorylation sites (Wang et al., 2008).

Effect of Mutating Identified and Predicted tBRI1 Phosphorylation Sites on *In Vitro* Kinase Function

To understand the biochemical function of the identified tBRI1 phosphorylation sites, we generated fourteen *in vitro* mutant constructs with putative tBRI1 cytoplasmic domain S, T and Y phosphorylated residues mutated to A (for S and T) or F (for Y) by site directed mutagenesis. All of these constructs were generated in the pFLAG MAC expression vector with an N-terminal FLAG epitope tag. The mutant constructs were overexpressed in *E. coli* BL-21 cells containing the pGTf-2 plasmid to ensure proper folding of the overexpressed protein. Overexpressed recombinant proteins were purified using FLAG M₂-agarose beads. The functional importance of each residue was assessed by determining the effect of each mutation on autophosphorylation of the tBRI1 cytoplasmic domain *in vitro* and on phosphorylation of a synthetic peptide (BR13) containing the optimal consensus sequence for BRI1 substrate phosphorylation. One microgram of affinity purified recombinant FLAG-tBRI1-CD from each of the 14 mutant constructs was incubated with [γ -³²P] ATP to test for autophosphorylation activity and 0.5 microgram of the purified proteins was used for peptide substrate phosphorylation assays. Mutating S-1040-A, S-1047-A and T-1054-A residues in the activation loop almost completely abolished kinase activity, with respect to

autophosphorylation and peptide substrate phosphorylation (Figure 6A and 6B). Mutation of the residues S-968-A in the JM and, S-1044-A and S-1049-A, in the activation loop significantly reduced kinase activity with respect to autophosphorylation.

Phosphorylation of the peptide substrate was also reduced by S-1044-A and S-1049-A mutations, but no significant effect on substrate phosphorylation was observed in the S-968-A mutation. Substrate phosphorylation was highly reduced in the S-1044-A mutation, with the average counts per min (CPM) of the mutant being even lower than T-1054-A, which itself almost completely abolished the autophosphorylation activity, indicating that S-1044 may be a very important residue for substrate phosphorylation (Figures 6A and 6B). Mutating residues Y-839-F, S-846-A and T-863-A in the JM and S-1173-A in the CT domain of tBRI1 didn't show much effect on the autophosphorylation activity of tBRI1-CD (Figure 6A). However Y-839-F and S-846-A mutations enhanced the peptide substrate activity of tBRI1-CD by 2.9 and 1.9 times respectively, compared to the WT protein. Almost no change in substrate phosphorylation was observed for the S-863-A and S-1173-A mutations (Figures 6A and 6B). A significant increase in kinase activity was observed as a result of mutating JM residues T-825-A, T-848-A and S-877-A, with the highest increase in autophosphorylation observed in the S-877-A mutation, followed by the T-825-A mutation (Figure 6A). Similar results were observed in peptide substrate phosphorylation by these mutations. Mutating S-877-A enhanced substrate phosphorylation by more than 20-fold compared to WT (tBRI1-CD), while mutating T-825-A and T-848-A residues increased substrate phosphorylation by 3.4 and 5.8 –fold respectively, compared to WT (tBRI1-CD). Interestingly, the T-877 residue

is equivalent to T-872 of Arabidopsis BRI1, which has been previously shown to enhance peptide substrate phosphorylation of Arabidopsis BRI1 by about 10-fold (Wang et. al, 2005). This residue is one of two JM sites that are highly conserved in all RD LRR-RLKs in Arabidopsis (Wang et. al, 2005). These results indicate that JM residues T-877, T-825-A and T-848-A may act as negative regulatory sites when phosphorylated.

Although the effects on the kinase activity of mutating specific S/T residues in tBRI1-CD were often similar to the effect of mutating conserved residues in Arabidopsis BRI1. However, there were differences as well (Wang et al., 2005). In tomato, for instance, mutating the catalytic domain residue S-1047, almost completely abolished the autophosphorylation activity of tBRI1-CD, whereas in Arabidopsis BRI1, mutating the conserved residue (T-1042) reduced, but did not completely abolish, autophosphorylation (Wang et al., 2005). In tomato, S-1040 looks to be a very important residue for the activity of tBRI1-CD, as its mutation completely abolished kinase activity of tBRI1. However, the effect of mutating the corresponding Arabidopsis residue (S-1035) on the autophosphorylation activity of Arabidopsis BRI1 was not observed.

Finally, mutating T-877-A in tBRI1 resulted in increased autophosphorylation (Figure 6A) compared to WT (tBRI1-CD), and mutating T-872-A in Arabidopsis BRI1 (equivalent to tBRI1, T-877 residue) also enhanced the autophosphorylation of the mutated protein compared to WT (BRI1) (Wang et al., 2005).

Genotypic and Phenotypic Analysis of Transgenic Tomato Overexpressing tBRI1-FLAG

Full-length tBRI1 containing a C-terminal FLAG tag was cloned into the binary plant transformation vector pBI121 (containing the CaMV 35S promoter for constitutive

overexpression) and transformed into WT tomato cv “Cobra”. Multiple tBRI1-FLAG transgenic lines were generated and PCR was used to verify transgene expression. Most of the transgenic lines checked by PCR contained truncated *tBRI1-FLAG*; however we were able to identify three lines expressing a full-length tBRI1-FLAG (Figures 7A and 7B). The expression of tomato BRI1-FLAG protein was also assessed by western blot analysis using anti-FLAG antibody (Figure 7C). One of the three positive lines, tBRI1-FLAG line #60, was advanced to obtain the R3 homozygous generation.

The phenotype of tBRI1-FLAG overexpressing line was very similar to that described by Holton et al. (2007). Plants overexpressing tBRI1-FLAG (line #60) had ovoid leaves with reduced serrations, and the internodal distance and hypocotyl length were greater than in WT plants (Figure 9). The germination of the 35S::tBRI1-FLAG line #60 was 1 to 2 days faster than WT, but the fruit size in the transgenic lines was much smaller than the fruit from the WT plants.

Identification of tBRI1 *In Vivo* Phosphorylation Sites

To identify *in vivo* autophosphorylation sites, tBRI1-FLAG overexpressing plants were grown for 20 days in a greenhouse and then treated with 24-epibrassinolide for 120 min before harvest. Total membrane proteins were extracted from the plants, immunoprecipitated, separated by SDS-PAGE, and the tBRI1-FLAG band was excised and subjected to Q-TOF LC/MS/MS or LC/MS^E analysis as described above. We were able to detect tBRI1-FLAG peptides with sequence coverage of about 52% using LC/MS^E analysis (Figure 8). However, we were unable to detect *in vivo* phosphorylation sites of tBRI1 using LC/MS^E analysis.

A number of peptides from non-specific proteins such as Rubisco and glyceraldehyde-3-phosphate were also detected along with tBRI1 peptides.

Identification of tBRI1-FLAG *In Vivo* interacting proteins

We used the Gel-MS^E approach to identify the tBRI1 interacting proteins that immunoprecipitate (IP) along with tBRI1-FLAG during anti-FLAG IP. To separate non-specific interacting proteins from the true tBRI1 interactors, we did a parallel experiment for comparison of proteins between WT and the tBRI1 overexpressing line # 60. Twenty day old WT and transgenic plants were treated with 24-epibrassinolide, total membrane protein was isolated and IP was performed as described above. Equal amounts of IP proteins from WT and tBRI1-FLAG plants were separated in adjacent lanes by 10% SDS-PAGE. Thirteen separate gel bands containing IP proteins were excised in parallel from both transgenic and WT lanes. All 26 excised bands were separately reduced, alkylated, digested with trypsin, and subjected to Q-TOF-LC/MS^E analysis for protein identification and quantification. Using this approach we were able to identify several different proteins that were specifically present in the tBRI1 transgenic line but absent in WT plants. The tBRI1-FLAG was specifically detected in band 4 from transgenic plants (Table 4).

Phenotypic comparison of Tomato Wild Type cv “Money Maker” and *cu3*^{-abs} Mutant

The *cu3*^{-abs1} mutant phenotype is less extreme than the *bri1* allele, *cu3* (Koka, et al., 2000) which is an extreme dwarf (Montoya, et al., 2002). Because the *cu3*^{-abs1} mutant is the ‘Money Maker’ genetic background, we characterized the difference in the growth and development between *cu3*^{-abs1} mutant and the wild type cv ‘Money Maker’.

A clear difference in the development of the WT and the mutant plants was observed. Both flowering and fruiting was delayed in *cu3^{-abs1}* mutant compared to the WT plants. In addition, numbers of flowers per plant were higher in WT compared to the mutant; on average there were more than 4 flowers per plant in WT at 59 days after planting (DAP) compared to average of about 1 flower per plant in the mutant. The maximum number of flowers in the WT plants was about 7 flower per plant at 90 DAP, whereas the in the mutant the maximum number of flowers was about 4 flowers per plant at 107 DAP. At 107 and 111 DAP, the mutant had higher average number of flowers per plant compared to WT plants of the same age (Figure 10).

The onset of fruiting in the mutant is delayed by 16 days compared to WT. At 76 DAP there were no fruits present in the mutant, whereas the WT plants had an average of more than 2 fruits per plant. Overall number of fruits per plant was higher in the WT compared to the mutant plants. At 111 DAP WT plants had an average of 5.5 fruits per plant, whereas the mutant plants had only 1 fruit per plant at the same age. A significant difference was observed between the height of WT and the mutant plants. Average height of WT plants was almost 2.9, 2.7, 2.4 and 2.3 times higher than the height of mutant plants at 59, 75, 95 and 111 DAP, respectively (Figure 10).

***In Vivo* Functional Analysis of tBRI1 Phosphorylation Sites**

To understand the importance of selected phosphorylation sites *in vivo*, we generated transgenic plants overexpressing *35S::tBRI1-FLAG* and *35S::tBRI1-FLAG* carrying mutations (T-1054-A and K-916-E) in the *curl3^{abs1}* mutant background. Phenotypic analysis of plants demonstrated partial to full rescue of the mutant plants to WT phenotype by

overexpressing WT (tBRI1-FLAG) in the *curl3^{abs1}* mutant (Figure 11). However, when a *tBRI1-FLAG* gene carrying the T-1054-A mutation was transferred into the *curl3^{abs1}* mutant background, it further enhanced the mutant phenotype of some transformed lines (Figure 11). A severe dwarf phenotype was also observed by transforming *tBRI1-FLAG* carrying kinase inactive (K-916-E) mutations.

Discussion

Brassinosteroid-deficient and insensitive mutants that display a characteristic dwarf phenotype were instrumental in understanding the underlying molecular mechanism of BR biosynthesis and signaling in the experimental plant *Arabidopsis*. Isolation of BR-mutants from important crop plants including tomato (Koka, et al., 2000), rice (Yamamuro, et al., 2000), barley (Chono, et al., 2003) and pea (Nomura, et al., 2003) clearly demonstrates the importance of this ubiquitous plant hormone. The molecular mechanism of BR signal transduction is best studied in *Arabidopsis*. However we need to understand the molecular details of this important pathway in crop plants, as well. Tomato, having a detailed genetic map, an extensive EST sequence database and relatively high transformation efficiency, is becoming an excellent model plant for studying horticultural crops.

In *Arabidopsis* several *in vitro* and *in vivo* autophosphorylation and transphosphorylation sites in BRI1 and BAK1 have been identified and their functional importance has been studied in detail (Oh et al., 2000; Wang et al., 2005; Wang et al., 2008; Oh et al., 2009).

Similarly, it is essential to identify the autophosphorylation sites of tomato BRI1 and BAK1 in order to understand the details of their kinase function in BR signal transduction.

We mapped individual *in vitro* autophosphorylation sites in tomato BRI1 and BAK1 and studied their biochemical functional role *in vitro* and *in planta*. We identified 12 *in vitro* autophosphorylation sites in tBRI1-CD and up to 7 autophosphorylation sites in tBAK1-CD. Most of these sites had been identified as phosphorylated sites on corresponding residues in Arabidopsis BRI1 and BAK1, but two of the tomato BRI1 and three of the tomato BAK1 sites have not been identified on corresponding residues in Arabidopsis. These results suggest a significant conservation but also possible differences in BRI1 and BAK1 downstream signaling events in different plants. We also identified a transphosphorylation site of tBAK1 (corresponding to Arabidopsis residue T-455). Interestingly, this site has been previously detected as one of the transphosphorylation site of Arabidopsis BAK1. This site is essential for BAK1 kinase function in Arabidopsis (Wang et al., 2008)

To study the biochemical function of tBRI1 *in vitro* autophosphorylation sites, we performed site-directed mutagenesis of each identified and predicted site. Autophosphorylation and substrate phosphorylation assays revealed that phosphorylation of highly conserved residues, T-1054, S-1047 and S-1040, present in the activation loop of tBRI1, was essential for tBRI1 kinase function *in vitro*. Mutations in the JM residues, T-825, T-848 and S-877 to their unphosphorylated counterparts, dramatically increased the autophosphorylation and peptide substrate phosphorylation. Mutation of the juxtamembrane residue, S-877-A, increased the peptide substrate activity by over 20 times compared to the WT (tBRI1-CD).

These results provide evidence for significant conservation in kinase function between Arabidopsis and tomato BRI1. However we observed some differences in the

function of some phosphorylated residues (such as T-1047 and T-877), which may suggest some variation in function between the two kinases and might result in some degree of divergence in the BRI1 downstream signaling events between these two species. Phenotypic analysis of the tomato transgenic lines expressing full-length 35S::tBRI1-FLAG constructs carrying mutation T-1054-A in the *curl3^{abs1}* (intermediate tomato *bri1* allele) mutant and wild-type tomato backgrounds, showed that this residue is essential for normal tBRI1 signaling *in planta* (Figure 11). This experiment clearly explains the *in vivo* importance of phosphorylated residues present in the activation loop of tBRI1 for normal functioning of tBRI1 kinase.

We made multiple attempts to identify *in vivo* autophosphorylation sites of tomato BRI. Although, we were able to detect tBRI1 peptides, we didn't identify any *in vivo* phosphorylation sites. LC-MS^E analysis identified several highly abundant plant proteins such as Rubisco and Glyceraldehyde-3-phosphate present in the IP sample along with tBRI1-FLAG. These non-specific proteins possibly reduced the binding of tBRI1 protein to anti-FLAG antibody during IP. Generally, the detection of phosphorylation sites by mass spectrometry in proteins is hindered by the low abundance, low stoichiometry, and poor ionization of phosphopeptides (Ficarro, et al., 2002). The identification of phosphopeptides in low abundance proteins such as tBRI1-FLAG, is even harder to detect using data dependent acquisition (DDA) mode, which selects the most abundant ions for collision induced dissociation (CID) (Blackburn, et al., 2010).

We identified several *in vivo* tBRI1 interacting proteins (Table 4). A number of these proteins or their subunits have been previously reported to play an important role in BR

biosynthesis and signal transduction. Tomato BRI1-interacting proteins include a WD-40 domain protein similar to Arabidopsis TGF- β Receptor Interacting Protein-1 (TRIP-1). TRIP-1 has been previously reported as a putative *in vivo* substrate of BRI1 (Ehsan, et al., 2005; Jiang and Clouse, 2001).

TCP transcription factors have been reported to control the morphology of shoot lateral organs in Arabidopsis (Koyama, et al., 2007). Recently, the role of TCP1 transcription factor in regulating the expression of the key biosynthetic gene *DWARF4* in *Arabidopsis thaliana* has been reported (Guo, et al., 2010). The fact that we identified a TCP transcription factor as a putative tBRI1 interacting protein is consistent with the important role these transcription factors might play in BR biosynthesis and signaling. 14-3-3 proteins play important role in signaling of at least three plant hormones, namely abscisic acid (ABA), BRs, and gibberellins (GA) (Oecking and Jaspert, 2009). A Recent study shows that in Arabidopsis, a 14-3-3 protein subunit binds to BRZ, a transcription factor involved in BR-mediated gene expression, and inhibits its nuclear localization (Gampala, et al., 2007). The identification of an omega subunit of 14-3-3 protein as a tBRI1 interacting protein, suggests a possible role for 14-3-3 proteins in tomato BR signaling.

Tang et al. (2008) performed a proteomic study to identify early response BR signaling proteins using two-dimensional (2-D) DIGE in *Arabidopsis*. Some of the proteins that they identified were similar to tBRI1 interactors that we have identified in this study, including BR-induced PM protein (DREPP). Overexpression of DREPP in a BR-deficient mutant, partially suppressed its phenotype, demonstrating that DREPP has a potentially important role in BR signaling (Tang, et al., 2008). We also identified an ABC transporter

protein that is a putative interactor of tBRI1 protein. Previous genetic analysis of an ABC transporter, TWD1, revealed that it is required for BR perception or signal transduction (Martinoia, et al., 2002). Overall, we have identified several proteins that are putative *in vivo* interactors of tBRI1. Some of these proteins have been previously associated in BR signaling but a number of these proteins are novel components whose role is not known. Additional work will be required to further understand the detailed role of these proteins in tomato BR signaling.

In summary, comparative functional analysis of tomato BRI1 and BAK1 phosphorylation sites with their Arabidopsis counterparts, revealed a high inter-species conservation but some divergence in receptor kinase mechanisms involved in BR signaling for regulating plant growth and development. A number of proteins that were identified as tBRI1 interacting proteins have been previously shown to be involved in BR signaling in Arabidopsis, however there are novel components that need to be further studied for their role in tomato BR signal transduction.

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Tables

Table 1. Identification of *in vitro* tBRI1 autophosphorylation sites

Peptide Sequence	Mascot Score	Identified Site(s)
EAL(pS)INLAAFEKPLR	36	S 863
EAALAYMDGSHSA(pT)ANSAWK	26	T 848
EAALYA(pY)MDGSHSA(pT)ANSAWK	21	Y 839, T 848
KL(pT)FADLLEATNGFHNDVSLVGGFGDVYK	28	T 877
LMSAMDTLHLSVSTLAG(pT)PGYVPPEYYQSFR	20	T 1054
LM(pSAMDpTHLpSVpSpT)*LAGTPGYVPPEYYQSFR	20	S 1040, T 1044, S 1047, S 1049, T 1050
DAQTNSSNNNNNNNNNLGIEGRE(T)K	12	T 825

(pS/pT/pY) = The identified phosphorylated residue

* = Up to three of the five S/T residues present within the parenthesis could be phosphorylated

Table 2. Comparison of *in vitro* autophosphorylation residues identified in Arabidopsis and tomato BRI1 cytoplasmic kinase domains

Tomato BRI1 Residue Number	Corresponding Arabidopsis BRI1 Residue Number	Protein Domain/Subdomain	Phosphorylation Status in Arabidopsis BRI1	Phosphorylation Status in Tomato BRI1
T-825	No S/T (M-817)	Juxtamembrane Region	N	ambiguous
Y-839	Y-831	Juxtamembrane Region	Y	Y
T-848	T-844	Juxtamembrane Region	Y	Y
S-863	S-858	Juxtamembrane Region	Y	Y
T-877	T-872	Juxtamembrane Region	Y	Y
S-968	S-963	Kinase Domain	N	Y
S-1040	S-1035	Kinase Subdomain VII/VIII Activation loop	ambiguous	ambiguous
T-1044	T-1039	Kinase Subdomain VII/VIII Activation loop	ambiguous	ambiguous
S-1047	T-1042	Kinase Subdomain VII/VIII Activation loop	ambiguous	ambiguous
S-1049	T-1044	Kinase Subdomain VII/VIII Activation loop	ambiguous	ambiguous
T-1050	T-1045	Kinase Subdomain VII/VIII Activation loop	ambiguous	ambiguous
T-1054	T-1049	Kinase Subdomain VII/VIII Activation loop	ambiguous	Y

Table 3. Identification of *in vitro* tBAK1 Auto/trans-phosphorylation sites

Peptide Sequence	PLGS Score	Mascot Score	Corresponding Arabidopsis Residue
G(pT)IGHIAPEYLSTGK	10.29	40	T 455
GTIGHIAPEYL(pS)TGK	n.d.	44	T465
ELQVA(pT)DNFSNK	7.44	22	S 286
ELQVATDNF(pS)NKNILGR	7.78	28	S 290
(pT)QGGELQFQTEVEMISMAVHR	6.84	n.d.	T 324
GTIGHIAPEYL(pSpT) ^a GK	6.43	42	S 465, T-466
LLVYPYMANG(pSVApS) ^a R	6.9	n.d.	S 370, S 373
LMDYKDTHV(pT)TAVR	7.12	50.00	T 449
LMDYKD(pTHVpTpT) ^a AVR	8.24	39.00	T 446, T 449, T 450
G(pT)*IGHIAPEYLSTGK	n.d.	28.00	T 455

(pS/T) = The identified phosphorylated residue

^a = one site within the parenthesis is detected as phosphorylated

* = Transphosphorylation site (by tBRI1)

n.d.= Phosphopeptide with same identified site not detected

Table 4. Tomato BRI1 interacting proteins identified using LC/MS^E

SGN ^a number	Protein annotation	PLGS Score of the matched peptide(s)	Sequence of matched peptides	Enrichment factor ^b
Band 1				
U313542	DREPP plasma membrane polypeptide	5.18	(K)VVEIYEAAA VEIK(S)	U
U344021	Tryptophan synthase beta subunit	4.90	(R)LTDYYKSLNK(G)	U
		4.61	(R)ETPLYFAQRLTDYYK(S)	
U313176	Phosphoglycerate kinase	5.24	(K)YSLAPLVPR(L)	U
		5.06	(R)LGFAGTVADPLFTNHVATKLR(S)	
U312485	Eukaryotic translation initiation	5.33	(R)VLITDLLAR(G)	U
		4.49	(K)IQGVFSATMPPEALEITRK(F)	
Band 3				
U345472	ABC transporter	4.52	(K)AQQQQNNLTFSGVISMATNTDYKK(R)	U
		4.51	(K)AGQVSICERVMVFLGLQR(V)	
Band 4				
U324170	BRI1	6.45	(R)LSNLAILK(L)	U
		5.92	(K)NVAILDLSYNR(F)	
		5.61	(K)LAGSIPELDFK(N)	
		5.39	(K)ITDVFDRE(E)	
		5.35	(K)LIHVSGQGDR(E)	
Band 5				
U329725	Pre mRNA cleavage complex factor	4.86	(K)EQDQIISNVAGFIYVTDVDLHRK(K)	U
		4.73	(R)IGGGPQAPRSALPIGAEPADPTR(L)	

a = Solanaceous Genomics Network number

b = Enrichment compared to protein identified in control (wild-type)

U= Uniquely identified in tomato BRI1 over expressing line

Table 4. (continued)

SGN ^a number	Protein annotation	PLGS Score of the matched peptide(s)	Sequence of matched peptides	Enrichment factor ^b
Band 6				
U313362	ATP synthase beta subunit	5.75	(K)IGLFGGAGVGK(T)	1.5
		5.03	(R)STAQRGGAIR(S)	
U328706	Kinase interacting family protein	5.33	(K)SEIFEAEENGILTKDIMLDR(V)	U
		4.48	(R)EQAESNNLVFDLWDTTSPVSK(A)	
Band 7				
U313176	Phosphoglycerate kinase	5.37	(R)LGFAGTVADPLFTNHVATKLR(S)	3.5
		5.33	(K)YSLAPLVPR(L)	
U313659	WD-40 repeat family protein / auxin-dependent protein (ARCA)	4.96	(R)LWDLQAGTTAR(R)	U
		4.61	(R)STLAGHSGYVNTVAVSPDGLCASGGK(D)	
Band 8				
U330878	SNF2 domain containing protein	5.62	(R)IGCTGLVQFFLASFYSIGGTK(Y)	U
U342110	DEAD box RNA helicase	5.36	(R)LIDLINNNTLK(L)	U
		5.27	(R)GVDVVVGTTPGR(L)	
Band 9				
U313929	FtsH protease	5.54	(K)GVLLVGPPTGK(T)	U
		5.47	(R)VQLPGLSQELLQK(F)	
U328342	TCP family transcription factor	5.00	(K)SNNAIKDSENTPQKEYSDGNKDVINSNNSSS(-)	U

a = Solanaceous Genomics Network number

b = Enrichment compared to protein identified in control (wild-type)

U= Uniquely identified in tomato BRI1 over expressing line

Table 4. (continued)

SGN ^a number	Protein annotation	PLGS Score of the matched peptide(s)	Sequence of matched peptides	Enrichment factor ^b
Band 10				
U317686	casein kinase I	5.31	(K)VLTDELNSYLNK(Y)	U
		5.08	(R)PGAPSKAR(V)	
Band 11				
U313245	ATP synthase gamma chain 1	7.27	(R)MSAMSSATDNASELKK(N)	U
		6.96	(K)VALVVVTGDR(G)	
U330878	SNF2 domain containing protein	5.92	(R)IGCTGLVQFFLASFYISGGTK(Y)	U
U314646	14 3 3 protein GF14 omega	5.99	(R)IISIEQKEESR(G)	U
		5.63	(R)NLLSVAYK(N)	
Band 13				
U313358	Nucleoside diphosphate kinase	5.48	(R)GLISEIVSR(F)	U
		5.41	(R)GDLAVVVGR(N)	
U324553	Protein kinase family	4.86	(K)EQVSLADWALACQR(K)	U
		4.59	(R)LEICIGSARGLHYLHTGAK(Y)	
U314885	Elongation factor 1 alpha	5.83	(K)IGGIGTVPVGR(V)	17.5
		5.33	(R)QTVAVGVVK(N)	
		5.06	(K)ARYDEIVK(E)	

a = Solanaceous Genomics Network number

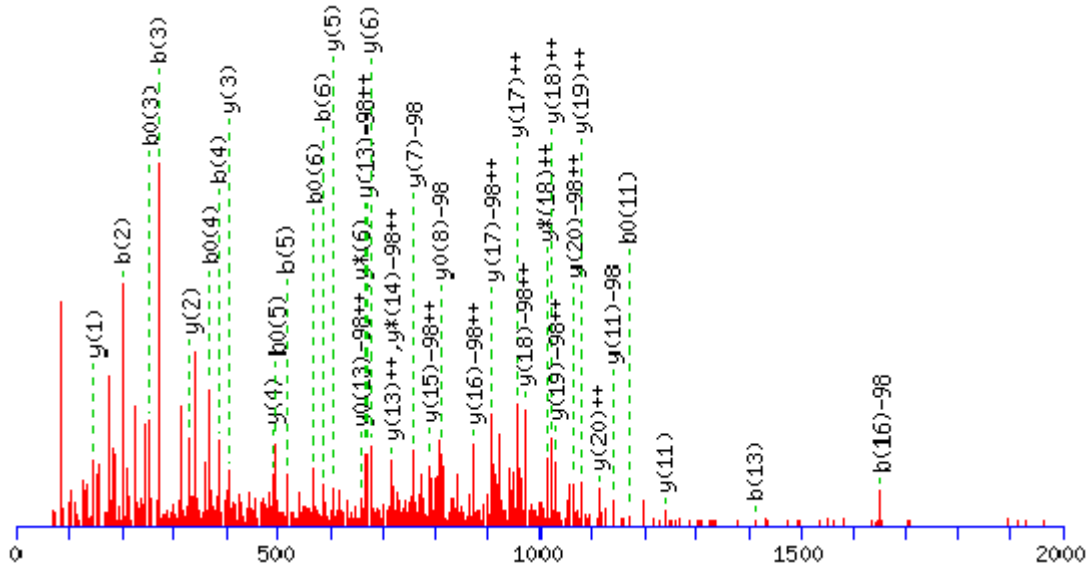
b = Enrichment compared to protein identified in control (wild-type)

U= Uniquely identified in tomato BRI1 over expressing line

Figures

Figure 1. Product (b and y ions) ion spectra of *in vitro* identified phosphorylation sites of tBRI1-CD using Q-TOF LC/MS/MS analysis. MBP-tBRI1-CD was *in vitro* autophosphorylated and tryptic peptides of MBP-tBRI1-CD were analyzed using a Waters NanoAcquity Premier Q-TOF mass spectrometer. Only product ion spectra above the Mascot probability threshold of 20 ($p < 0.05$) are shown. All the site assignments were also verified by manual inspection. Phosphorylation sites are enclosed within parenthesis and highlighted red. **(A)** Q-TOF product ion spectrum identifying T 848 as an *in vitro* phosphorylation site in MBP-tBRI1-CD. **(B)** Q-TOF product ion spectrum identifying Y 839 and T 848 as *in vitro* phosphorylation sites in MBP-tBRI1-CD. **(C)** Q-TOF product ion spectrum identifying S 863 as an *in vitro* phosphorylation site in MBP-tBRI1-CD. **(D)** Q-TOF product ion spectrum identifying T 1054 as an *in vitro* phosphorylation site in MBP-tBRI1-CD. **(E)** Q-TOF product ion spectrum identifying up to three of the five sites, T 1040, T 1044, S 1047, S 1049 and T 1050, enclosed within parenthesis, as *in vitro* phosphorylation sites in MBP-tBRI1-CD. **(F)** Q-TOF product ion spectrum identifying T 877 as an *in vitro* phosphorylation site in MBP-tBRI1-CD.

A. LC/MS/MS Fragmentation of EAALEAYMDGSHSA(pT)ANSAWK



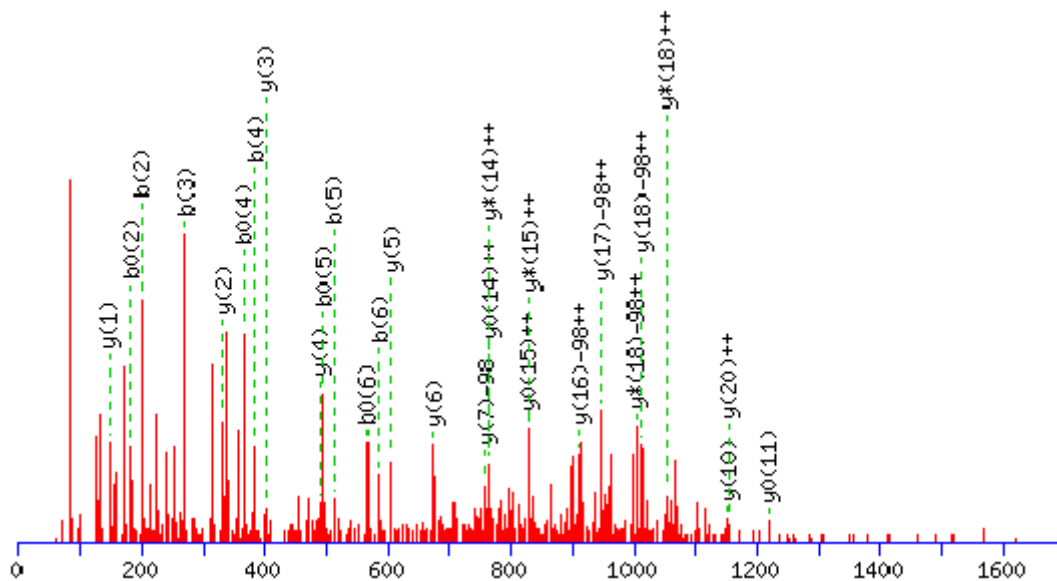
Monoisotopic mass of neutral peptide Mr(calc): 2425.9998

Variable modifications:

T16 : Phospho (ST), with neutral losses 97.9769

Ions Score: 26 Expect: 0.022

B. LC/MS/MS Fragmentation of EAALEA(pY)MDGSHSA(pT)ANSAWK



Monoisotopic mass of neutral peptide Mr(calc): 2505.9661

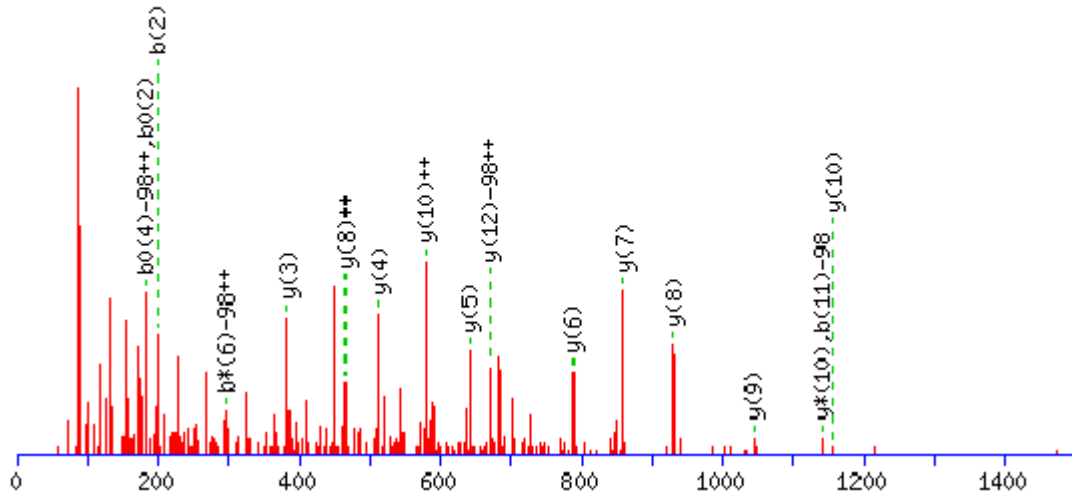
Variable modifications:

Y7 : Phospho (Y)

T16 : Phospho (ST), with neutral losses 97.9769

Ions Score: 21 **Expect:** 0.046

C. LC/MS/MS Fragmentation of EAL(pS)INLAAFEKPLR



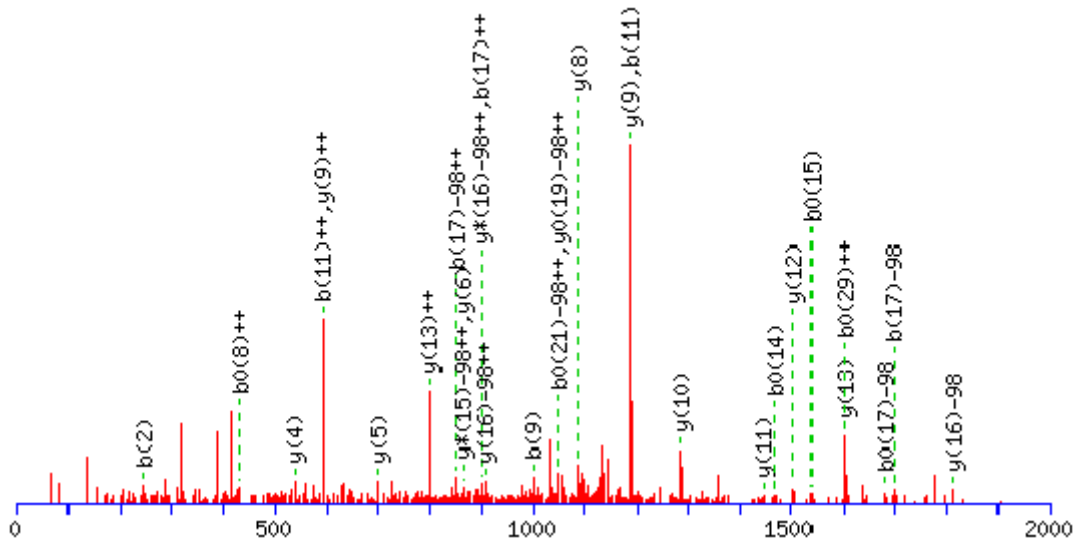
Monoisotopic mass of neutral peptide Mr(calc): 1750.9018

Variable modifications:

S4 : Phospho (ST), with neutral losses 97.9769

Ions Score: 36 **Expect:** 0.002

D. LC/MS/MS Fragmentation of LMSAMDTHLSVSTLAG(pT)PGYVPPEYYQSFR



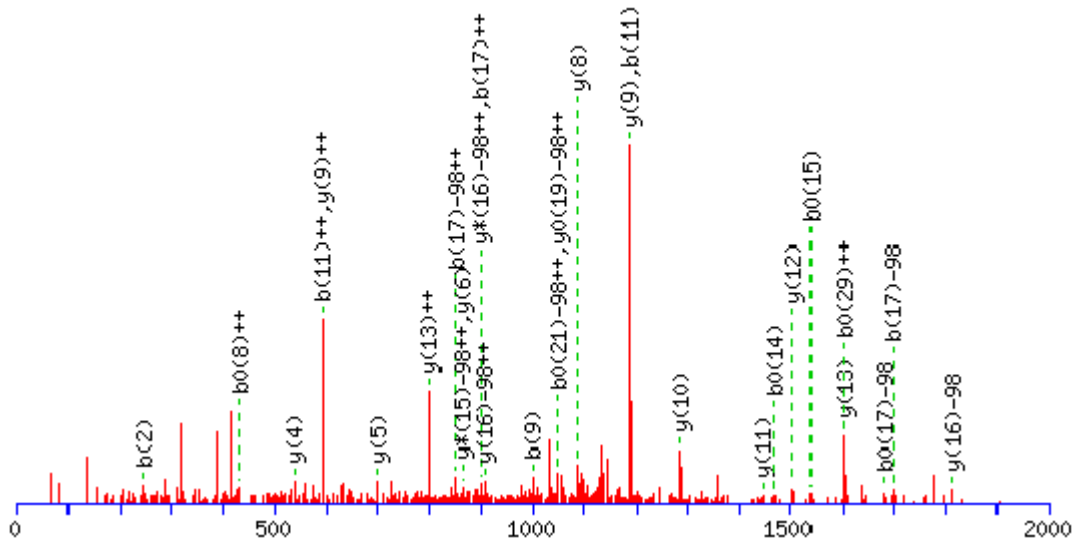
Monoisotopic mass of neutral peptide Mr(calc): 3397.5400

Variable modifications:

T17 : Phospho (ST), with neutral losses 97.9769

Ions Score: 20 **Expect:** 0.15

E. LC/MS/MS Fragmentation of LM(pSAMDpTHLpSVpSpT)LAGTPGYVPPEYYQSFR

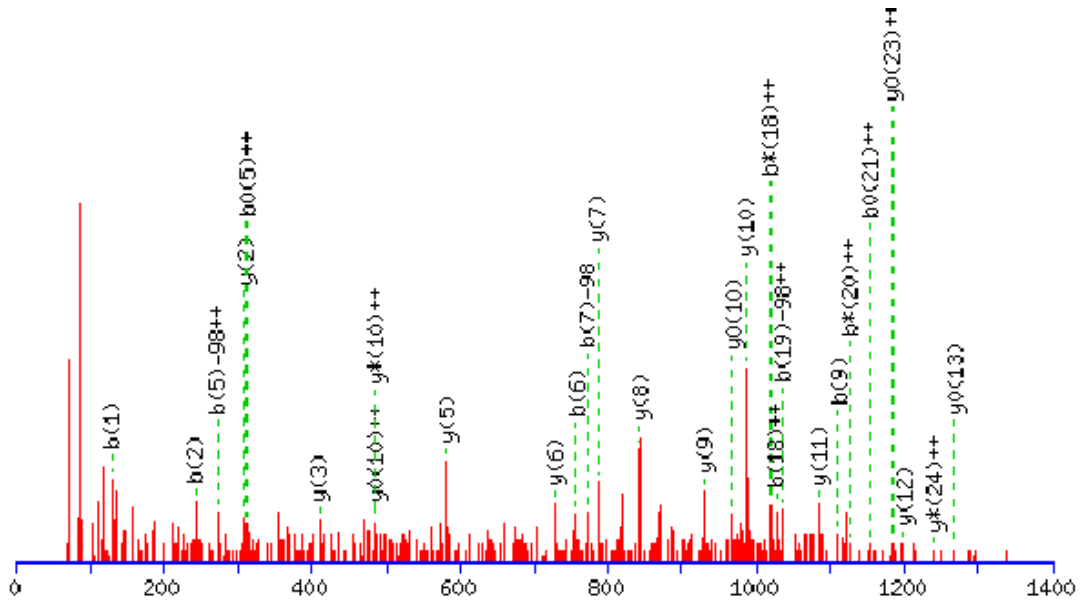


Monoisotopic mass of neutral peptide Mr(calc): 3397.5400

Variable modifications:
Assigned based on manual inspection

Ions Score: 20 Expect: 0.15

F. LC/MS/MS Fragmentation of KL(pT)FADLLEATNGFHNDSLVGSGGFGDVYK



Monoisotopic mass of neutral peptide Mr(calc): 3251.5176

Variable modifications:

T3 : Phospho (ST), with neutral losses, 97.9769

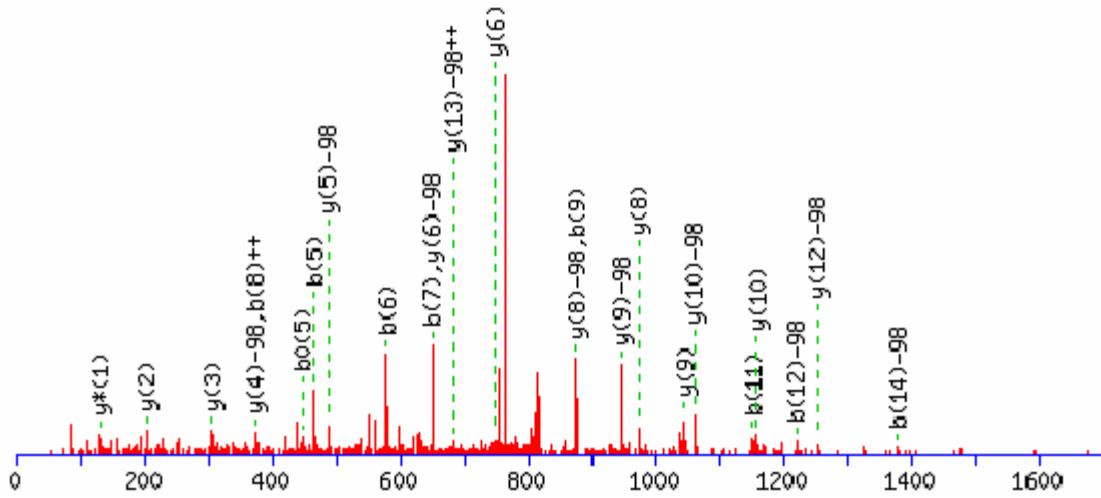
Ions Score: 28 **Expect:** 0.016

<i>E</i>	<i>T</i>	<i>K</i>	<i>K</i>	<i>R</i>	<i>R</i>	<i>R</i>	<i>K</i>	<i>K</i>	<i>E</i>	<i>A</i>	<i>A</i>	<i>L</i>	<i>E</i>	<i>A</i>	<u><i>Y</i></u>	<i>M</i>	<i>D</i>	<i>G</i>	<i>H</i>	843
<i>S</i>	<i>H</i>	<i>S</i>	<i>A</i>	<u><i>T</i></u>	<i>A</i>	<i>N</i>	<i>S</i>	<i>A</i>	<i>W</i>	<i>K</i>	<i>F</i>	<i>T</i>	<i>S</i>	<i>A</i>	<u><i>R</i></u>	<i>E</i>	<i>A</i>	<i>L</i>	<u><i>S</i></u>	863
<i>I</i>	<i>N</i>	<i>L</i>	<i>A</i>	<i>A</i>	<i>F</i>	<i>E</i>	<i>K</i>	<i>P</i>	<i>L</i>	<i>R</i>	<i>K</i>	<i>L</i>	<u><i>T</i></u>	<i>F</i>	<i>A</i>	<i>D</i>	<i>L</i>	<i>L</i>	<i>E</i>	883
<i>A</i>	<i>T</i>	<i>N</i>	<i>G</i>	<i>F</i>	<i>H</i>	<i>N</i>	<i>D</i>	<i>S</i>	<i>L</i>	<i>V</i>	<i>G</i>	<i>S</i>	<i>G</i>	<i>G</i>	<i>F</i>	<i>G</i>	<i>D</i>	<i>V</i>	<i>Y</i>	903
<i>K</i>	<i>A</i>	<i>Q</i>	<i>L</i>	<i>K</i>	<i>D</i>	<i>G</i>	<i>S</i>	<i>V</i>	<i>V</i>	<i>A</i>	<i>I</i>	<i>K</i>	<i>K</i>	<i>L</i>	<i>I</i>	<i>H</i>	<i>V</i>	<i>S</i>	<i>G</i>	923
<i>Q</i>	<i>G</i>	<i>D</i>	<i>R</i>	<i>E</i>	<i>F</i>	<i>T</i>	<i>A</i>	<i>E</i>	<i>M</i>	<i>E</i>	<i>T</i>	<i>I</i>	<i>G</i>	<i>K</i>	<i>I</i>	<i>K</i>	<i>H</i>	<i>R</i>	<i>N</i>	943
<i>L</i>	<i>V</i>	<i>P</i>	<i>L</i>	<i>L</i>	<i>G</i>	<i>Y</i>	<i>C</i>	<i>K</i>	<i>V</i>	<i>G</i>	<i>E</i>	<i>E</i>	<i>R</i>	<i>L</i>	<i>L</i>	<i>V</i>	<i>Y</i>	<i>E</i>	<i>Y</i>	963
<i>M</i>	<i>K</i>	<i>Y</i>	<i>G</i>	<u><i>S</i></u>	<i>L</i>	<i>E</i>	<i>D</i>	<i>V</i>	<i>L</i>	<i>H</i>	<i>D</i>	<i>R</i>	<i>K</i>	<i>K</i>	<i>I</i>	<i>G</i>	<i>I</i>	<i>K</i>	<i>L</i>	983
<i>N</i>	<i>W</i>	<i>P</i>	<i>A</i>	<i>R</i>	<i>R</i>	<i>K</i>	<i>I</i>	<i>A</i>	<i>I</i>	<i>G</i>	<i>A</i>	<i>A</i>	<i>R</i>	<i>G</i>	<i>L</i>	<i>A</i>	<i>F</i>	<i>L</i>	<i>H</i>	1003
<i>H</i>	<i>N</i>	<i>C</i>	<i>I</i>	<i>P</i>	<i>H</i>	<i>I</i>	<i>I</i>	<i>H</i>	<i>R</i>	<i>D</i>	<i>M</i>	<i>K</i>	<i>S</i>	<i>S</i>	<i>N</i>	<i>V</i>	<i>L</i>	<i>L</i>	<i>D</i>	1023
<i>E</i>	<i>N</i>	<i>L</i>	<i>E</i>	<i>A</i>	<i>R</i>	<i>V</i>	<i>S</i>	<i>F</i>	<i>G</i>	<i>M</i>	<i>A</i>	<i>R</i>	<i>L</i>	<i>M</i>	<u><i>S</i></u>	<i>A</i>	<i>M</i>	<i>D</i>	<i>1043</i>	
<i>T</i>	<i>H</i>	<i>L</i>	<u><i>S</i></u>	<u><i>V</i></u>	<u><i>S</i></u>	<u><i>T</i></u>	<i>L</i>	<i>A</i>	<u><i>G</i></u>	<u><i>T</i></u>	<i>P</i>	<i>G</i>	<i>Y</i>	<i>V</i>	<i>P</i>	<i>E</i>	<i>Y</i>	<i>Y</i>	<i>1063</i>	
<i>Q</i>	<i>S</i>	<i>F</i>	<i>R</i>	<i>C</i>	<i>S</i>	<i>T</i>	<i>K</i>	<i>G</i>	<i>D</i>	<i>V</i>	<i>Y</i>	<i>S</i>	<i>Y</i>	<i>G</i>	<i>V</i>	<i>L</i>	<i>L</i>	<i>E</i>	<i>1083</i>	
<i>L</i>	<i>L</i>	<i>T</i>	<i>G</i>	<i>K</i>	<i>Q</i>	<i>P</i>	<i>T</i>	<i>D</i>	<i>S</i>	<i>A</i>	<i>D</i>	<i>F</i>	<i>G</i>	<i>D</i>	<i>N</i>	<i>N</i>	<i>L</i>	<i>V</i>	<i>G</i>	1103
<i>W</i>	<i>V</i>	<i>K</i>	<i>L</i>	<i>H</i>	<i>A</i>	<i>K</i>	<i>G</i>	<i>K</i>	<i>I</i>	<i>T</i>	<i>D</i>	<i>V</i>	<i>F</i>	<i>D</i>	<i>R</i>	<i>E</i>	<i>L</i>	<i>L</i>	<i>K</i>	1123
<i>E</i>	<i>D</i>	<i>A</i>	<i>S</i>	<i>I</i>	<i>E</i>	<i>I</i>	<i>E</i>	<i>L</i>	<i>L</i>	<i>Q</i>	<i>H</i>	<i>L</i>	<i>K</i>	<i>V</i>	<i>A</i>	<i>C</i>	<i>A</i>	<i>C</i>	<i>L</i>	1143
<i>D</i>	<i>D</i>	<i>R</i>	<i>H</i>	<i>W</i>	<i>K</i>	<i>R</i>	<i>P</i>	<i>T</i>	<i>M</i>	<i>I</i>	<i>Q</i>	<i>V</i>	<i>M</i>	<i>A</i>	<i>M</i>	<i>F</i>	<i>K</i>	<i>E</i>	<i>I</i>	1163
<i>Q</i>	<i>A</i>	<i>G</i>	<i>S</i>	<i>G</i>	<i>M</i>	<i>D</i>	<i>S</i>	<i>T</i>	<i>S</i>	<i>T</i>	<i>I</i>	<i>G</i>	<i>A</i>	<i>D</i>	<i>D</i>	<i>V</i>	<i>N</i>	<i>F</i>	<i>S</i>	1183
<i>G</i>	<i>V</i>	<i>E</i>	<i>G</i>	<i>G</i>	<i>I</i>	<i>E</i>	<i>M</i>	<i>G</i>	<i>I</i>	<i>N</i>	<i>G</i>	<i>S</i>	<i>I</i>	<i>K</i>	<i>E</i>	<i>G</i>	<i>N</i>	<i>E</i>	<i>L</i>	1203
<i>S</i>	<i>K</i>	<i>H</i>	<i>L</i>	- 1207																

Figure 2. Summary of *in vitro* autophosphorylation sites of tBRI1-CD. Juxtamembrane and C-terminal regions are italicized and shown in blue and brown, respectively. The six confirmed *in vitro* phosphorylation sites are marked red and underlined. The kinase domain activation loop is marked with brackets, and an additional three sites of phosphorylation occur within this activation loop, but their exact assignment among the five residues in red is currently unclear.

Figure 3. Product (b and y ions) ion spectra of *in vitro* identified phosphorylation sites of tBAK1-CD using Q-TOF LC/MS/MS or Q-TOF LC/MS^E analysis. MBP- tBAK1-CD was *in vitro* autophosphorylated and tryptic peptides of MBP-tBAK1-CD were analyzed by using a Waters NanoAcquity Premier Q-TOF mass spectrometer. All the site assignments were also verified by manual inspection. Phosphorylation sites are enclosed within parenthesis and highlighted red. **(A)** Q-TOF LC/MS/MS product ion spectrum identifying the residue corresponding to Arabidopsis BAK1 S-465 as an *in vitro* phosphorylation site in MBP-tBAK1-CD. **(B)** Q-TOF LC/MS/MS product ion spectrum identifying the residue corresponding to Arabidopsis BAK1 T-449 as an *in vitro* phosphorylation site in MBP-tBAK1-CD. **(C)** Q-TOF LC/MS^E product ion spectrum identifying residue corresponding to Arabidopsis BAK1 T-455 as an *in vitro* phosphorylation site in MBP-tBAK1-CD. **(D)** Q-TOF product ion spectrum identifying the residue corresponding to Arabidopsis BAK1 S-286 as an *in vitro* phosphorylation site in MBP-tBAK1-CD. **(E)** Q-TOF product ion spectrum identifying one of the two residues corresponding to Arabidopsis BAK1 residues, T-286 or S-290, enclosed within parenthesis, as an *in vitro* phosphorylation site in MBP-tBAK1-CD. **(F)** Q-TOF LC/MS^E product ion spectrum identifying the residue corresponding to Arabidopsis BAK1 S-290 as an *in vitro* phosphorylation site in MBP-tBAK1-CD. **(G)** Q-TOF product ion spectrum identifying one of the three residues corresponding to Arabidopsis BAK1 residues, T-446, T-449, or T-450, enclosed within parenthesis, as an *in vitro* phosphorylation site in MBP-tBAK1-CD. **(H)** Q-TOF product ion spectrum identifying one of the two residues corresponding to Arabidopsis BAK1 residues, T-449 or T 450, enclosed within parenthesis, as an *in vitro* phosphorylation site in MBP-tBAK1-CD.

A. LC/MS/MS Fragmentation of GTIGHIAPEYL(pS)TGK



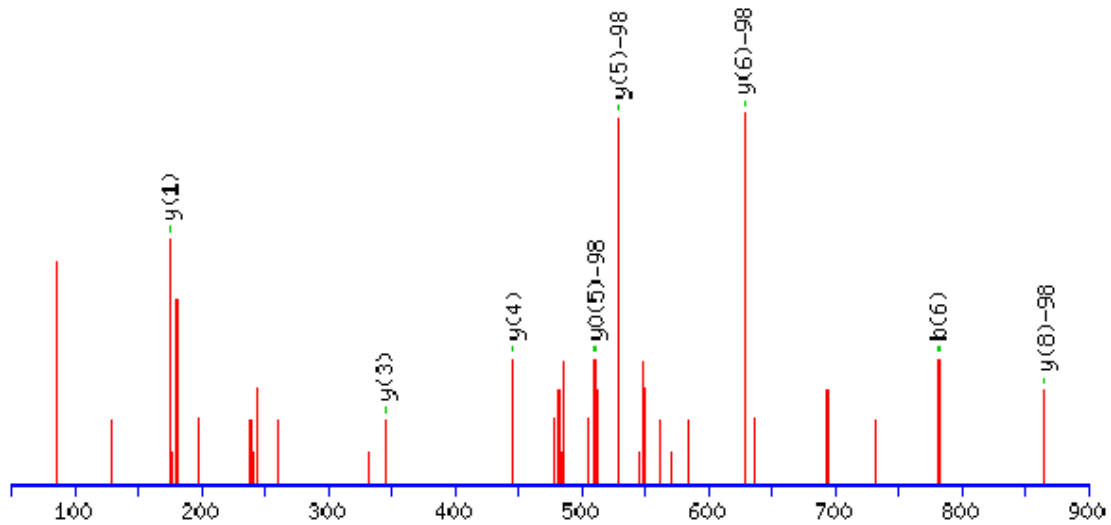
Monoisotopic mass of neutral peptide Mr(calc): 1622.7705

Variable modifications:

S12 : Phospho (ST), with neutral losses 97.9769

Ions Score: 44 **Expect:** 0.0003

B. LC/MS/MS Fragmentation of LMDYKDTHV(pT)TAVR



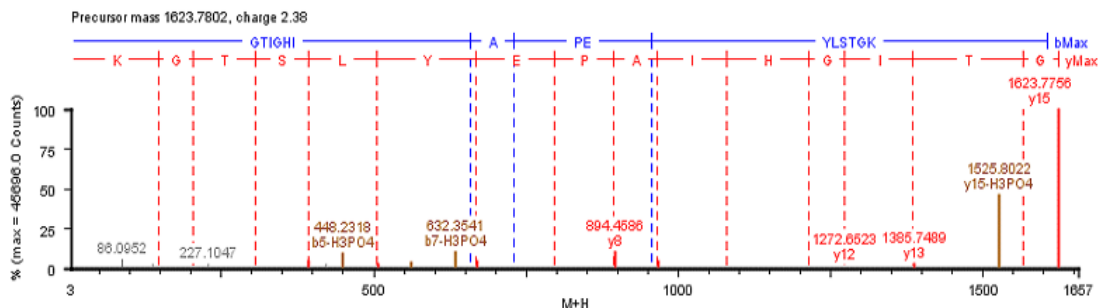
Monoisotopic mass of neutral peptide Mr(calc): 1744.7855

Variable modifications:

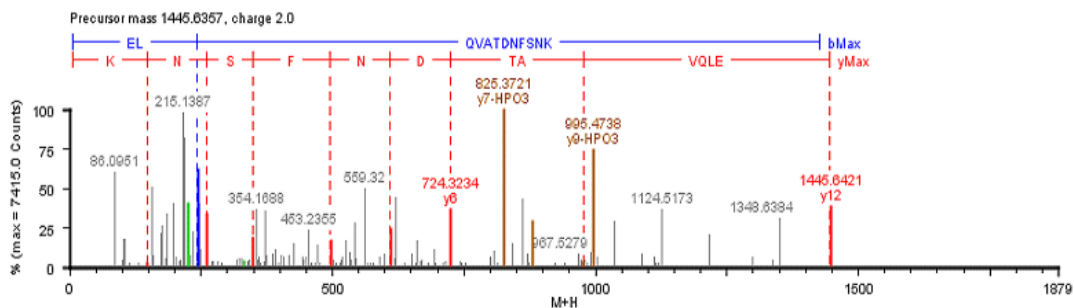
T10 : Phospho (ST), with neutral losses 97.9769

Ions Score: 50 **Expect:** 9.6e-05

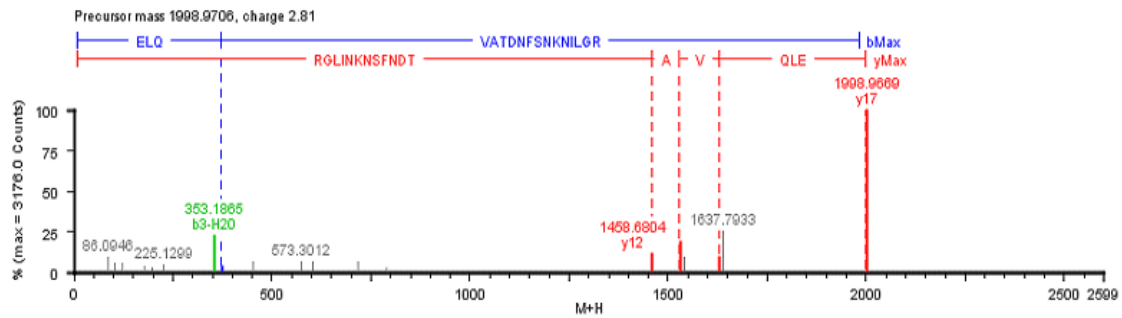
C. LC/MS^E Fragmentation of G(pT)IGHIAPEYLSTGK (PLGS Score 10.29)



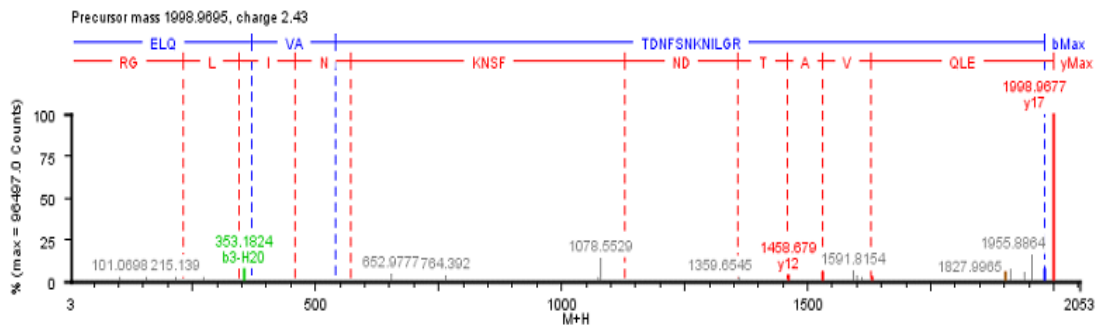
D. LC/MS^E Fragmentation of GELQVA(pI)DNFSNK (PLGS Score 7.44)



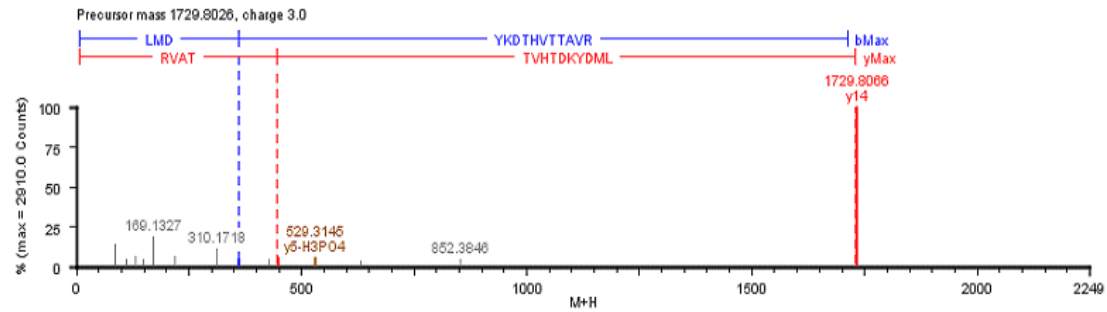
E. LC/MS^E Fragmentation of ELQVA(pTDNFpS)NKNILGR (PLGS Score 8.55)



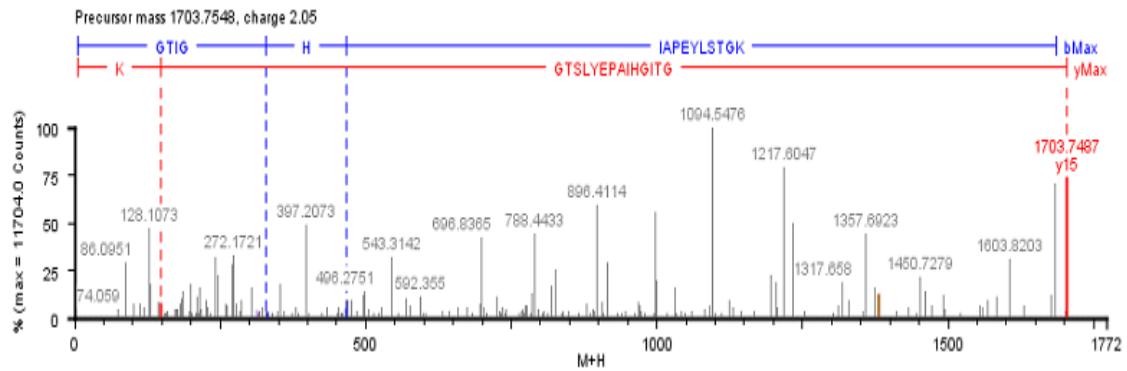
F. LC/MS^E Fragmentation of ELQVATDNF(pS)NKNILGR (PLGS Score 7.78)



G. LC/MS^E Fragmentation of LMDYKD(pTHVpTpT)AVR (PLGS Score 8.24)



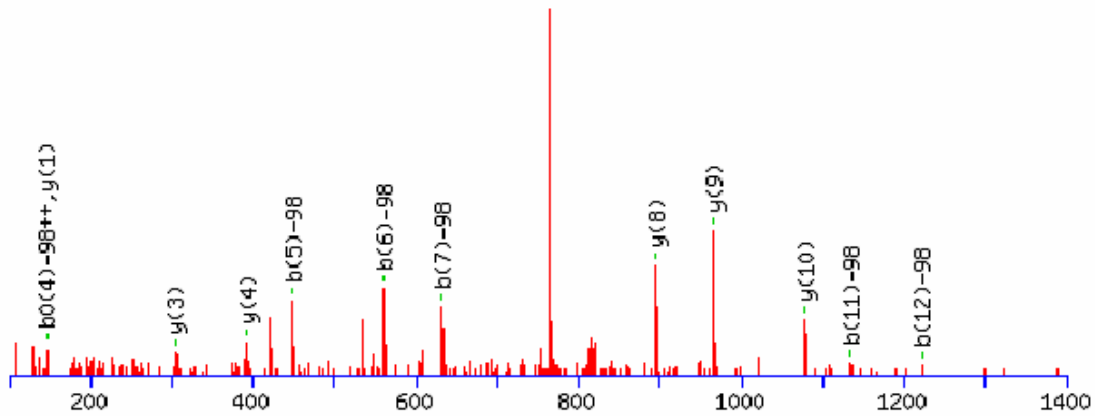
H. LC/MS^E Fragmentation of GTIGHIAPEYL(pSpT)GK (PLGS Score 6.43)



Arab.	WRRKKPQDHFFDVPAEEDP EVHLGQLKRFS LRELQVA <u>S</u> DNF <u>S</u> NKNILGRGGFGKVYKGRL	308
Tomato	FRRRKPEDHFFDVPAEEDP EVHLGQLKRFS LRELQVA <u>T</u> DNFSNKNILGRGGFGKVYKGRL	308
Arab.	ADG <u>T</u> LVAVKRLKEERTQGGELQFQTEVEMI SMAVHRNLLRLRGFCMTPTERLLVYPYMAN	368
Tomato	ADG <u>S</u> LVAVKRLKEER <u>T</u> QGGELQFQTEVEMI SMAVHRNLLRLWGFMTATERLLVYPYMAN	368
Arab.	GSVASCLRERPESQPPLDWPKRQRIALGSARGLAYLHDHCDPKIIHRDVKAANILLDEEF	428
Tomato	GSVASRLRERPESDPPLGWPIRKICIALGSARGLAYLHDHCDPKIIHRDVKAANILLDEEY	428
Arab.	EAVVGDFGLAKLMDYKD <u>THV</u> <u>TT</u> AVRG <u>T</u> IGH IAPEYLS T GKSS EKTDVFGYGVMLLELITG	488
Tomato	EAVVGDFGLAKLMDYKD <u>THV</u> <u>T</u> AVRG <u>T</u> IGH IAPEYL S TGKSS EKTDVFGYGVMLLELITG	488
Arab.	QRAFDLARLANDDDVMLLDWVKGLLKEKKLEALVDVDLQGNYKDEEVEQLIQVALLCTQS	548
Tomato	QRAFDLARLANDDDVMLLDWVKGLLKEKY ETLVDADLQGNYN EEEE VKLIQVALLCTQS	548
Arab.	SPMERPKMSEVVRMLEGDGLAERWEEWQKE EMFRQDFNYPTHHPAVSGWIIIGDSTSQIEN	608
Tomato	SPMERPKMSEVVRMLEGDGLAERWEEWQKE EMFRQDFNH-AHHPHTD-WIIADSTYNLRP	606
Arab.	EYPSGPR- 615	
Tomato	DELSGPR- 613	

Figure 4. Sequence alignment of tomato and Arabidopsis BAK1 cytoplasmic domains with all possible phosphorylation sites indicated in red for tomato and orange for Arabidopsis. Tomato BAK1 transphosphorylation site is underlined.

LC/MS/MS Fragmentation of G(pT)IGHIAPEYLSTGK



Monoisotopic mass of neutral peptide Mr(calc): 1744.7855

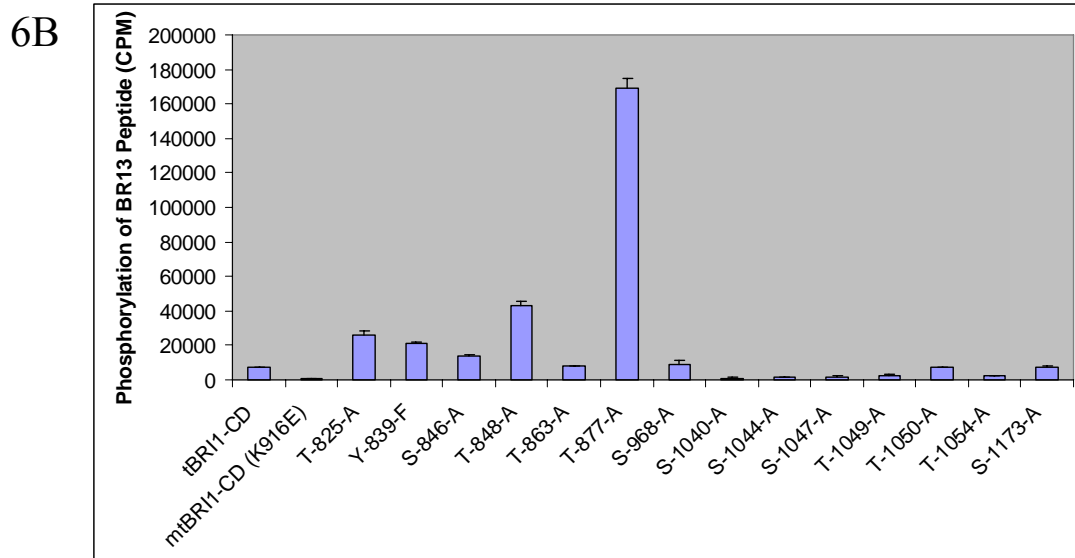
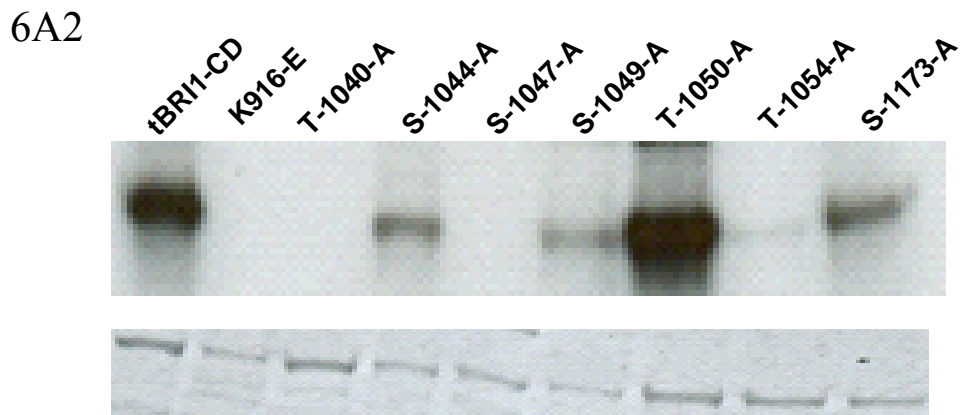
Variable modifications:

T2 : Phospho (ST), with neutral losses 97.9769

Ions Score: 28

Figure 5. Product (b and y ions) ion spectrum of an *in vitro* identified transphosphorylation site of tBAK1-CD by tBRI1-CD using Q-TOF LC/MS/MS. MBP- mtBAK1-CD (K317E) was incubated with FLAG-tBRI1-CD *in vitro* and autophosphorylated. Tryptic peptides of MBP-mtBAK1-CD (K317E) were analyzed with a Waters NanoAcquity Premier Q-TOF mass spectrometer. The tomato BAK1 residue corresponding to Arabidopsis BAK1 S-455 is an *in vitro* transphosphorylation site.

Figure 6. Effect of mutating specific S and T residues of FLAG-tBRI1-CD on *in vitro* autophosphorylation and substrate phosphorylation. **(A)** Autoradiograph showing autophosphorylation of recombinant FLAG-tBRI1-CD (wild type) and a range of mutants, including constructs in which specific S and T residues are mutated to A by site-directed mutagenesis, and a kinase-inactive mutant (mtBRI1-CD(K 916 E)). Equal loading amounts of recombinant protein (as shown by the stained gel in the bottom panel) were incubated with [γ - 32 P] ATP and separated by SDS-PAGE, followed by autoradiography (top panel). The experiment was repeated three times with consistent results. **(B)** Phosphorylation of a synthetic peptide (BR13) containing the consensus sequence for optimum Arabidopsis BRI1-CD substrate phosphorylation. A typical 20- μ L reaction mixture contained 0.5 μ g of affinity-purified FLAG-tBRI1-CD, 0.8 μ ci [γ - 32 P] ATP (888 cpm/pmol), and 0.10 mg/mL of synthetic peptide in kinase buffer. Reactions were incubated for 20 min at room temperature and incorporation of 32 P into the synthetic peptide was quantified by binding to P81 phosphocellulose paper, followed by liquid scintillation spectrometry. Error bars are SE, $n = 3$.



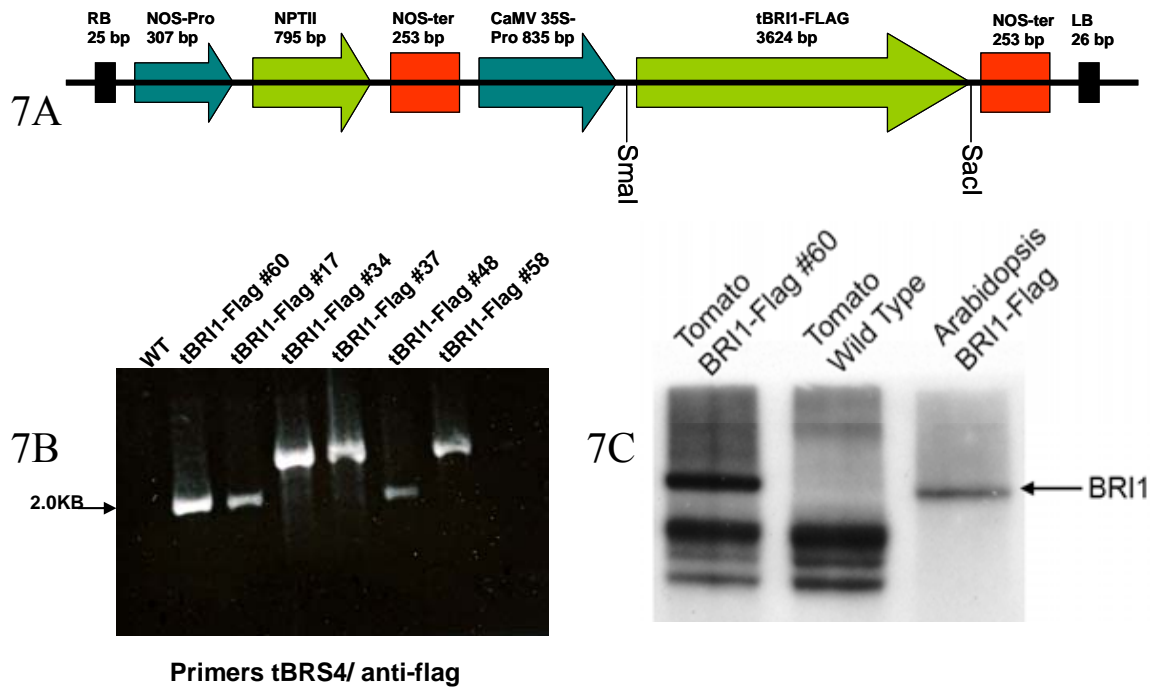


Figure 7. Cloning of 35S::tBR1-FLAG into plant transformation vector and analysis of the transgenic lines. (A) Map of the binary vector pBI121 showing restriction enzymes, *SmaI* and *SacI* that were used for cloning tBR11-FLAG (B) Gel picture of PCR products from six independent transgenic lines, performed to verify the expression of the transgene in different transgenic lines. The positive band corresponding to the size (2.0 kb) of *FLAG-tBR11-1* transgene is indicated (C) Western blot analysis to identify protein in tomato transgenic line # 60 overexpressing tBR11-FLAG using anti-FLAG antibody. A band corresponding to the size (130 KDa) of Arabidopsis BRI1 (used as positive control) is observed in tomato line # 60

8A

tBri1_Flag Coverage Map

1	MQARETYFRQ	HPLSLMKLFF	VLLLIFFLPP	ASPAASVNGI	YQDSQQLLSF
51	KAALFFIPTL	LQHWLSSTGP	CSFTGVSKRN	SRVSSIDLSN	TFLSYDFSLV
101	TSYLLPLSNL	ESLVKMANL	SGSLTSAAKS	QCQVTLDSID	LAENTISGFI
151	SDISSFGVCS	NLKSIMLSKN	FLDPPGCEML	KAATFSLQVL	DLSYMNISGF
201	NLFFWVSSMG	FVELEFFSLK	GKLAGSIFE	LDFENLSYLD	LSAMNFSTVF
251	PSFKDCSNLQ	HLDLSSNKFY	GDIGSSLSSC	GKLSFLNLTN	NQFVGLVPKL
301	PSSELQYLVL	RGMDFGGVYP	NQLADLCKTV	VELDLSYNNF	SGNVFESLGE
351	CSSELEVDIS	YMNFGKLPV	DTLSKLSNIK	TMVLSFNKPY	GGLPDSFSNL
401	LKLETLDMS	NNLTGVIPSG	ICEDFMNLE	VLYLQNNLPE	GPIPDLSLNC
451	SQIVSLDLSF	MYLTGSIPTS	LGSLSKLQDL	ILWLNQLSGE	IQQLMYLQA
501	LEMILDFND	LTGPIPASLS	NCTFLNHSIL	SNNQLSGEIP	ASLGRLSNLA
551	ILFLGNNSIS	GNIPAELGNC	QSLINWDLMT	NFLNGSIPFP	LFRQSGNIAY
601	ALLTGERVYV	IKMDGSKECH	GAGNLEFGG	IRQEQDRIS	TRHPCMFTRV
651	YRGITQPTFN	HMGSMIFLDL	SYNKEGSIPT	KELGAMYYLS	ILNLGNDLS
701	GMIQQQLGGL	KMVAILDLSY	NRFDGTFIMS	LTSITLIGEI	DLSMNNLSGM
751	IPESAPFDTF	PQYRFAMNSL	CGYPLIPCS	SGPKSDANQH	QKSHRQASL
801	AGSVANGLLF	SLFCIFGLII	VAIETKCFRR	KKEAALEAYH	DGSHSHTAN
851	SAWKFTSARE	ALSINLAATP	YPIRELTTFAD	LLEATNGFMN	DSLVGGGGFG
901	DVYKAGLKDQ	SVVAEKCLIH	VSGQGDREFT	AENETIGKIK	HRNLVPLLCY

8B

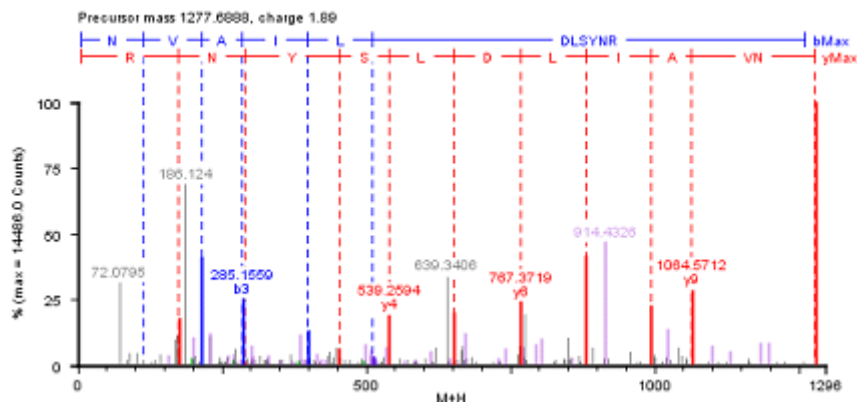


Figure 8. Identification of *in vivo* tBRI1-FLAG using Q-TOF LC/MS^E analysis (A)

Coverage map of the identified tBRI1-FLAG peptides (percent coverage of tBRI1-FLAG protein was 51.7%). (B) Q-TOF LC/MS^E product ion spectrum of one of the identified tBRI1-FLAG peptides.

9A



9B



Figure 9. Phenotypic comparison of wild-type (WT) cv Cobra vs tBRI1-FLAG overexpressing line. (A) Picture of 25 day old WT plant with serrations in the leaves. (B) Picture of 30 day old 35S::tBRI1-FLAG line # 60 plant with more ovoid leaves with very less serrations on the leaves.

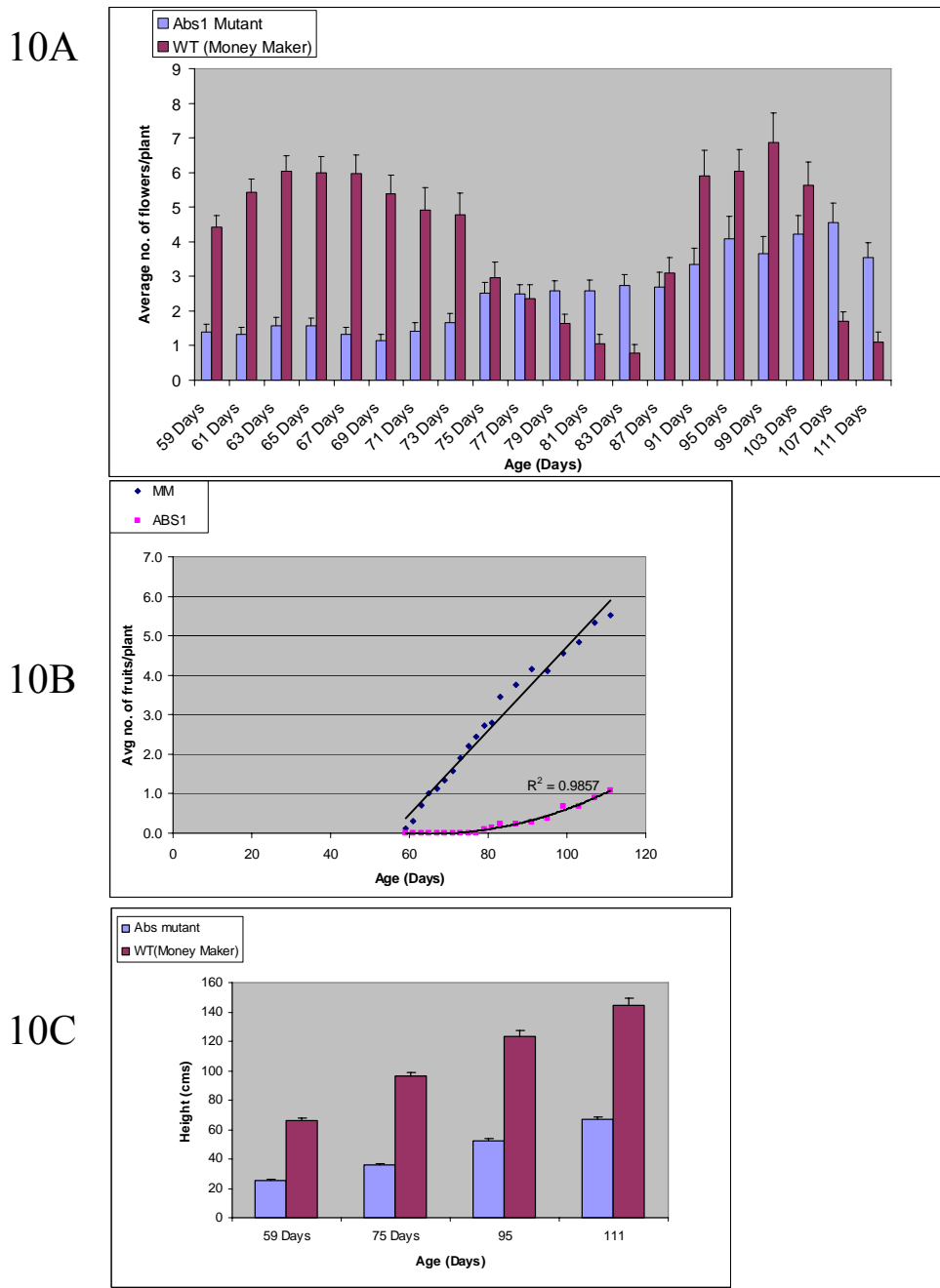


Figure 10. Growth properties of wild-type (WT) vs. *curl3^{abs1}* mutant plants as measured over 111 days. **(A)** Average number of flowers per plant. **(B)** Average number fruits per plant. **(C)** Average height per plant. Error bars are SE, n = 33.

Figure 11. Effect of mutating T-1054 residue of the tBRI1 cytoplasmic domain on rescue of the *bri1-5* mutant. Transgenic constructs 35S::tBRI1-FLAG (*tBRI1-FLAG/ curl3^{abs1}*) and 35S::tBRI1-FLAG, carrying mutation T-1054-A (*T-1054-A/ curl3^{abs1}*) were transformed into the *curl3^{abs1}* background. All lines were grown under the same greenhouse conditions and are the same age (50 d). **(A)** Phenotypic comparison of *curl3^{abs1}* and *T-1054-A/ curl3^{abs1}* expressing independent transgenic lines. **(B)** Phenotypic comparison of WT, *tBRI1-FLAG/ curl3^{abs1}curl3^{abs1}*, and *T-1054-A/ curl3^{abs1}*, independent lines.

11A



**T-1054-A/
*curl3-^{abs1}***

curl3-^{abs1}

11B



WT

***tBRI1-FLAG/
*curl3-^{abs1}****

curl3-^{abs1}

**T-1054-A/
*curl3-^{abs1}***

Chapter Four: Cloning and Biochemical Properties of Tomato TRIP-1

Introduction

Brassinosteroids (BRs) are polyhydroxylated plant steroid hormones that are essential for a variety of developmental programs including cell elongation, vascular differentiation, seed germination, fertility and senescence (reviewed by Clouse and Sasse, 1998; Mandava, 1988; Sasse, 2003). In contrast to animal steroid hormones, which are primarily perceived by nuclear receptors, BRs are perceived at the cell surface by the receptor kinase BRI1 (BRASSINOSTEROID-INSENSITIVE 1) (Clouse, 2002). The BR signaling pathway in plants has similarities with both animal receptor tyrosine kinase and transforming growth factor- β (TGF- β) receptor mediated signaling. The TGF- β family receptors regulate proliferation, lineage determination, cell differentiation and cell death, and therefore play a critical role in the development and homeostasis of organisms from fruit flies to humans (Massague, 1998). Similar to BRI1/BAK1 heterodimerization, TGF- β receptors form a heterotetrameric complex between type I (RI) and type II (RII) receptor kinase pairs. Binding of the TGF- β peptide ligand to RII initiates the heterotetrameric complex formation between RI and RII, which results in trans-phosphorylation of RI by RII on specific Ser/Thr residues. The activation of RI by RII leads to phosphorylation of the Smad proteins, which then move into the nucleus, associate with transcription factors and thus result in altered gene expression (Massague, 1998; Shi and Massague, 2003). In addition to Smads, the RI protein also interacts with other cellular proteins, which include a WD-domain protein known as, TGF- β Receptor Interacting Protein-1 (TRIP-1) (Chen et al., 1995; Choy and Derynck, 1998).

WD-domain proteins are a large family of eukaryotic proteins whose members contain highly conserved repeating units usually ending with Trp-Asp (WD). This conserved domain generally repeats four to eight times in each polypeptide (reviewed by Neer et al., 1994). The WD-repeat domain, also known as the WD-40 repeat or GH-WD repeat was first identified in the β -subunit of hetero-trimeric GTP-binding proteins (G proteins) (Fong, et al., 1986). WD-domain proteins regulate numerous important cellular functions such as cell division, cell-fate determination, gene transcription, transmembrane signaling, mRNA modification and vesicle fusion. They are also involved in protein–protein interaction, and WD proteins often exist in multi-subunit protein complexes, interacting with other proteins via WD-repeats that form circular structures known as β -propellers (Neer et al., 1994; Ehsan et al., 2005; Jiang and Clouse, 2001)

TRIP-1 (also known as eIF3i) was also identified as a subunit of eukaryotic translation initiation factor 3 (eIF3), giving it a dual function in signaling and eukaryotic translation initiation (Asano et al., 1997). eIF3 is the largest initiation factor known, composed of more than 10 subunits and having a molecular weight of 600 kDa. TRIP-1 (eIF3i) is one of the core subunits of eIF3 in eukaryotes (Burks et al., 2001). eIF3 plays an essential role in initiation of protein synthesis and is required for binding of mRNA to 40S ribosomal subunits, maintaining stability of ternary complex binding to 40S subunits, and dissociation of 80S ribosomes (Jiang and Clouse, 2001; Hu et al., 2008; Browning, 2004; Hinnebusch, 2006).

Jiang and Clouse (2001) identified TRIP-1 homologs in bean and Arabidopsis. The antisense suppression of *TRIP-1* in Arabidopsis resulted in extremely dwarfed plants with

altered leaf morphology, delayed senescence and bushy phenotype resembling in some aspects the phenotype of BR-insensitive or BR-deficient plants (Jiang and Clouse, 2001). Ehsan et al. (2005) showed there was *in vivo* interaction between Arabidopsis TRIP1 and BRI1. They also demonstrated that BRI1 can transphosphorylate TRIP-1 on three residues (T14, T89, and T197 or S198) *in vitro*, providing evidence for TRIP-1 being a putative *in vivo* cytoplasmic substrate of BRI1 that is required in BR signaling.

The objective of this study was to clone the tomato ortholog of Arabidopsis *TRIP-1* and to begin characterization of its biochemical role in BR signal transduction. We report here the cloning of the tomato *TRIP-1* (*tTRIP-1*) gene. Kinase assays revealed that tTRIP-1 can be transphosphorylated *in vitro* by both tomato BRI1 (tBRI1) and tomato BRI1 ASSOCIATED RECEPTOR KINASE 1 (tBAK1) cytoplasmic kinase domains (CDs). Tomato BRI1-CD can also phosphorylate synthetic peptides that contain Arabidopsis TRIP-1 (AtTRIP-1) sequences shown previously to be phosphorylated by BRI1. Liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis identified a putative tBRI1 *in vitro* transphosphorylation site in tTRIP1. PCR analysis verified the presence of the epitope tagged *tTRIP-1* transgene in transgenic tomato lines.

Materials and Methods

Expression and Purification of Recombinant tTRIP-1 Protein

Total RNA was isolated from 100 mg of leaves from wild type tomato plants (cv. ‘Cobra’) by grinding in liquid N₂ followed by RNA purification using the RNeasy kit (Qiagen, Valencia, CA). One µg of total RNA was used for first strand cDNA synthesis using the ImProm-II Reverse Transcription system with oligo-dT primer (Promega, Madison,

WI). One μL of cDNA was used in a 20 μL PCR reaction, containing buffer [4 μL 10X pfx amplification buffer (2X final conc.)], 1 mM MgSO_4 , 300 μM dNTPs, 50 ng of tomato genomic DNA, 5 μM of forward primer 5'-cgataagcttaggccaatattgatgaaggcc-3' and reverse primer 5'-gactctcgagctaaatcctgatgttgaagtaatcc -3' primer, plus 5 units of cloned pfx DNA polymerase (Invitrogen, Carlsbad, CA). After pre-incubation at 94°C for 2 min, 31 cycles (of 94°C, 20 s; 58°C, 30 s; 68°C, 60s) were performed followed by an extension step of 7 min at 68°C to amplify the *tTRIP1* sequence. The PCR product was gel-purified using the QIAquick Gel Extraction Kit (Qiagen). The purified PCR product was digested with *HindIII/XhoI* (Promega, Madison, WI), gel-purified and ligated with *HindIII/XhoI* digested, and gel-purified pFLAG-MAC (Sigma, St. Louis, MO) to obtain FLAG-*tTRIP1*. The construct was confirmed by DNA sequencing.

The FLAG-tBRI-CD construct was transformed into *E. coli* BL 21 (DE3) cells (Stratagene, La Jolla, CA). Expression of the recombinant protein was induced by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and cells were incubated for 4 h at 30°C in LB medium.

The cell pellet was frozen at -20°C overnight, and lysis buffer [20 mM Tris-HCl, pH 7.4; 200 mM NaCl; 1mM EDTA, 0.25 mg/ml lysozyme] was added to the frozen pellet. The pellet was solubilized in lysis buffer using a paintbrush and incubated at room temperature for 5 min, followed by sonication for 2.5 min. Samples were centrifuged for 20 min at 9000 \times g after sonication and the supernatant was transferred to new tubes. Flag-tagged protein was isolated by incubating the soluble total protein extract was FLAG M₂ agarose beads at 4°C overnight on a rotating mixer. FLAG elution buffer [20 mM Tris-HCl, pH 7.4;

200 mM NaCl; 1 mM EDTA, 0.25 mg/ml FLAG peptides] was used for elution of FLAG-tTRIP-1. The purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue (CBB) staining.

Transphosphorylation Assay

For tTRIP-1 transphosphorylation assays a 40 μ L reaction containing 1 μ g each of affinity purified recombinant kinase CDs was added to 5 μ g of affinity purified FLAG-tTRIP-1 and incubated with 20 μ Ci of [γ - 32 P] ATP in kinase buffer (50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-KOH, pH 7.9, 10 mM MnCl₂, 1.0 mM dithiothreitol, and 0.2 mM unlabeled ATP). The samples were incubated at room temperature for 1 h. Reactions were terminated by adding 20 μ L of 2X Laemmli loading buffer (Laemmli, 1970) and analysed by 10% (w/v) SDS-PAGE and autoradiography.

Peptide Substrate Assay

Peptide substrate assays were performed as described (Oh, et al., 2000) with some changes in reaction volumes and protein amounts. 20 μ L reactions contained 0.10 mg/mL synthetic peptide, 0.5 μ g FLAG-tBRI1-CD, 0.8 μ Ci [γ - 32 P] ATP and 0.1 mM unlabelled ATP in a kinase buffer consisting of 50 mM MOPS [3-(*N*-morpholino) propanesulfonic acid], pH 7.4; 10 mM MgCl₂, and 0.2 mM CaCl₂. Following a 20-min incubation at room temperature, 20 μ L of the reaction solution was spotted onto a 2 \times 2-cm piece of P81 phosphocellulose paper. The paper was washed four times in 75 mM H₃PO₄ (4 min per wash) and 32 P incorporation into the peptide was determined by liquid scintillation counting of the washed squares. Each reaction point was determined in triplicate, and the results are presented as means \pm SE. The sequence of the peptides substrates are follow:

Trip 1 – GKLIKESDKESGHKK

Trip 2 – SGHKKTVTSLAKSA

Trip 3 – DGSHFLTGSLDKSA

Trip 5 – SGKELFTFKFNAPTR

Determination of in vitro Transphosphorylation Sites of tTRIP-1

Affinity purified recombinant FLAG-tBRI1-CD (1 μ g) and FLAG-tTRIP-1 (5 μ g) were incubated together for 1h at room temperature in kinase buffer [50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-KOH, pH 7.9, 10 mM $MnCl_2$, 1.0 mM dithiothreitol, and 2.0 mM unlabeled ATP] and then separated by electrophoresis on a 10% Bis-Tris NU-PAGE gel (Invitrogen).

The tTRIP-1 protein bands were excised with a blade. In-gel reduction, alkylation and trypsin digestion was performed on the excised bands as described (Rowley et al., 2000). After digestion, peptides were extracted in buffer containing 5% (v/v) formic acid; 50% (v/v) acetonitrile; and 45% (v/v) 50mM NH_4HCO_3 . The peptide samples were filtered after extraction and then injected into a nanoACQUITY Ultra Performance LC (UPLC) coupled to a Premier Q-ToF mass spectrometer (Waters Corporation, Milford, MA) equipped with a nanolockspray ion source (Waters). The injected peptides were first desalted and concentrated on a 300 μ m x 1 cm C18 trapping column at a flow rate of 10 μ l/min. The trapped peptides were then separated by passage through an in-line 25cm x 75 μ m Bridged Ethyl Hybrid (BEH) C18 matrix column (Waters) using mobile phase A=0.1% formic acid in water and B=0.1% formic acid in acetonitrile for the gradient. The peptides were eluted at flow rate of 300nl/min for 60 min with a gradient of 7%B to 40%B. After the

sample run, the C18 BEH columns were equilibrated at initial conditions (2% mobile phase B) for 22 min prior to injecting the next sample.

Phosphopeptide Enrichment by Immobilized Metal Ion Affinity Chromatography (IMAC)

Peptides extracted after in-gel trypsin digestion were enriched for phosphopeptides using Phos-select Iron affinity gel (Sigma). The peptides were dried completely vacuum centrifugation and resuspended in 50 μ L of resuspension/ wash buffer [250 mM glacial acetic acid, 30% (v/v) acetonitrile]. Ten μ L of Phos-select resins prewashed with wash buffer was added to the peptide sample. The sample was incubated at room temperature for 1 h on a shaker. After incubation, the resins were collected by centrifugation, washed extensively using wash buffer, and eluted with 50 μ L of basic elution buffer containing 400 mM of NH_4OH .

Data Analysis for Phosphopeptide Identification

The peptide samples were analyzed by LC/MS/MS on the Q-ToF Premier in data dependent acquisition (DDA) mode (Blackburn et al., 2010). The top eight most intense ions in each survey scan were selected and subjected to collision-induced dissociation (CID) to obtain MS/MS spectra. A predigested standard, rabbit phosphorylase B, was run between samples to verify instrument sensitivity and performance. The raw data files obtained from LC/MS/MS analysis were searched against an *E. coli* database with the addition of tomato MBP-BRI1 and tTRIP-1 protein sequences, using the Mascot 2.2 search algorithm (Matrix Science, London, UK) running on an in-house server. The dynamic mass modification of 80 Da for phosphorylation on Ser, Thr and Tyr residues was searched.

Cloning of Tomato *TRIP-1* gene into Plant Transformation Vector

A small portion of the FLAG-tTRIP-1 ligation reaction (from FLAG-tTRIP-1/pFLAG-MAC) was PCR amplified with the primers 5'-cgatggatccatggactacaaggacgacgatgac-3' and 5'-gactgagctcctaaatcctgatgttgaagtaac-3'. This PCR product was gel-purified, digested with *BamHI* and *SacI* and introduced into the binary vector pBI121 (Clontech, Palo Alto, CA, USA) that had also been digested with *BamHI/SacI* and gel purified to remove the *uidA* gene. The resulting plasmid (35S: FLAG-tTRIP1/pBI121) was transformed into *Agrobacterium* strain GV3101.

Another pair of primers, forward 5'-cgatcccgggagttttatttttaattttcttcaaatacttccatcatgaggccaatattgatgaagg-3' and reverse 5'-gactgagctcctattgtcatcgtcgtcctttagtcaatcctgatgttgaagtaatcc-3' were used to amplify tTRIP1-FLAG from tomato cDNA. This PCR product was gel-purified, digested with *SmaI* and *SacI* and introduced into the binary vector pBI121 (Clontech) that had also been digested with *SmaI/SacI* and gel purified to remove the *uidA* gene. The resulting plasmid (35S:tTRIP1-FLAG/pBI121) was transformed into *Agrobacterium* strain GV 3101.

Transformation of Tomato cv. 'Money Maker' with *tTRIP-1* containing N and C terminal FLAG tags.

Agrobacterium-mediated transformation was performed as described by Krasnyanski et al. (2001) with modifications. Seeds of tomato cv. 'Money Maker' were obtained from Thompson and Morgan Seeds, Inc. (Buffalo, NY) and sterilized with 70 % ethanol for 1 min and 50% Clorox (commercial bleach) for 30 min. After sterilization, seeds were rinsed four times with sterile distilled water (3-4 min per rinse), and then placed on tomato seedling

medium (1X MS salts + Gamborg's (B5) vitamins, 10 g/L Sucrose, 12 g/L Agar; pH 5.6) in glass jars, to germinate. Eight-day-old seedlings grown under the light (16 h light: 8 h dark) at 25 °C were used as a source of cotyledon explants.

Agrobacterium strain GV3101 containing 35S: FLAG-tTRIP-1/pBI121 or 35S: tTRIP-FLAG /pBI121 plasmids was cultured in 25 ml of liquid YEP media containing [100 mg/l kanamycin, 50 mg/l rifampicin, 50 mg/l gentamicin] and grown overnight on a shaker at 220 rpm at 28°C. The overnight *Agrobacterium* culture was centrifuged at 1000×g to precipitate the cells. The cell pellet was resuspended in 25 ml of liquid resuspension (LR) medium [1x MS salts + MS vitamins, 30 g/L Sucrose, 2.0 g/L Glucose, 700 mg/L MES; pH 7.6] with 40 mg/L acetosyringone and grown for another 1-2 hours at 28°C. The suspension was then diluted to an OD₆₀₀ of 0.3-0.4 with LR medium containing 40 mg/L acetosyringone.

Cotyledons (well shaped but not curly) of eight-day-old seedlings were used for explants. The explants were cut and placed in Petri plates containing tomato co-cultivation (TCC) medium [1x MS salts + MS vitamins, 30 g/L Sucrose, 2.0 g/L Glucose, 0.5 mg/L IAA, 1.0 mg/L BA, 700 mg/L MES, 40 mg/L acetosyringone, 12 g/L Agar; pH 5.6] and wounded with multi-pronged needles. Wounded cotyledons were submerged in the diluted liquid *Agrobacterium* suspension for 15 min with shaking at 50 rpm. Following inoculation, cotyledons were blotted dry on sterile paper towels and placed back on TCC medium for 48 h in the dark at 25 C.

After co-cultivation, explants were washed 2-3 times with LR medium containing 300 mg/L timentin, blotted dry on sterile paper towels and were placed (15-20 explants per plate) on tomato shoot regeneration (TSR) medium [1x MS salts + MS vitamins, 30 g/L Sucrose,

0.1 mg/L IAA, 2.0 mg/L zeatin, 300 mg/L timentin, 100 mg/L kanamycin, 40mg/L acetosyringone, 8.0 g/L Agar; pH 5.6]. After one week on TSR medium, all explants were re-positioned to achieve full contact with regeneration medium. After two weeks on TSR medium, the explants were subcultured onto fresh TSR medium and further cultured under 16 h photoperiod (provided by 20 watt cool-white fluorescent tubes yielding a light intensity of 30 $\mu\text{mol}/\text{m}^2/\text{sec}$) at 25 °C.

Five to six weeks later, one to two cm long shoots were excised and placed on a Tomato Rooting medium (TR) [1x MS salts + MS vitamins, 30 g/L Sucrose, 300 mg/L Timentin, 50 mg/L Kanamycin, 8.0 g/L Agar; pH 5.6] with kanamycin for secondary selection. Tomato plants with well developed roots were transferred into small plastic pots with sterile soil and covered with clear plastic covers for a week of acclimatization in a growth chamber. Following acclimatization, plants were transferred to the greenhouse for further growth and fruiting.

PCR to Verify *tTRIP-1* Transgene Expression

Total RNA was isolated from 100 mg of leaves harvested from transgenic tomato plants. Tissue was ground in liquid N₂ and total RNA isolated using the RNAeasy kit (Qiagen). One μg of total RNA was used for synthesis of first strand cDNA with the ImProm-II Reverse Transcription system using oligo dT primer (Promega). One μL first strand cDNA was used in a 20- μL PCR reaction (as described above) except that the extension time of 1.2 min was used to amplify *tTRIP-1* sequence.

Results

Identification, Expression and Purification of *E.coli* Recombinant tTRIP-1

To identify tomato *TRIP-1*, we performed a blast search of Arabidopsis *TRIP-1* (At2g46280) against the public tomato EST database. This search identified an EST contig, TC195665, whose open reading frame shared 81% identity (90% similarity) to Arabidopsis TRIP-1 for the entire length (328 amino acids) of the TRIP-1 protein. Another BLAST comparison of tomato unigene SGN-U575556 showed 82% identity with TRIP-1 a WD-40 domain containing protein, and only 30% identity to the next most related protein, transducin (At1g15470). The unigene, SGN-U575556 is also present in the tomato BAC genome clone C11HBA0064J132, which contains 5'UTR, 3'UTR and the entire open reading frame sequence of tTRIP-1. The 3' end sequence of *tTRIP-1* open reading frame was determined by sequencing the tomato cDNA clones. The forward primer 5'-cgataagcttaggccaatattgatgaaggcc-3' and reverse primer 5'-gactctcgagctaaatcctgatgttgaagtaatcc -3' primer, were designed to isolate *tTRIP-1* from tomato.

To characterize the functional role of tomato TRIP-1 in BR signal transduction, tTRIP-1 was cloned into the *E. coli* expression vector, pFLAG-MAC to get *tTRIP-1* fused with an N-terminal FLAG (*FLAG-tTRIP-1*) (Figure 1). The *FLAG-tTRIP-1* containing constructs were verified for the presence of *tTRIP-1* by restriction enzyme digestion (Figure 1A) and sequencing. The sequence of our cloned *tTRIP-1* showed two single nucleotide polymorphisms (G222A and T711C) compared to the published sequence of the tomato BAC

genome clone C11HBA0064J13, although there was no change in the encoded amino acid sequence (Figure 2). The FLAG-tTRIP-1 construct was transferred into BL-21 (DE3) cells for overexpression of the protein. Recombinant FLAG-tTRIP-1 protein was purified using FLAG M₂-agarose beads and examined by gel electrophoresis with CBB staining (Figure 1B).

Sequence Alignment of tTRIP-1 Protein with TRIP-1 Proteins from Other Organisms

To determine how conserved tomato (tTRIP-1) protein is with other species, we performed a multiple sequence alignment of tomato TRIP-1 with bean (PvTRIP-1, accession no. AF335551); Arabidopsis (AtTRIP-1, AAC62878.1); Drosophila (DmTRIP-1, AAF52183); human (HsTRIP-1, S60335); and fission yeast (Sum1, P79083) using the ClustalW 1.81 (Thompson et al., 1994) multiple sequence alignment tool (Figure 3). The sequence alignment scores indicated highest conservation between tomato and bean TRIP-1 proteins with 87% identity (93% similarity). Sequence alignment between tomato and Arabidopsis TRIP-1 protein showed a similarly high 81% identity (90% similarity). The residues conserved among all TRIP-1s are shown by a star (Figure 3). Tomato TRIP-1 shares sequence similarity with human (HsTRIP-1); Drosophila (DmTRIP-1) and fission yeast (Sum1) with sequence alignment identity of 46%, 47% and 44% respectively. The sequence in the WD domains is highly conserved among all plant TRIP-1 sequences examined (Figure 3).

PredictProtein (Rost, et al., 2004) predicted that the secondary structure of tTRIP-1 is composed of β -sheets (55.5%) and loop structures (44.5%). An Interproscan (Zdobnov and

Apweiler, 2001) search confirmed the presence of five WD40-repeat domains in the protein. The three dimensional structure of tTRIP-1, as modeled by protein homology/analogy recognition engine (Phyre) (Kelley and Sternberg, 2009), predicted to be a 7-bladed beta-propeller similar to the tertiary structure of the WD 40 region of the beta1-subunit of signal-transducing G protein heterotrimer from cow (d1tbga) (Figures 4A and 4B).

Transphosphorylation of tTRIP-1 by tBRI1-CD and tBAK1-CD

When tomato BRI1 and BAK1 CDs are expressed in *E. coli*, active kinases are obtained that can autophosphorylate and transphosphorylate each other. Tomato BRI CD can also phosphorylate synthetic peptides (Chapter 2). The Arabidopsis TRIP-1 homolog has been shown previously to be a putative *in vivo* cytoplasmic substrate of BRI1 that is phosphorylated by BRI1 kinase domain on three specific residues (Ehsan et al., 2005).

To see if tomato BRI and BAK1 can significantly phosphorylate the tomato TRIP-1 ortholog, a kinase assay was performed to assess tTRIP-1 transphosphorylation by tBRI1 and tBAK1 (Figure 5). As expected, FLAG-tTRIP-1 does not show any autophosphorylation activity of its own as determined by [γ - 32 P] ATP kinase assays (Figure 5, lane 1). However, when mixed with MBP-tBRI1-CD, FLAG-tBRI1-CD or MBP-tBAK1-CD, significant transphosphorylation was observed on tTRIP-1 (Figure 5, lanes 2, 4 and 5). In order to see if tTRIP-1 transphosphorylation is dependent on active tBRI1 and tBAK1 kinases, FLAG-tTRIP-1 was mixed with kinase inactive forms of FLAG-tBRI1-CD (K916E) and MBP-tBAK1-CD (K317E) and no transphosphorylation activity was detected (Figure 5, lanes 3 and 6).

Overall, these data confirm that tTRIP-1 can be transphosphorylated by both tBRI and tBAK1. This is similar to Arabidopsis TRIP-1 in terms of transphosphorylation by BRI1 (Ehsan et al., 2005), however in Arabidopsis BAK1 does not significantly phosphorylate TRIP-1 *in vitro*, whereas significant transphosphorylation was observed on tTRIP-1 by tBAK1-CD.

Tomato BRI1 can Phosphorylate Peptide Substrates

We previously showed that tBRI-CD can phosphorylate synthetic peptide substrates (Chapter 2, Figure 5). Arabidopsis BRI1-CD can phosphorylate synthetic peptides with an optimal sequence recognition motif of [RK]-[RK]-X(2)-[ST]-X(3)-[LMVIFY]-[RK], where residues in brackets indicate the amino acid that can occur at that position (Oh et al., 2000). The preliminary recognition motif for tBRI1-CD was similar to that identified for Arabidopsis BRI1-CD.

To see if tBRI1-CD recognizes short peptides corresponding to Arabidopsis TRIP-1, a peptide substrate assay was performed by incubating tBRI1-CD with four TRIP-1 peptides. These assays confirmed that TRIP-1 peptides are phosphorylated by tBRI1-CD. The highest phosphorylation activity was observed in the Trip5 peptide followed by the Trip2 peptide. In comparison, Trip1 and Trip3 peptides appear to be poor substrates of tBRI1-CD (Figure 6). The level of phosphorylation for Trip5 was almost equal to the BR13 and BR15 peptides, two peptides that show the highest phosphorylation activity in the tBRI1-CD peptide substrate assays.

Trip5 peptide is also one of the highest phosphorylated substrate peptides of Arabidopsis BRI-CD (Ehsan et al., 2005). The Thr residue present in the Trip5 peptide that was identified as one of the Arabidopsis TRIP-1 phosphorylation sites is also present at the conserved position in tTRIP-1.

Although these peptides were based on the Arabidopsis TRIP-1 sequence and were not designed to exactly match the tTRIP-1 sequence, still they provide a good indication of the requirements for tBRI1-CD substrate phosphorylation due to the conserved sequences of tTRIP-1 in the region of these peptides. This data also provides additional evidence that tTRIP-1 is an *in vitro* substrate of the tBRI1 cytoplasmic kinase domain.

Tomato TRIP-1 Transphosphorylation Sites

To identify the specific transphosphorylation sites of tTRIP-1 by tBRI1-CD, we analyzed tryptic peptides transphosphorylated by tBRI1-CD *in vitro* by LC/MS/MS and LC/MS^E with and without IMAC. The sequence coverage of the tTRIP-1-cytoplasmic domain was 81% (Figure 7A). Based on a Mascot score of 45, one of the two sites (T-110, T-111) could be phosphorylated in the tTRIP-1 peptide 108-LAVITTDPFMGLTSAIHIK-125 (Fig. 7B). However due to poor ion coverage of the fragmented peptide and a higher expect value, we cannot unambiguously assign the phosphorylation site. Interestingly, T-110 and T-111 residues are highly conserved in plants, with T-111 being present at the conserved position among all species examined, while T-110 is replaced by S-110 in human and *Drosophila* TRIP-1 proteins and is not conserved in yeast (Figure 7C).

Genotypic and Phenotypic Analysis of Tomato Transgenic Plants Overexpressing tBRI1-FLAG

Full length tTRIP-1 with either an N or a C-terminal FLAG epitope tag was cloned into the binary plant transformation vector pBI121 (containing a 35S promoter for constitutive overexpression) and transformed into WT tomato cv 'Money Maker'. Multiple tTRIP-1 transgenic lines were generated. PCR using a 35S Forward primer 5'-aaacctcctcggattccat-3' and 5'-gactgagctcctaaatcctgatgtgaagtaac-3 as reverse primer was used to identify lines expressing N-FLAG-tTRIP-1. Out of the seven lines checked one was identified as positive for FLAG-tTRIP-1 expression (Figure 8). The phenotype of this FLAG-tTRIP-1 overexpressing line appeared to be similar to the WT control with respect to visible morphological features and overall growth rates.

Discussion

We cloned tomato TRIP-1, a WD-domain protein with high sequence similarity to plant (PvTRIP-1 and AtTRIP-1) as well as other eukaryotic TRIP-1s, including *Drosophilla* (DmTRIP-1), Human (HsTRIP-1) and yeast (Sum1). Tomato TRIP-1 is composed of five WD-repeats that are present at the same conserved location in all eukaryotic species. The high sequence similarity of tTRIP-1 with TRIP-1 from other eukaryotic organisms, especially plant TRIP-1s, suggests a functional similarity as well.

TRIP-1 (eIF3i) is an essential component of the eIF3 complex involved in eukaryotic translation initiation. Besides its role in translation, TRIP-1 plays other independent roles as well. For example, in yeast, TRIP-1, known as TIF34 (p39) in *S. cerevisiae* (Verlhac et al., 1997) and Sum1 in *S. pombe* (Humphrey and Enoch, 1998), is required for cell proliferation,

and mutation of TIF34 or Sum1 leads to cell deaths (Humphrey and Enoch, 1998). Moreover, loss of the TIF34 subunit in *S. cerevisiae* causes degradation of all other eIF3 subunits, suggesting that TIF34 is an essential component for preserving the structure of the eIF3 complex (Naranda et al., 1997). In vertebrates, TRIP-1 serves as a cytoplasmic substrate for TGF- β receptor kinase subunits (Chen et al., 1995). Overexpression of TRIP-1 in human cells enhanced oncogenesis and tumor growth, suggesting its importance in regulating cell division (Ahlemann et al., 2006; Dong and Zhang, 2006). In Arabidopsis, TRIP-1 also plays an essential role in growth and development. Antisense suppression of AtTRIP-1 resulted in plants with extreme dwarfism, altered leaf morphology, delayed senescence and a bushy phenotype in mature plants (Jiang and Clouse, 2001). Some of the morphological characters of these plants resembled the phenotype of BR-deficient and insensitive mutants (Ehsan, et al., 2005; Jiang and Clouse, 2001). While we currently have no data on the phenotype of TRIP-1 mutants in tomato, our biochemical analysis of the tBRI1-CD interactions with tTRIP-1 presented in this chapter, provides a foundation for further functional analysis of the role of TRIP-1 in tomato growth and BR signal transduction.

The potential role of AtTRIP-1 in BR signaling, as a putative cytoplasmic substrate of AtBRI1, was demonstrated by Ehsan et al. (2005). They found that AtTRIP-1 can interact with BRI1 *in vivo* and can be phosphorylated by BRI1 CD *in vitro*. Data presented here show that similar to AtTRIP1, tTRIP-1 is an *in vitro* substrate of tBRI1 and can be phosphorylated by tBRI1-CD on specific Thr residues. These results confirm the similarity in BRI1 kinase function and substrate recognition between tomato and Arabidopsis. However, there were some differences observed in transphosphorylation of TRIP-1 by BAK1 in these two species.

In tomato, BAK1 can significantly transphosphorylate tTRIP-1, whereas AtBAK1 doesn't significantly phosphorylate AtTRIP-1 *in vitro* (Ehsan, et al., 2005). This difference could be the result of some divergence in the interaction between BAK1 and downstream substrates between the two species. It would be interesting to test TRIP-1 phosphorylation by BAK1 in other plant species such as bean (PvTRIP-1), which shows higher sequence similarity to tTRIP-1 than AtTRIP-1. The difference in BAK1/ TRIP-1 interactions between Arabidopsis and tomato might also be the result of variation in the WD-repeat domain sequences, involved in protein-protein interactions.

The phosphorylation of TRIP-1 recombinant protein as well as synthetic peptide substrates by tBRI1-CD, and the identification of a putative tTRIP-1 transphosphorylation site, confirms that tTRIP-1 is a good *in vitro* substrate of tBRI1. Tomato transgenic plants overexpressing tTRIP-1 were generated to further characterize the *in vivo* interaction of tTRIP-1 and tBRI1, and to help understand the function of tTRIP-1 *in planta*. *In vivo* substrates of BRI1, including BRI1-interacting protein, BKI1, a negative regulator of BR signaling (Wang and Chory, 2006) and BR-signalling kinase 1, BSK1, a positive regulator of BR signaling (Kim et al., 2009) have been identified using molecular genetics and biochemical approaches. Similar approaches can be employed in the future to determine if tTRIP-1 is a true *in vivo* substrate of tBRI1, required for BR signaling.

Since TRIP-1 is a subunit of the eIF3 complex, it would be interesting to see if other subunits of eIF3 also serve as BRI1 substrates. Several subunits of plant eIF3 have been cloned from Arabidopsis and wheat (Burks et al., 2001). Phosphorylation plays a critical role

in the regulation of several plant and animal eIF3 subunits (Homma et al., 2005; Maiti et al., 2003; Shenberger et al., 2005; Holz et al., 2005; Shi et al., 2003; Pincheira et al., 2001; Langland et al., 1996). This suggests the possibility that BR-dependent phosphorylation of TRIP-1 and other eIF3 subunits might play an important role in regulating eIF3 activity and therefore overall global protein translation in plants, thereby providing an alternative pathway by which BR signal transduction regulates plant growth and development (Figure 9).

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Figures

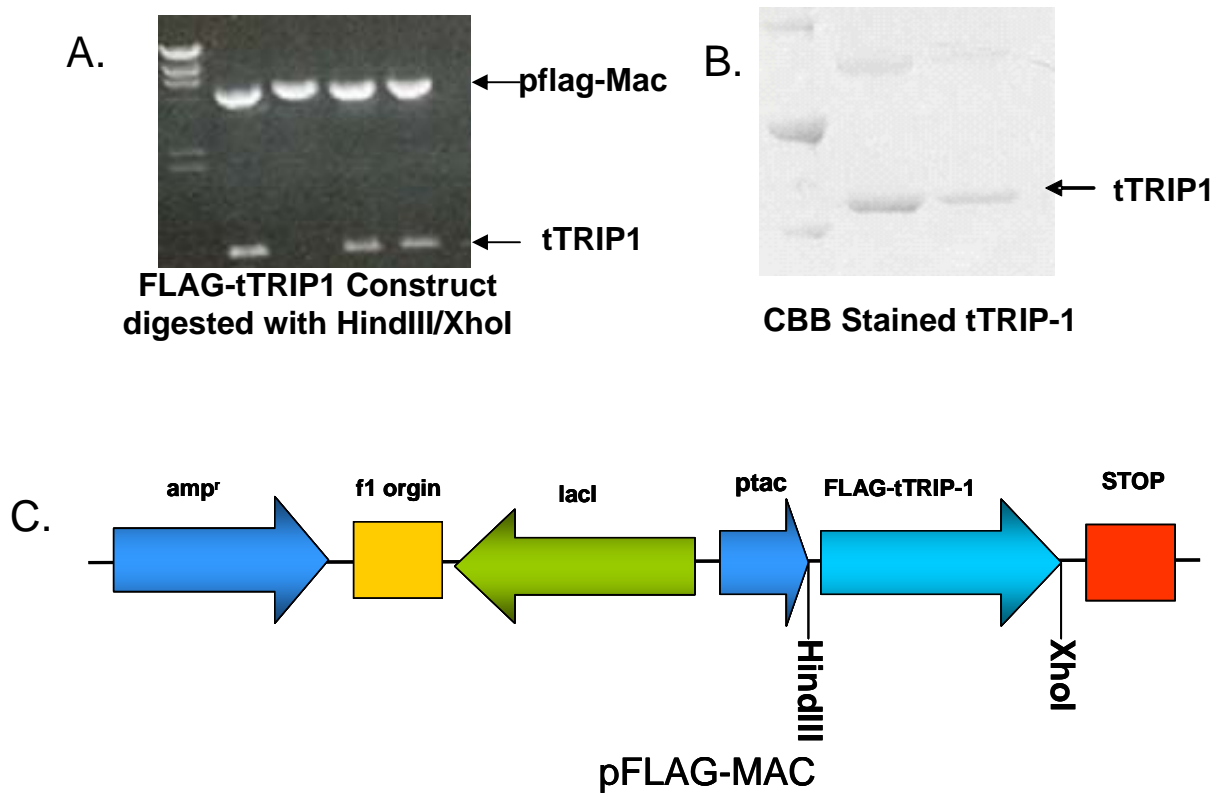


Figure 1. Cloning of tTRIP-1 into pFLAG-MAC expression vector. **(A)** Digestion of the construct with restriction enzymes (*XhoI*/*HindIII*) to verify the presence of tTRIP-1. **(B)** Gel picture of CBB stained, purified FLAG-tTRIP-1 protein. **(C)** Map of the expression vector pFLAG-MAC showing the restriction enzymes, *HindIII* and *XhoI* that were used for cloning tTRIP-1.

Figure 2. Nucleotide sequence of the full-length cDNA of *tTRIP-1* with derived amino acid sequence. Two nucleotides that exhibited polymorphism (G222A and T711C) without any change in amino acid sequence compared to the published sequence of BAC clone C11HBa0064J13, are highlighted in red and overscored.

ATGAGGCCAATATTGATGAAGGGCCATGAAAGGCCGTTAACTTTTTCTAAAGTACAACAGA	60
M R P I L M K G H E R P L T F L K Y N R	20
GATGGAGATCTGCTCTTCTCTTTCGCGCTAAGGACCATAACCCCTACCGTTTGGTTTGCCGAT	120
D G D L L F S C A K D H T P T V W F A D	40
AACGGCGAGCGCCTCGGCACTTACCGTGGCCATAACGGTGCCGTTTGGTGCTGTGACGTT	180
N G E R L G T Y R G H N G A V W C C D V	60
TCTCGGGATTTCGTCCAGGCTAATAACTGGAAGTGCGGATCAACTGCAATGTTGTGGGAT	240
S R D S S R L I T G S A D Q T A M L W D	80
GTCCAAACTGGTGCCAGCTGCACACGTTTACCTTTGACTCCCCTGCTAGGTCTGTTGAT	300
V Q T G A Q L H T F T F D S P A R S V D	100
TTTTCAGTTGGCGATAAACTCGCAGTGATCACCCTGATCCTTTTTATGGGACTGACATCT	360
F S V G D K L A V I T T D P F M G L T S	120
GCTATCCATATCAAAAATATCAGCAAAGATCCCAGTGAACAAATGAGTGAGTCGGTGCTC	420
A I H I K N I S K D P S E Q M S E S V L	140
GTCTTAAAGGGTCCCCAGGGAAGAATCAACAGAGCTGTGTGGGGACCCCTGAATAAAACA	480
V L K G P Q G R I N R A V W G P L N K T	160
ATTATAAGTGCTGGTGAAGATGCTGTAATACGTATCTGGGATGCTGAGACTGGAAAGGTA	540
I I S A G E D A V I R I W D A E T G K V	180
CTGAAGGAGTCTGACAAAGAAATCGGTCATAAAAAGGGTATTACATCGCTACAAAATCA	600
L K E S D K E I G H K K G I T S L Q K S	200
GTGGATGGTTTCGCACTTCATTACTGGTTCTCTTGATAAATCTGCAAAGCTCTGGGACATC	660
V D G S H F I T G S L D K S A K L W D I	220
AGAAGTTTGACACTTATCAAGAACTATACGACCGAACGACCCGTGAATGCGTCACAATG	720
R S L T L I K N Y T T E R P V N A V T M	240
TCGCCGCTTCTTAATCATGTGGTTCTGGGAGGTGGTCAAGACGCTTCAGCTGTCACCACT	780
S P L L N H V V L G G G Q D A S A V T T	260
ACTGACCATCGTGCTGGAAAGTTTGGAGCCAAGTTCTATGACAAGATTCTTACTGAAGAA	840
T D H R A G K F E A K F Y D K I L T E E	280
ATAGGAGGTGTCAAAGGGCATTTTGGACCAATAAATGCTTTGGCTTTCAATCCTGATGGG	900
I G G V K G H F G P I N A L A F N P D G	300
AAAAGTTTTGCAAGTGGTGGTGAAGATGGATATGTGAGATTGCATCATTTTTGACCAGGAT	960
K S F A S G G E D G Y V R L H H F D Q D	320
TACTTCAACATCAGGATTTAG	981
Y F N I R I -	327

Figure 3. Multiple sequence alignment of tomato tTRIP-1 with bean (PvTRIP-1, accession no. AF335551); Arabidopsis (AtTRIP-1, AAC62878.1); Drosophila (DmTRIP-1, AAF52183); human (HsTRIP-1, S60335); and fission yeast (Sum1, P79083) using ClustalW 1.81 multiple sequence alignment tool. WD-domains are over-scored and numbered. “*” indicate that the amino acids in that column are identical in all sequences in the alignment, “:” indicates that conserved substitutions have been observed, “.” indicates that semi-conserved substitutions are observed.

(i)

tTRIP1 MRPILMKGHERPLTFLKYNRDGDLLFSCAKDHTPTVWFADNGERLGTYRGHNGAVWCCDV 60
PvTRIP1 MRPILMKGHERPLTFLKYNRDGDLLFSCAKDHNPTVWFADNGERLGTYRGHNGAVWCCDV 60
AtTRIP1 MRPILMKGHERPLTFLRYNREGDLLFSCAKDHTPTLWFADNGERLGTYRGHNGAVWCCDV 60
DmTRIP1 MRPLMLQGHESITQIKYNREGDLLFSCSKDQKPNVWYSLNGERLGTYDGHQGAVWCLDV 60
HsTRIP1 MKPILLQGHESITQIKYNREGDLLFTVAKDPIVNVWYSVNGERLGTYMGHTGAVWCVDA 60
Sum1 MRPIILQGHESITQIKYNHGDGDLFSCAKDKVINVWFVSHNGERLGTYEHTGAIWTCDI 60
:

(ii)

tTRIP1 SRDSSRLITGSADQTAMLWDVQGTGAQLHTFTFDSPARSVDVSVGDKLAVITTDPFMGLTS 120
PvTRIP1 SRDSGRLITGSADQTAKLWNVQGTGQQLFTFNFDSPARSVDVSVGDKLAVITTDPFMELPS 120
AtTRIP1 SRDSSRLITGSADQTAKLWDVKSGKELFTFKFNAPTRSVDFAVGDR LAVITTDHFVDRTA 120
DmTRIP1 DWESRKLITGAGDMTAKIWDVEYGTVIASIPKSSVRTSNFSFSGNQAAAYSTDKAMGQSC 120
HsTRIP1 DWDTKHVLTGSADNSCRLWDCETGKQLALLKTN SAVRTCGFDFGGNIIMFSTDKQMGYQC 120
Sum1 NKSSTLMVSGAADNTMRLWDVKTGKQLYKWEFPTAVKRVEFNEDDTRILAVTEERMGYAG 120
.: :*:*. * : **: : * : :..: * .. **: :

(iii)

tTRIP1 AIHIKNISKDPSEQMSESVLVLKGPQG=-RINRAVWGPLNKTIISAGEDAVIRIWD AETG 178
PvTRIP1 AIHVKRIANDPTEQTGDSVVLKGPQG--RINRAIWGPLNRTIISAGEDAVIRIWDSETG 178
AtTRIP1 AIHVKRIAEDPEEQDAESVVLHCPDGKKRINRAVWGPLNQTIIVSGGEDKVIRIWD AETG 180
DmTRIP1 ELFLIDVRNADSSLS-EQEPTLRIPMTESKITSMLWGPLDETIITGHDN GNIAIWDIRKG 179
HsTRIP1 FVSFFDLR-DPSQID-NNEPYMKIPCNDSKITSAVWGPLGECIIAGHESGELNQYSAKSG 178
Sum1 TVTVFRVPISESDAAETPLYVITRE-SKATVAGWSYLSKFLFTGHEDGSVSR YDAITG 179
: . : . : : . : . *.*.. :. : . : . *

(iv)

tTRIP1 KVLKESDKEIGHKKGITSLQKSVDSHFITGSLDKSAKLWDIRSLTLIKNYTTERPVNAV 238
PvTRIP1 KLIKESDKESGHKKTVTSLAKSADGSHFLTGSLDKSARLWDTRTLTLIKTYVTERPVNAV 238
AtTRIP1 KLLKQSDDEEVGHKKDITSLCKAADD SHFLTGSLDKTAKLWDMRTLTL LKTYTTVVPVNAV 240
DmTRIP1 QKVVDS--GTDHSAGINDMQLSKDGTMFV TASKDTTAKLFDSESLMCLKTYKTERPVNSA 237
HsTRIP1 EVLVN---VKEHSRQINDIQLSRDMTMFV TSKDNTAKLFDSTTLEHQKTFRTERPVNSA 235
Sum1 -EFVESKQVHNSGSTITDLQFYPRDYFIT SCKDTTAKAIDVDSFEVIKTYLTD TPLNTS 238
. : :..: * :*:*..*:*:* * : : * : * *:*:






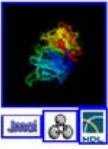
tTRIP1 TMSPLLNHVVLGGGQDASAVTTT DHRAGKFEAKFYDKILTEEIGGVKGHFGPINALAFNP 298
PvTRIP1 AMSPLL DHVVLGGGQDASAVTTT DHRAGKFEAKFYDKILQEEIGGVKGHFGPINALAFNP 298
AtTRIP1 SLSPLL NHVVLGGGQDASAVTTT DHRAGKFEAKFYDKILQEEIGGVKGHFGPINALAFNP 300
DmTRIP1 AISPIMDHVVLGGGQDAMEVTTT STKAGKFD SRFFHLYEEEEFARLKGHFGPINS LAFHP 297
HsTRIP1 ALSPNYDHVVLGGGQEAMDVTTT STRIGKFEARFFHLAFEEEEFGRVKGHFGPINS VAFHP 295
Sum1 SFTPVQDFVILGGGQEARDVTTT TAARQKFEARFYHAILEEELGRVKGHFGPINTI AVHP 298
:::* :.*:*:*:*:* * * * : *:*:*:*:* * * : * : * * * * * : * : *

(v)

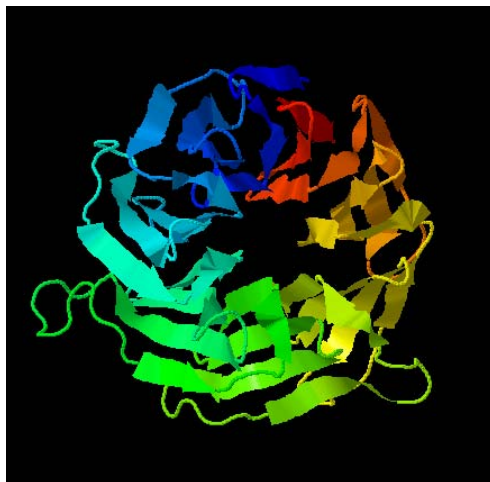
tTRIP1 DGKSFASGGEDGYVRLHHFDQDYFNIRI-- 326
PvTRIP1 DGKSFSSGGEDGYVRLHHFDPDYFNIRI-- 326
AtTRIP1 DGKSFSSGGEDGYVRLHHFDSDYFNIRI-- 328
DmTRIP1 DGKSYASGGEDGFVRVQTFDSTYFENIFE- 326
HsTRIP1 DGKSYSSGGEDGYVRIHYFDPQYF EF EF EFA 325
Sum1 KGTGYASGGEDGYVRVHFFDKNYFDFKYTL 328
.*..:*****:*:*:* * * * :

Figure 4. Structural parameters of tTRIP-1 as predicted by the protein homology/analogy recognition engine (Phyre) (Kelley and Sternberg, 2009). **(A)** Cropped view of three hits in the primary table of fold recognition, including descriptors of fold and superfamily. **(B)** Images of the tertiary structure models of tTRIP-1; (i) Frontal view WD 40 region of c2g9A, protein/DNA binding protein; (ii) Top view of WD 40 region of beta1-subunit of the signal-transducing G protein heterotrimer from Cow (d1tbga).

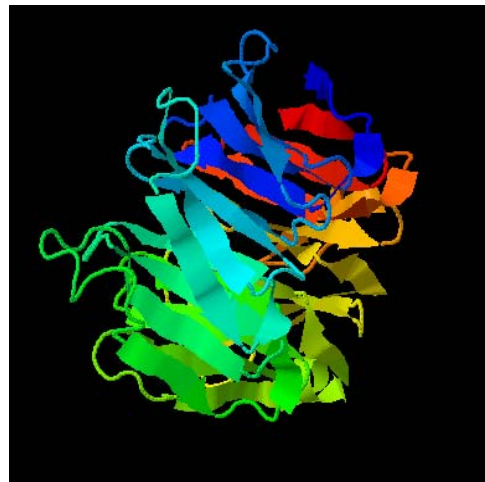
A. QuickPhyre Results for Job tTRIP-1 (Top three hits)

Fold Recognition								
View Alignments	SCOP Code	View Model	E-value	Estimated Precision	BioText	Fold/PDB descriptor	Superfamily	Family
	c2g9aA (length:311) 21% i.d.		1.4e-45	100 %	n/a	PDB header: structural protein/dna binding protein	Chain: A; PDB Molecule: wd-repeat prote n 5;	PDBTitle: structural bas s for the specific recognition of methylated2 histone h3 lysine 4 by the wd-40 protein wdr5
	c2anqA (length:336) 20% i.d.		4.2e-45	100 %	n/a	PDB header: transcription	Chain: A; PDB Molecule: wd-repeat prote n 5;	PDBTitle: structure of wdr5
	d1tbga (length:340) 16% i.d.		9.8e-45	100 %	n/a	7-bladed beta-propeller	WD40 repeat-like	WD40-repeat

B.



(i) Front View (c2g9A)



(ii) Top View (d1tbga model)

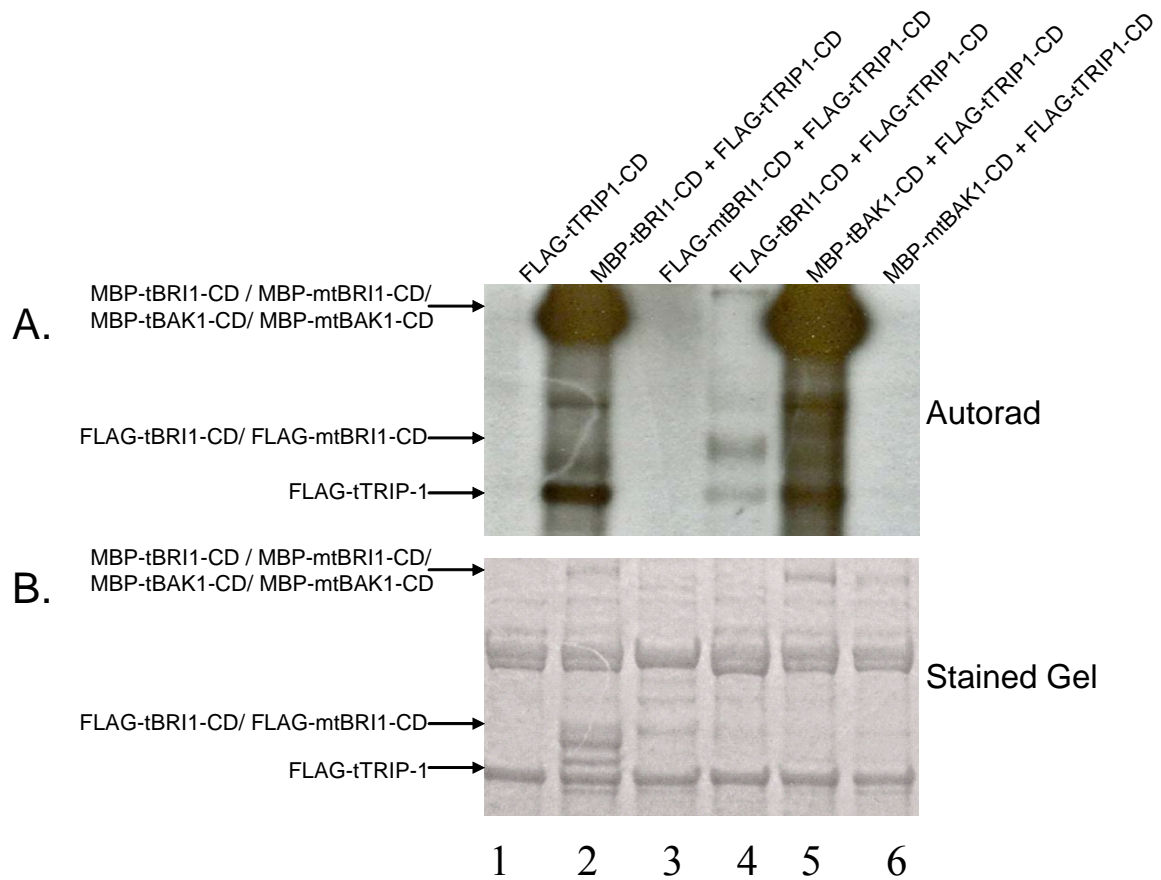


Figure 5. Both tomato BRI1-CD and BAK1-CD can transphosphorylate tTRIP-1 *in vitro*.

(A) Autoradiographic results of protein samples after exposing to X-ray film for 1 hr.

Proteins were incubated with [γ - 32 P] ATP in kinase buffer for 1hr followed by separation on

10% SDS-PAGE gel. Each lane represents a different reaction. (B) CBB stained protein

samples from the same gel.

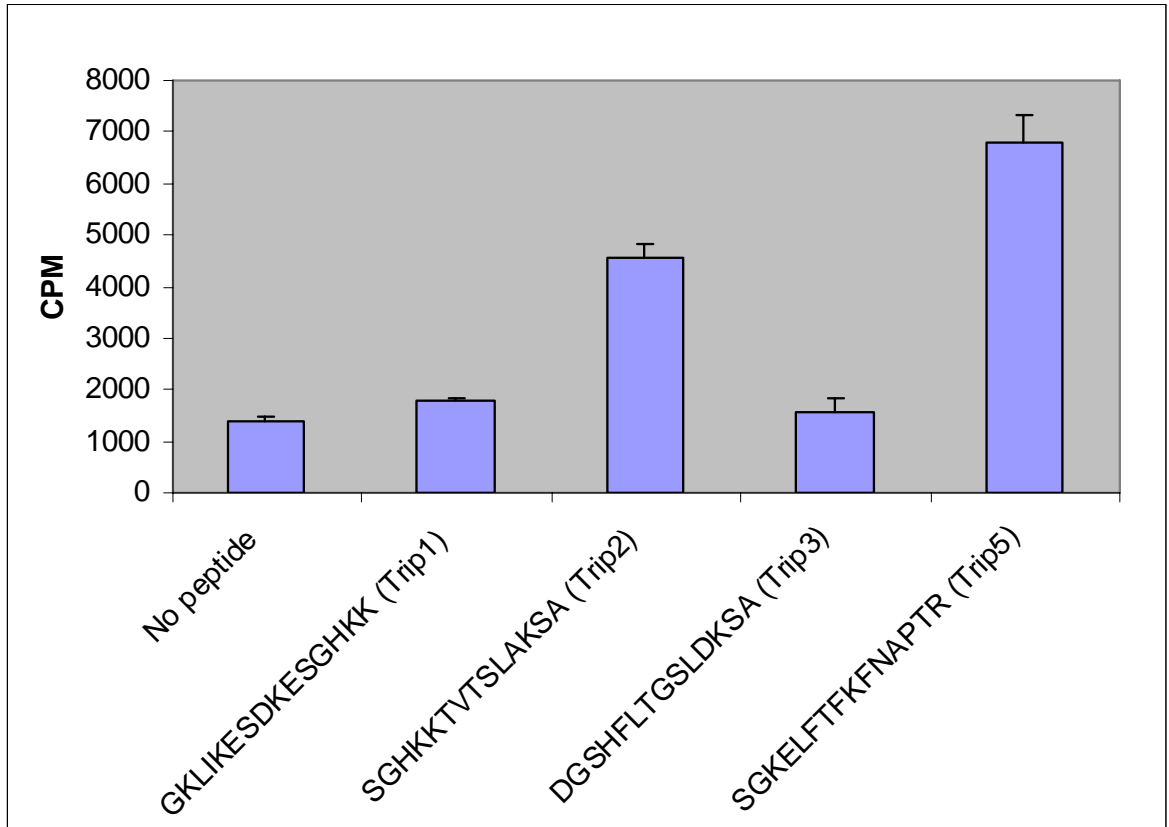
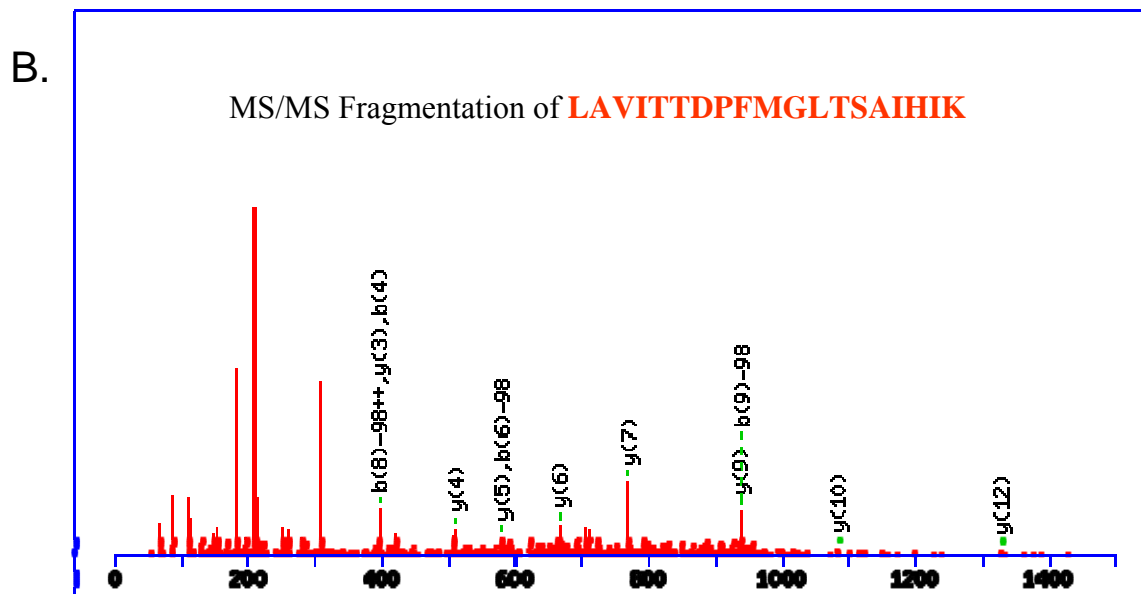


Figure 6. Phosphorylation of TRIP-1 synthetic peptides *in vitro* by FLAG-tBRI1-CD. A typical 20- μ L reaction mixture contained 0.5 μ g of affinity-purified FLAG-tBRI1-CD, 0.8 μ Ci [γ - 32 P] ATP (888 cpm/mol), and 0.10 mg/mL of synthetic peptide in kinase buffer. Reactions were incubated for 20 min at room temperature and incorporation of 32 P into the synthetic peptide was quantified by binding to P81 phosphocellulose paper, followed by liquid scintillation spectrometry. Error bars are SE, $n = 3$.

Figure 7. LC/MS/MS analysis of *in vitro* transphosphorylation sites of FLAG-tTRIP by tBRI1-CD. **(A)** Mascot search result showing sequence coverage of tTRIP-1, peptides that matched to tTRIP-1 shown in bold red, and putative phosphorylated residues highlighted in green. **(B)** Mascot LC/MS/MS spectra of the putative phosphopeptide showing b and y ions that matched the peptide, monoisotopic mass of the peptide, ion score and expect value (shown below the spectra). **(C)** Sequence alignment of the putative phosphopeptide of tTRIP-1 with bean (PvTRIP-1, accession no. AF335551); Arabidopsis (AtTRIP-1, AAC62878.1); Drosophila (DmTRIP-1, AAF52183); human (HsTRIP-1, S60335); and fission yeast (Sum1, P79083) using ClustalW 1.81 multiple sequence alignment tool.

A. 1 MDYKDDDDKR PILMKGHERP LTFLKYNRDG DLLFSCAKDH TPTVWFADNG
 51 ERLGTYRGHN GAVWCCDVSR DSSRLITGSA DQTAMLWDVQ TGAQLHTFTF
 101 DSPARVDFS VGDKLAVI**TT** DPFMGLTSAI HIKNISKDPS EQMSESVLVL
 151 KGPQGRINRA VWGPLNKTII SAGEDAVIRI WDAETGKVLK ESDKEIGHKK
 201 GITSLQKSVD GSHFITGSLD KSAKLWDIRS LTLIKNYTTE RPNNAVTMSP
 251 LLNHVVLGGG QDASAVTTTD HRAGKFEAKF YDKILTEEIG GVKGHFGPIN
 301 ALAFNPDGKS FASGGEDGYV RLHFFDQDYF NIRI



MS/MS Fragmentation of **LAVITDPFMGLTSAIHK**
 Monoisotopic mass of neutral peptide Mr(calc): 2123.0738
 Variable modifications:
 T6 : Phospho (ST), with neutral losses 97.9769
 M10 : Oxidation (M), with neutral losses 0.0000
 Mascot Ions Score: 45 Expect: 0.00029

C. tTRIP1 108-LAVITDPFMGLTSAIHK-125
 AtTRIP1 108-LAVITTDHFVDRTAAIHVK-125
 PvTRIP1 108-LAVITDPFMELPSAIHVK-125
 DmTRIP1 108-QAAYSTDKAMGQSCFLI-125
 HsTRIP1 108-IIMFSTDKQMGYQCFVSFF-125
 Sum1 108-RILAVTEERMGYAGTVTVF-125

*

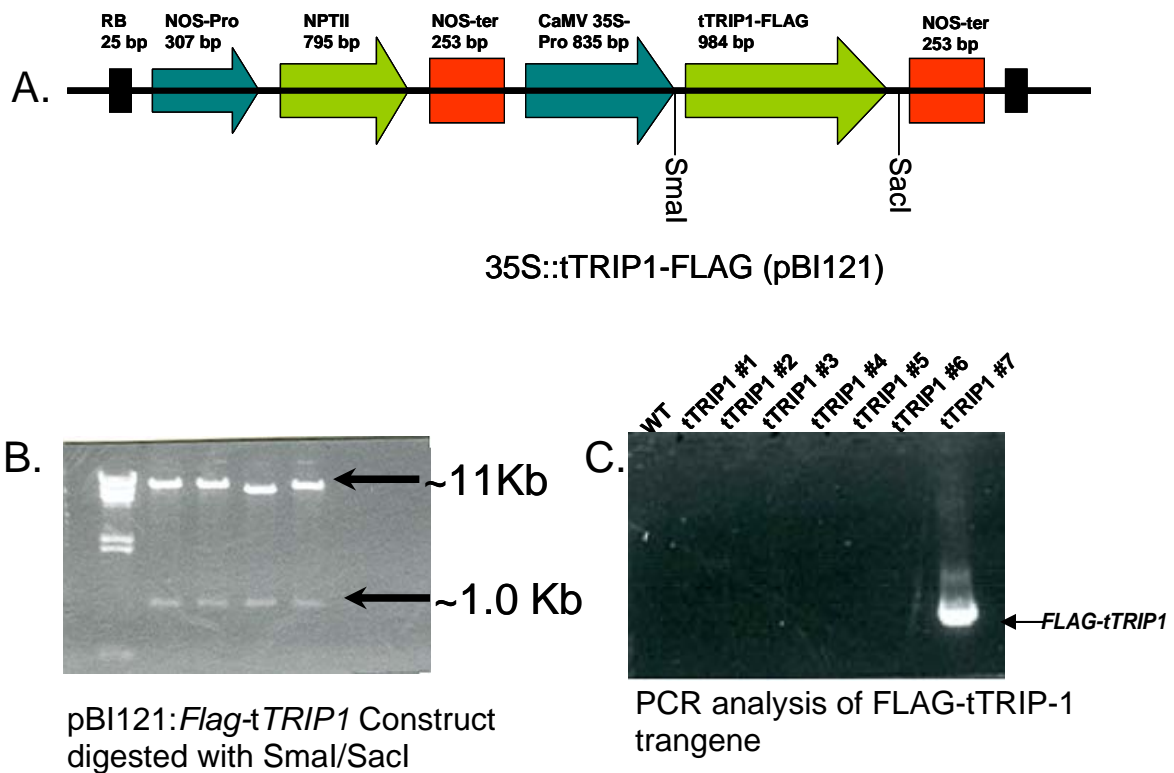


Figure 8. Cloning of tTRIP-1 into plant transformation vector and analysis of the transgenic lines. (A) Map of the binary vector pBI121 showing restriction enzymes, *SmaI* and *SacI* that were used for cloning tTRIP-1 (B) Digestion of the construct with restriction enzymes (*SmaI/ SacI*) to verify the presence of tTRIP-1 (C) Gel picture of PCR products from seven independent transgenic lines, performed to verify the presence of in different transgenic lines. The positive band corresponding to the size of *FLAG-tTRIP-1* transgene is indicated

Figure 9. A model for BR-dependent phosphorylation of TRIP-1 by both, BRI1 and BAK1. In this model, BL binds directly to BRI1, resulting in activation of BRI1. The activated BRI1 then associates with and transphosphorylates inactive BAK1. Once activated by BRI1, BAK1 transphosphorylates back onto juxtamembrane and c-terminal domain residues of BRI1, thereby quantitatively enhancing the phosphorylation of BRI1 to its downstream substrates which eventually leads to BR regulated gene expression. Both BRI1 and BAK1 can transphosphorylate TRIP-1 *in vitro*, however, the *in vivo* functional role of TRIP-1 in BR signal transduction is not yet clear. It is proposed that TRIP-1 might be involved in BR-regulated translation initiation and therefore, plant growth and regulation. For simplicity, all discovered components of BR signaling are not shown.

