**Corrections**

**BIOCHEMISTRY.** For the article “Arg-302 facilitates deprotonation of Glu-325 in the transport mechanism of the lactose permease from *Escherichia coli*” by Miklos Sahin-Toth and H. Ronald Kaback, which appeared in number 11, May 22, 2001, of *Proc. Natl. Acad. Sci. USA* (98, 6068–6073; First Published May 15, 2001; 10.1073/pnas.111139698), Fig. 1 was printed incorrectly due to a printer’s error. The correct figure and its legend appear below.

![Model for H⁺ translocation during lactose/H⁺ symport via lac permease.](image)

**PLANT BIOLOGY.** For the article “BRSl, a serine carboxypeptidase, regulates BR11 signaling in *Arabidopsis thaliana*” by Jia Li, Kevin A. Lease, Frans E. Tax, and John C. Walker, which appeared in number 10, May 8, 2001, of *Proc. Natl. Acad. Sci. USA* (98, 5916–5921; First Published April 24, 2001; 10.1073/pnas.091065998), the authors note the following. In Fig. 1B, all eight places marked “brl–5” for genotype should read “brl1–5.”
BRS1, a serine carboxypeptidase, regulates BRI1 signaling in Arabidopsis thaliana

Jia Li*, Kevin A. Lease*, Frans E. Tax†, and John C. Walker‡

*Division of Biological Sciences, University of Missouri, Columbia, MO 65211; and †Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721

Edited by Clarence A. Ryan, Jr., Washington State University, Pullman, WA, and approved March 9, 2001 (received for review February 8, 2001)

Brassinosteroid-insensitive 1 (BRI1) of Arabidopsis thaliana encodes a cell surface receptor for brassinosteroids. Mutations in BRI1 severely affect plant growth and development. Activation tagging of a weak bri1 allele (bri1-5) resulted in the identification of a new locus, brs1-1D. BRS1 is predicted to encode a secreted carboxypeptidase. Whereas a brs1 loss-of-function allele has no obvious mutant phenotype, overexpression of BRS1 can suppress bri1 extracellular domain mutants. Genetic analyses showed that brassinosteroids and a functional BRI1 protein kinase domain are required for suppression. In addition, overexpressed BRS1 missesense mutants, predicted to abolish BRS1 protease activity, failed to suppress bri1-5. Finally, the effects of BRS1 are selective: overexpression in either wild-type or two other receptor kinase mutants resulted in no phenotypic alterations. These results strongly suggest that BRS1 processes a protein involved in an early event in the BRI1 signaling.

Brassinosteroids (BR) are general regulators of plant growth and development. BR biosynthetic and response mutants have a similar complex phenotype characterized by dwarfed stature, round and curled leaves, reduced male fertility, and delayed flowering and senescence (1–5). The BR biosynthetic mutants can be rescued by the application of exogenous BR. The signal perception or transduction mutants, on the other hand, cannot be rescued by exogenous BR and are known as brassinosteroid-insensitive (bri) mutants (1). Interestingly, all of the over 20 bri mutants reported to date represent a single mutant locus, bri1 (1–5), which suggests that bri1 is the only nonredundant or viable mutant locus in the BR signaling pathway. BRI1 has been cloned via a position-based strategy, which demonstrated that it encodes a leucine-rich repeat receptor-like protein kinase (LRR-RLK) (3). Whereas LRRs have been defined as a protein–protein interaction domain in many cases (6), the function of the LRRs of BRI1 remains uncertain.

The role of the BRI1 LRR domain in BR signaling has been examined by expression of a chimeric receptor, containing the BRI1 extracellular domain and the protein kinase domain of Xa21, in rice cells (7). Xa21 is an LRR-RLK from rice that has been cloned via a position-based strategy, which demonstrated that it encodes a leucine-rich repeat receptor-like protein kinase (LRR-RLK) (3). Whereas LRRs have been defined as a protein–protein interaction domain in many cases (6), the function of the LRRs of BRI1 remains uncertain.

The activation–tagging transgenic lines were generated in a bri1-5 (Ws-2 ecotype; ref. 4) background with construct pSKI015 (9) via floral dipping Agrobacterium-mediated transformation technique (10). Plants were grown at 23°C under continuous illumination (~100 μmol·m−2·sec−1). bri1-5 brs1-1D was identified in a screen of 2,500 transgenic plants. Homozygous bri1-5 brs1-1D plants were used for all of the experiments described in this paper. Genomic DNA was isolated from 2-week-old Arabidopsis plants with Nucleon Phytopure Plant DNA Extraction Kit (Amersham Pharmacia). For BRI1 cloning, 10 μg of genomic DNA was digested with XhoI in a 100-μl volume. After digestion, the enzyme was heat-inactivated, and the reaction was passed through a CONCERT Nucleic Acid Purification System (GIBCO/BRL). A ligation was set up in a 100-μl volume, and 4 μl of the ligated solution was used as the template for inverse PCR. The ligated DNA yielded a 2-kb inverse PCR product with two inverse PCR primers (T3 long primer: 5′-ATAATACCT-CACCTAAAGGGAACAAAAG; ACTRB primer: 5′-GTTT-TCTAGATCAGGAACACTAGTT). For reverse transcription (RT)-PCR, 2 primers were used (BR5AbDNAFoward: 5′-TCTGTACCATGCAAGAACCCATT; BR5AbDNAREverse: 5′-TCCTGTCTCCTAAATGATCTCTG). The RT-PCR product (BRS1 cDNA) was cloned into pBluescript (Stratagene) SK+ at KpnI and SacI sites.

Total RNA Isolation and Northern Analyses. Total RNA samples were isolated from 4-week-old above ground tissues with an Rneasy Midi Kit (Qiagen, Chatsworth, CA). Ten micrograms of total RNA were used for each lane in the Northern analyses. After hybridization, the blots were exposed to x-ray film for 5 days to detect the BRS1 expression in wild-type and bri1-5 plants.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: BR, brassinosteroid; BRI1, brassinosteroid insensitive 1; BR5Ab, bri1 suppressor 1; RLK, receptor-like protein kinase; LRR, leucine-rich repeat; RT-PCR, reverse transcription-PCR; T-DNA, portion of the Ti (tumor-inducing) plasmid that is transferred to plant cells.

To whom reprint requests should be addressed. E-mail: WalkerJ@missouri.edu.

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Vector Construction, Site-Directed Mutagenesis, and Gene Transformation. To recapitulate the overexpression phenotype, the BRS1 cDNA was cloned into the KpnI and SacI sites of pBIB-KAN (11) with a dual enhanced 35S promoter. S181F and H438A mutants were introduced with a QuickChange Site-Directed Mutagenesis Kit (Stratagene) in a pBluescript SK+ vector. The mutagenized BRS1 cDNA was then cloned into pBIB-KAN.

Generation of Double and Triple Mutants. brl-5 and brl-5 brs1-1D were crossed with dwf-4 or det2-101. The F1 were allowed to self-fertilize to generate a segregating F2. In the F2, double and triple mutants were identified by genotyping individuals by PCR and DNA sequencing. The plants containing brs1-1D have a BAR resistance gene (9). The plants surviving application of 0.25 g/liter glutosate-ammonium (Finale, AgrEvo Environmental Health, Montvale, NJ) were confirmed by PCR genotyping, by using a T7 primer (from the T-DNA of SKI075; T7: 5'-GTGATGAAATCGTACTATAGGGCGAATTG) and a 3' end BRS1 specific primer (brs1/cDNA3'SacI: 5'-TCTTGAACCTCCTAGTTCAAGTCTCAGAGAAGACTTTTTCAAGCTTC), and bri1/cDNA3'SpI (5'-TCGTGACACCTCAATGTCTCTTCTAGGAAACTTC). The PCR product was then used as the template for sequencing by using primer bri1/CDS5'KpnI, and bri1/cDNA3'SpI KpnI was genotyped by PCR by using two primers flanking the T-DNA insertion as described previously (12).

Results

Identification of brs1-1D Locus. Genetic analyses of BR signaling have thus far failed to identify other BR perception/transduction regulatory components. We hypothesized that some components of the BR signaling pathway have not been identified in previous screens because of functional redundancy. Genetic modifier screens using activation tagging allow for the identification of new regulatory proteins that are functionally redundant (9). Taking advantage of a fertile partial loss-of-function brl allele, brl-5 (4), we generated transgenic plants harboring an activation tagging T-DNA, designed to activate expression of genes in the vicinity of the insertion. From 2,500 transgenic plants, we identified a single plant in which the brl mutant phenotype was suppressed. Genetic analysis showed that the suppression is dominant and cosegregates with the single T-DNA insertion in this line. The mutant was named brs1-1D for brl suppressor-Dominant.

brs1-1D Suppresses Multiple brl-5 Defects. Physiological studies demonstrated that multiple brl-5 defects are suppressed by brs1-1D (Fig. 1A). The primary inflorescence stems of brl-5 brs1-1D plants are twice as long as those with brl-5 alone; the rosettes are wider, because the leaves are bigger and not curled (Fig. 1A and B). In addition, brs1-1D suppresses the seconded inflorescence branch length phenotype of brl-5 (Fig. 1B). Furthermore, brs1-1D suppresses the late flowering of brl-5 (Fig. 1B). Other traits, which are unaffected in brs1-1D relative to wild-type plants such as flower number, coflorescence number and branch number, were not altered in brs1-1D plants (data not shown).

BRS1 Encodes a Serine Carboxypeptidase-Like Protein. We cloned the genomic DNA flanking the brl-5 brs1-1D T-DNA by inverse PCR (Fig. 2 A and B). Sequence analysis revealed that 1.1 kb from the right border of the T-DNA is a gene encoding a putative serine carboxypeptidase. This predicted protein has homology with wheat and barley serine carboxypeptidase II proteins, and with yeast Kex1p (13–15). These proteases belong to the S10 family and are in a group named carboxypeptidase D (E.C. 3.4.16.6; ref. 16). The proteases of this group preferentially remove Arg or Lys from the C terminus of a peptide. There is a typical N-terminal signal peptide in the predicted protein that would direct the protein to the secretory pathway (Fig. 2C). The serine carboxypeptidase “catalytic triad,” S181, D386, and H438 are conserved with other carboxypeptidase D group proteases. Sequence alignment also revealed a possible cleavage linker peptide, indicating that this serine carboxypeptidase itself may be processed within the endoplasmic reticulum (ER)-trans Golgi network to form a mature protease, similar to other plant serine carboxypeptidases (17).

Overexpression of a BRS1 cDNA Recapitulates the Suppression Phenotype. Based on the gene annotation, we cloned the cDNA encoding this serine carboxypeptidase by RT-PCR. Northern blots showed that steady-state level of the serine carboxypeptidase mRNA in brl-5 brs1-1D plants is about 30 times higher than in wild-type or brl-5 plants (Fig. 3). Expression of this serine carboxypeptidase mRNA in wild-type plants or brl-5 plants is relatively low (Fig. 3). To confirm that brl-5 suppression is due to the overexpression of this serine carboxypeptidase, the cDNA clone was placed under the control of a CaMV 35S promoter. S181F and H438A mutants were introduced with a QuickChange Site-Directed Mutagenesis Kit (Stratagene) in a pBluescript SK+ vector. The mutagenized BRS1 cDNA was then cloned into pBIB-KAN.
A

Chromosome 4

BRS1

gene for signal recognition particle receptor-like protein

gene for hypothetical proteins

T-DNA from pSK1015

4X 35S enhancers

4X 35S enhancers

ATG

TAG

2 kb

2 kb

1 kb

1 kb

BRST1 cDNA

MARTPH1FLLVLALLSTTPSSSSREQKDIXKALPGQ
KVAF50YSGVYVH0QGRLALYLYLSSPSISPKPILLL
WLLNGPPCSS1AYASEEEIFGPR1KXSNNLLNRFPAWNR
DANLL1PLEPSAGVYSGTYVSDLGKGDGERTQCNLTLF
IWRFLSLQFYKQFDYFGASEGASCYHVPLNKKINDKNA
FSKPI1INLKGPLQGNAIVDNSGDITVTYWTHAIISR
SYKSLIKYLNQVENDCCDNAVNYAMNFEDGIDQYSI
YFTPCUAQQKRT7GDFVRMKNLARRLA3CT1ES1
YADCFYFSPRSYDPRAMTVTDLGCWYHAFYK617KDS
DKTMLP1YKELAASGLWRIWFGSDT7SQVTPATRFSLSH
LNLFVKTNPYCTDQVQTWTEVKGLTFTVRAGHEV
PLLFEFPR1LLIRSRPLAGKELRSTY

Fig. 2. The BRS1 gene encodes a protein with homology to serine carboxypeptidases (54% and 53% identity with wheat and barley serine carboxypeptidase II proteins; 28% identity with yeast Kex1p protein). (A) The flanking sequence of the T-DNA was cloned via inverse PCR. The T-DNA insert localizes to the bottom part of chromosome IV, 5' of the T-DNA, 6.5 kb from the 4 X 35S enhancers, there is a gene encoding a signal recognition particle-receptor-like protein. Overexpression of BRS1 was confirmed as the suppressor, BRS1. (B) Comparison of the cDNA and genomic sequences indicated that BRS1 has 9 exons and 8 introns. (C) Deduced amino acid sequence of BRS1. A possible signal peptide cleavage site is identified by an arrow. Five potential N-linked glycosylation sites are marked in the open boxes. The asterisks below an amino acid indicate the three putative "catalytic triad" amino acids, S181, D386, and H438. A possible cleavage linker peptide is underlined. The BRS1 sequence was obtained from GenBank (accession no. AL161577; reference GI: 7269962).

BRST1 May Be Functionally Redundant. We hypothesized that BRS1 is functionally redundant, in which case the loss-of-function brs1 mutant would have no phenotype. The Wisconsin Arabidopsis T-DNA insertion lines (20) were screened, and a plant in which a single T-DNA is inserted in the first intron of BRS1 was identified. This line lacks any detectable BRS1 mRNA by Northern or RT-PCR analysis but is phenotypically normal (data not shown). BLAST searches (21) indicated that BRS1 is part of a gene family. At least 36 carboxypeptidase-D-like/carboxypeptidase-II-like genes are encoded in the Arabidopsis genome. Only 5 of them, however, are closely related to BRS1 and share 52% to 73% amino acid sequence identity with BRS1. Four of the five closely related gene products contain putative signal peptides. Taken together, these findings support the hypothesis that one or more genes may be functionally redundant with BRS1.

BRST1 Selectively Regulates the BRI1 Signaling Pathway, and a Functional BRI1 Kinase Domain Is Required for Suppression. To determine whether BRS1 acts selectively in the BRI1 signaling pathway, a 35S::BRS1 cDNA construct was transformed into bri1-9 (4), bri1-1 (5), cdl-1 (22), Ler (Landsberg erecta; ref. 23), and wild-type plants (Table 1). The transformation results indicated that overexpression of BRS1 suppresses a second bril allele, bri1-9. Both bril-5 and bri1-9 have missense mutations in the BRI1 extracellular domain (4). In bri1-5, a cysteine is replaced by a tyrosine residue (C69Y). In bril-9, a serine is changed to phenylalanine (S662F). Overexpression of BRS1 in a BRI1 cytoplasmic kinase domain mutant, bri1-1 (A909T; ref. 5) did not result in any suppression phenotype (Table 1), indicating that a functional BRI1 kinase domain is required for the regulation provided by BRS1. In addition, overexpression of BRS1 in wild-type plants resulted in no obvious phenotypic alterations, although the expression of BRS1 is elevated in the WT bri1-1D plants (Fig. 5). These data suggest that BRS1 does not act as a general growth regulator. Transformation data also showed that the clavata1 and erecta RLK mutants of Arabidopsis are not suppressed by BRS1 (Table 1). Together, these results indicate that BRS1 selectively regulates BRI1 signaling.

BR Plays an Essential Role for BRS1’s Action. Recent studies from the Chory laboratory indicated that BR regulates the perception part of the BRI1-mediated signal transduction pathway (7). To investigate the role of BR in the regulation conferred by BRS1, we created bri1-5 dwf4-1 double and bri1-5 dwf4-1 brs1-1D triple mutants. dwf4 mutants are defective in BR biosynthesis (12). The triple mutant plants did not show any suppression phenotype compared with the double mutant plants. This result suggests BR is essential for BRS1 action (Fig. 6). Similar results were observed when another BR biosynthetic mutant, det2-101 (24), was used to replace dwf4-1 in the double and triple mutant analyses (data not shown).

Carboxypeptidase Activity Is Required for BRS1 Function. To test whether the serine carboxypeptidase activity is required for suppression, we created missense mutants in the "catalytic triad," known to be essential for serine carboxypeptidase enzymatic activity (16). bri1-5 plants overexpressing these missense BRS1 mutants, S181F and H438A, did not show the suppressed phenotype as summarized in Table 1. The expression of BRS1 mutants has been confirmed by Northern analyses (data not shown). Thus, the serine carboxypeptidase activity of BRS1 is necessary for suppression of bril mutant phenotypes.

Discussion Because BRS1 overexpression suppresses multiple bril defects, BRS1 might play an important role in an early stage of the BRI1 signaling pathway. The presence of an N-terminal signal peptide in BRS1 predicts that the protein should enter the secretory pathway. Sequence analysis failed to identify any endoplasmic reticulum or Golgi apparatus retention or retrieval sequences. Therefore, BRS1 is likely to be a secreted protein. In addition, BRS1 can suppress two weak BRI1 extracellular domain mu-
tants, bri1-5 and bri1-9, but failed to suppress a cytoplasmic kinase domain mutant, bri1-1. Finally, the presence of BR is essential for BRS1’s suppression activity because BRS1 cannot phenotypically affect the mutants harboring homozygous BR-deficient loci, dwf4-1 and det2-101. These results are consistent with the hypothesis that BRS1 regulates an early event in BRI1 signaling. The observation that transgenic plants carrying missense mutations in the BRS1 protease “catalytic triad” failed to suppress the bri1-5 phenotype suggests that carboxypeptidase activity is required for the suppression.

Proteases are required in many signaling pathways. For example, in yeast, both Kex1p and Kex2p are required for the excision of signaling peptides, α-mating pheromone, and K1 killer toxin, from their inactive precursors (15, 25). Kex2p is a membrane-bound endoprotease that specifically cleaves on the carboxyl side of pairs of basic amino acids (e.g., KR or RR). After the action of Kex2p, Kex1p, a type D serine carboxypeptidase, selectively trims off the flanking amino acids from the C terminus of processing intermediates.

In addition to peptide ligand processing, there are also examples of receptor proteolytic processing. One case of this processing is the insulin receptor. The insulin proreceptor is synthesized as inactive precursor. It is then processed at a RKRR site by an endoprotease in the trans Golgi network to form a mature receptor (26).

In plants, there are few reports concerning the processing of ligand-like peptides or receptor-like proteins. In response to wounding, tomato systemin is processed from its inactive form, preprosystemin (27). Also in tomato, a secreted leucine-rich repeat protein (LRP), which was thought to be involved in a plant defense response, is proteolytically processed during pathogenesis (28). It is not clear whether prosystemin is cleaved by a subtilisin-like endoprotease, but it has been found that systemin physically interacts with a subtilisin-like endoprotease (27). LRP is likely processed by a subtilisin/Kex2p-like endoprotease. Additionally, the functions of two Arabidopsis Kex2p-like genes have been determined: AIR3 is involved in the regulation of auxin-induced lateral root formation (29) and SDD1 functions in guard cell development (30). The regulatory roles of serine carboxypeptidases in plants have not yet been investigated.

Does BRS1 process the BRI1 receptor? In a recent report from He and colleagues (7), the BRI1 extracellular domain was fused with the intracellular kinase domain of Xa21 to test the hypothesis that the extracellular domain of BRI1 is the recognition domain for BR. In this study, the functional chimera was the molecular weight expected of a full-length receptor, suggesting that BRI1 is not proteolytically processed.

Does BRS1 process a proteinaceous proligand or an extracellular BR binding protein? Whereas ligands that function in two other RLK-signaling pathways, CLAVATA3 (31) and SCR (32),
contain possible Kex2p-processing sites, a proteinaceous ligand for BRI1 has not yet been described. However, at least two putative steroid-binding proteins, which contain signal peptides, could be the substrates of BRS1. The proteolytic processing may resemble the actions of yeast Kex1p and Kex2p, in which an Arabidopsis Kex2p-like endoprotease may recognize and cleave a dibasic site in its substrate. After cleavage, BRS1 trims the intermediate and releases an active BR binding protein. The BR–BR binding protein complex (ligand complex) then binds to the extracellular domain of BRI1 and triggers a series of cellular responses. Because of the extracellular domain mutation in bri1-5, ligand activation of the BRI1 receptor may be rate limiting. Elevated expression of BRS1 would increase the amount of the active steroid binding protein, which would favor formation of ligand complex-BRI1 receptor binding. Increase in ligand binding would enhance BRI1-5 receptor activity and the signal transduction pathway. As a result, multiple bri1-5 defects are suppressed.

The results reported in this study show that the serine carboxypeptidase encoded by BRS1 is involved in the BRI1 signaling pathway. Although it is not clear whether proteases function in other RLK-signaling pathways, the identification of BRS1 suggests that protein processing/proteolysis in plant signal transduction is important.

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