Arabidopsis BRS1 Is a Secreted and Active Serine Carboxypeptidase*

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The Arabidopsis BRS1 gene encodes a serine carboxypeptidase II-like protein. Its biological role in the brassinosteroid signaling pathway was first established by its capability to specifically suppress a weak brassinosteroid insensitive 1 (bri1) allele, bri1-5, when overexpressed. To gain additional insights into the molecular mechanisms of BRS1 function, the subcellular localization and the biochemical characteristics of BRS1 were determined by using transgenic plants harboring a 35S-BRS1-GFP construct and fusion proteins purified from 35S-BRS1-FLAG transgenic plants. The BRS1-GFP protein was mainly secreted and accumulated in the extracellular space. Immunological data suggest that BRS1 is proteolytically processed by an unknown endoproteinase in planta. Affinity-purified BRS1-FLAG from transgenic plants show strong hydrolytic activity with a broad P1 substrate preference including basic and hydrophobic groups on either side of the scissile bond. The hydrolytic activity of BRS1 can be strongly inhibited by a serine protease inhibitor, phenylmethylsulfonyl fluoride. The pH and temperature optimas for the hydrolytic activity of BRS1 are pH 5.5 and 50 °C, respectively. These data demonstrate that BRS1 is a secreted and active serine carboxypeptidase, consistent with the hypothesis suggested by our previous genetic evidence that BRS1 may process a protein involved in an early event in the BRI1 signaling pathway.

Serine carboxypeptidases (Ser-CPs) are widely distributed proteases identified in most higher organisms. The major structural characteristic of these proteins is that they contain a conserved amino acid triad, Ser-His-Asp, catalytically essential for enzyme activity (1). In mammals, Ser-CPs are largely involved in producing active peptide hormones from their inactive precursors. This process usually requires two consecutive steps. First, a larger precursor is cleaved at selective sites by an endopeptidase such as prohormone convertase 1, prohormone convertase 2, or furin. The Ser-CPs are then responsible for trimming off the exposed carboxyl-terminal amino acids and transforming the inactive intermediates into active hormones (2, 3).

In plants, extensive studies of Ser-CPs have been mainly focused on their functions in turnover and mobilization of storage proteins using as nitrogen and carbon resources during seed germination and senescence.

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The abbreviations used are: Ser-CP, serine carboxypeptidase; BR, brassinosteroid; CIAP, calf intestinal alkaline phosphatase; FAA, furylacryloyl; GFP, green fluorescent protein; GI5, β-glucuronidase; LRR, leucine-rich repeat; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MES, N-morpholineethanesulfonic acid; MS, mass spectrometry; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RLK, receptor-like protein kinase; RT, reverse transcription.
cloned into a binary vector pBIG-HYG (14). The resulting construct was named pHYG-BRSp-GLUS. Transgenic plants harboring 3SS-BRS1-GFP, 3SS-BRS1-FLAG, and 3SS-BRS1(H438A)-FLAG were generated in a br1-5 background. Transgenic plants harboring BRS1p-GLUS were generated in wild type Arabidopsis (ecotype WS2) plants. In each case, the homozygous transgenic plants were selected from the T3 generation and used for the analyses described in this paper except as otherwise specified. All plants were grown in a 16-h light and 8-h dark growth chamber at 20 °C, whereas seedlings were grown under continuous light at 20 °C.

RT-PCR Analyses—Total RNA was isolated using the RNaseasy plant mini kit (catalog number 74904) from Qiagen (Germantown, MD). For reverse transcription, SuperScript II RNase H− reverse transcriptase from Invitrogen was used (catalog number 18064-014). Two μg of total RNA was reverse-transcribed to the first strand of the cDNA in a 20-μl volume. A 1-μl volume of the RT product was used as a PCR template. Thirty cycles were used for amplifying BRS1 and BRI1 cdNA, and 20 cycles were used for amplifying the quantity control, EF1-α. Twenty-one PCR cycles were used to compare the BRS1 expression level in inflorescence stems.

GLU Staining of phyG-BRS1p-GLUS Transgenic Plants—T3 transgenic plants harboring BRS1p-GLUS were used for histochemical GLU staining. Plant tissues were vacuum-infiltrated in X-Gluc solution (15) and incubated at 37 °C for 6 h followed by destaining with 70% ethanol.

Confocal Microscopy—Root tips from 3–5-day-old T3 transgenic br1-5 plants harboring 3SS-BRS1-GFP were used for confocal microscopy analysis. Seeds were planted vertically on semi-solid one-half Murashige and Skoog medium and used for the analyses described in this paper except as otherwise specified. All plants were grown in a 16-h light and 8-h dark growth chamber at 20 °C, whereas seedlings were grown under continuous light at 20 °C.

Protein Deglycosylation and Dephosphorylation—Hydrolytic activities of BRS1-FLAG toward different dipeptides were determined according to a method adapted from Plummer and Kimmel (16) and Latchinian-Sadek and Thomas (17). Furylacryloyl (FA)-dipeptides were purchased from Bachem BioSciences. The anti-FLAG M2 agarose affinity gel was run on a 12.5% SDS-polyacrylamide gel and blotted to a Biotrans nylon membrane (catalog number M1821, Promega, Madison, WI) treatment experiments. The gel was stained with 1 μl of CIAP incubated at 37 °C for 2 h. In the control experiment, the dye used was enhanced GFP, and the laser output was 2, the dye used was enhanced GFP, and the laser output was 2. The resulting solution was spun at 6000 × g for 6 min at 4 °C and the supernatant was collected as the total crude protein sample. The supernatant was further spun at 100,000 × g for 25 min at 4 °C. The resulting supernatant was saved as the soluble protein sample. The pellet was resuspended and further homogenized in membrane solubilization buffer (10 mM Tris-HCl (pH 7.3), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 μM pepstatin, and 10 μM E-64) to immunodeect the BRS1 protein. The solution was spun at 100,000 × g for 25 min at 4 °C to separate solubilized membrane proteins (supernatant) from the insoluble membrane fraction (pellet).

Protein samples from different protein fractions were mixed with 1× PBS prewashed anti-FLAG M2 agarose affinity gel (catalog number A2220; Sigma). After overnight shaking at 4 °C, the gel was washed 5 times with cold 1× PBS (PBS and 0.1% Tween 20). Immunoprecipitated protein was eluted in 2× SDS sample buffer and run on a 7.5% SDS-polyacrylamide gel. The presence of BR51-FLAG in different protein fractions was demonstrated by Western blotting.

Protein Affinity Purification—Two-week-old seedlings of br1-5, bri1-5 3SS-BRS1-FLAG or br1-5 3SS-BRS1(H438A)-FLAG were harvested and ground to fine powder in liquid N2, respectively. The suspension formed after the addition of membrane solubilization buffer as mentioned above was further homogenized to release the soluble and membrane proteins. After spinning at 100,000 × g for 25 min at 4 °C, soluble and membrane proteins were collected for affinity purification. The anti-FLAG M2 agarose affinity gel was transferred to a column (catalog number 731-1550; Bio-Rad), and the gel was washed with at least 20× volumes of 1× PBS. Then the protein samples were loaded into the column, and the flow-through was collected. Loading of the flow-through was repeated five times. The gel was washed with at least 20× volumes of 1× PBS, and finally the BRS1-FLAG was eluted with 4× 1.5-column volumes of FLAG peptide (100 ng/μl, prepared in 1× PBS; catalog number F3290, Sigma) by competition. The protein concentration was measured with a Bradford assay kit (catalog number 500-0006, Bio-Rad). Eluted protein was used for SDS-PAGE (12.5%) gel analysis and enzyme activity assay.

Protein Deglycosylation and Dephosphorylation—Soluble proteins isolated from br1-5 and bri1-5 3SS-BRS1-FLAG seedlings (5 mg) were affinity-purified with anti-FLAG M2 agarose affinity gel and used for endoglycosidase H (catalog number 1088726; Roche Diagnostics Corporation) and calf intestinal alkaline phosphatase (CIAP; catalog number M1821, Promega, Madison, WI) treatment experiments. The gel was washed with 280 μl of water, and 15-μl aliquots of the gel were used for different treatments. Endoglycosidase treatment involved 50 μM potassium phosphate buffer (pH 5.8) and 250 milliunits of endoglycosidase H incubated at 37 °C for 3 h. CIAP treatment involved 1× CIAP buffer and 10 μl of CIAP incubated at 37 °C for 2 h. In the untreated control, an equal volume of water was added instead of endoglycosidase H or CIAP. After the treatment, proteins were eluted in 2× SDS sample buffer.

Mass Spectrophotometric Assay—Affinity-purified protein with anti-FLAG M2 agarose affinity gel was run on a 12.5% SDS-polyacrylamide gel. The gel was Coomassie Blue-stained and destained in 10% ethanol and 10% acetic acid in H2O. The chosen bands were excised. In-gel trypsin digestion and MALDI-TOF mass spectrometry were conducted at the proteomics and mass spectrometry facility at the Donald Danforth Plant Science Center in St. Louis, MO.

Enzyme Activity Assay—Hydrolytic activities of BR51-FLAG toward different dipeptides were determined according to a method adapted from Plummer and Kimmel (16) and Latchinian-Sadek and Thomas (17). Fucyacycloyl (FA)-dipeptides were purchased from Bachem BioSciences Inc., King of Prussia, PA.

For the time course of BRS1 hydrolytic activity, ∼400 ng of purified BRS1-FLAG protein was incubated in 1 ml of 25 mM MES (pH 5.5) containing 0.1% Triton X-100 and 1 mM FA-Arg-Leu at 37 °C for 1h.
The reduction of absorption at 342 nm was monitored every 10 min using a spectrophotometer (GENESYS 5, Spectronic Instruments, Inc. Rochester, NY). The PMSF treatment was performed as described by Latchinian-Sadek and Thomas (17).

**Substrate, pH, and Temperature Profiles**—Affinity-purified BRS1-FLAG was used for the substrate, pH, and temperature profile assays. All of the reactions except the optimal temperature experiment were done at 37 °C. The same amount of purified BRS1-FLAG and reaction buffer as described above were used for optimal substrate and temperature assays. The concentration of the substrate was 1 mM in all the reactions. The reduction of absorption at 342 nm was recorded after 1 h of incubation. One mM FA-Phe-Ala was used for the optimal pH and temperature experiments.

The optimal pH for the hydrolytic activity was tested over the pH range of 4.0–7.0. The ion strength for all buffers was 25 mM. Sodium citrate buffer (pH 4.0–5.0), MES buffer (pH 5.5), and Bis-Tris buffer (pH 6.0–7.0), respectively, were used for the assays.

**RESULTS**

**BRS1 Exhibits a Broad Expression Pattern**—brs1-1D (bri1 suppressor 1-Dominant 1) is a dominant bri1-5 suppressor identified by our previous activation-tagging genetic screen (6). Overexpression of BRS1 can specifically suppress bri1 extracellular domain mutants instead of an intracellular kinase-dead mutant, suggesting that BRS1 may play a role in the early events of the BR signaling pathway. Because BRI1, the gene encoding the BR receptor, is expressed mainly in young tissues but less abundantly in mature tissues (18, 19), we predict that BRS1 may have a similar or overlapping expression pattern. RT-PCR analyses indicated that BRS1 is expressed in almost all of the tissues tested (Fig. 1A). Unlike BRI1, however, BRS1 can only be detected at lower levels in roots and


stems. Consistently, transgenic plants harboring BRS1p-GUS show that BRS1 is mainly expressed in young tissues such as cotyledons, young leaves, unopened flowers, and meristems (Fig. 1, B–E). These results clearly indicate that BRS1 and BRI1 have overlapping expression domains.

Interestingly, we observed that most of the bri1-5 35S-BRS1-FLAG lines show ~3-fold the height of the bri1-5 plants, whereas bri1-5 brs1-1D plants are only about two times the height of bri1-5. To test whether the BRS1 expression level is the cause of the plant height difference, total RNA was extracted from primary inflorescence stems of bri1-5, bri1-5 brs1-1D, and one representative line of bri1-5 35S-BRS1-FLAG, and RT-PCR analysis was conducted. As expected, the more the BRS1 is expressed, the taller the transgenic plants grow (Fig. 1F). These results are consistent with the idea that the reduced BR perception caused by the point mutation in bri1-5 can be partially restored by the overexpression of BRS1.

**BRS1-GFP Is Localized to the Exterior of the Cell**—It was suggested from our previous genetic data that BRS1 may be involved in an early step in BR signaling. Because the BR perception occurs extracellularly, we predict that BRS1 should be a secreted protein. Sequence analysis indicated that BRS1 contains a typical N-terminal signal peptide but failed to identify any endoplasmic reticulum or Golgi retention signals, implying that BRS1 may be secreted from cells. To confirm this hypothesis, we generated transgenic bri1-5 plants harboring a BRS1-GFP fusion protein. These transgenic plants exhibit the bri1-5 suppression phenotype similar to that of the original bri1-5 brs1-1D suppressor and the bri1-5 35S-BRS1-FLAG transgenic plants, suggesting that the BRS1-GFP fusion protein is functionally equivalent to BRS1 in planta (Figs. 2A and 4A). Therefore, the localization revealed by BRS1-GFP should represent that of native BRS1. Using a GFP antibody to analyze the total protein extracts from the transgenic plants, several specific bands were detected. Among them, an 85-kDa band may represent the intact BRS1-GFP protein, and a 44-kDa band may represent the C-terminal BRS1 fragment attached to GFP after a predicted processing step (6). Other smaller bands seen in the Western assay may result from partial digestion of the BRS1-GFP. Further confirmation of BRS1-GFP processing will be discussed in the latter part of this section (“Results”).

Root tips from 3–5-day-old bri1-5 35S-BRS1-GFP seedlings (T2) were prepared for confocal microscopy assay. bri1-5 and bri1-5 35S-BAK1-GFP plants from similar developmental stages were used as negative and positive controls, respectively. BAK1 (BRI1-associated receptor kinase), another crucial component in BR signal transduction pathway, was demonstrated to be a plasma membrane protein (20, 21). Without any treatment, the localization of the green fluorescence signals for both BRS1-GFP and BAK1-GFP are indistinguishable, as both are apparently localized on the cell surface; after plasmolysis was induced with 0.8 M mannitol, the green fluorescence signal of BAK1-GFP moved with the plasma membrane, indicating its plasma membrane localization. The majority of the green fluorescence signal of BRS1-GFP, however, stayed in the cell wall, and only part of the green fluorescence signal moved with the plasma membrane. These observations suggest that BRS1 protein is mainly secreted and that some of the BRS1-GFP protein may associate with yet unknown membrane proteins (Fig. 2B).

**BRS1 Is a Glycoprotein**—To further confirm that BRS1 is mainly a secreted protein and may be partially associated with membrane proteins as seen in the confocal microscopy results, the distribution of the BRS1-FLAG protein in different protein fractions was investigated. Different protein fractions were isolated from bri1-5 and homozygous bri1-5 35S-BRS1-FLAG seedlings. BRS1-FLAG is a glycoprotein in planta, because bri1-5 plants harboring 35S-BRS1-FLAG show a similar suppression phenotype as that of bri1-5 brs1-1D (Fig. 4A). Equal amounts of protein samples from different protein fractions were immunoprecipitated with anti-FLAG M2 agarose affinity gel overnight.
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FIGURE 4. BRS1 protein is proteolytically processed in vivo. A, phenotypes of 3-week-old plants. Wild type BRS1-FLAG is functional in bri1-5, whereas the mutation H438A in BRS1 abolishes its function. Bar, 1 cm. B, BRS1-FLAG protein is cleaved to two chains. Protein was immunopurified from bri1-5; bri1-5 35S-BRS1(H438A)-FLAG, and bri1-5 35S-BRS1-FLAG plants. Left section shows Coomassie blue-stained protein samples. About 300 ng of eluted protein was loaded for each lane. M, molecular mass marker; F1 and F2, two cleaved bands. Right section shows the Western blot result with anti-FLAG antibody (about one-fifth of the protein was loaded in this gel). Arrow, full-length BRS1-FLAG protein; asterisk, the C-terminal BRS1-FLAG peptide. Protein was run on a 12.5% SDS-polyacrylamide gel. C, deduced amino acid sequence of BRS1. Five tryptic peptides in a quadrupole-time-of-flight assay of F1 are shown in red. The putative cleavage linker peptide is marked as blue and italic. Arrowhead shows the predicted signal peptide cleavage site. D, MS/MS spectrum of peptide ALPGQPK in protein fragment F1. E, BRS1 is processed in vivo. Total protein was extracted with SDS buffer with (+) or without (−) mercaptoethanol (β-ME). Fifteen μg of protein was loaded per lane. The gel was hybridized with anti-FLAG antibody. Arrow shows chain B; triangle shows the A and B complex. F, BRS1 is not self-processed. Immunopurified BRS1-FLAG was incubated at 37 °C in 25 mM MES buffer, pH 5.5, with 0.1% Triton X-100. For the PMSF treatment, 1 mM PMSF was added to the protein and incubated on ice for 2 h before the incubation at 37 °C. About 50 ng of protein was loaded on each lane. The gel was hybridized with anti-FLAG antibody.

at 4 °C and eluted in 2× SDS sample buffer. The eluted protein samples were run on SDS-polyacrylamide gel and analyzed by Western analysis. Consistent with the subcellular localization of BRS1-GFP, Western results showed that BRS1-FLAG can be detected in both total crude and soluble protein fractions and that a small amount of BRS1-FLAG can be processed in vivo. Total protein was extracted with SDS buffer with (+) or without (−) mercaptoethanol (β-ME). Fifteen μg of protein was loaded per lane. The gel was hybridized with anti-FLAG antibody. Arrow shows chain B; triangle shows the A and B complex. F, BRS1 is not self-processed. Immunopurified BRS1-FLAG was incubated at 37 °C in 25 mM MES buffer, pH 5.5, with 0.1% Triton X-100. For the PMSF treatment, 1 mM PMSF was added to the protein and incubated on ice for 2 h before the incubation at 37 °C. About 50 ng of protein was loaded on each lane. The gel was hybridized with anti-FLAG antibody.

Characterization of BRS1 protein in vivo

Many plant serine carboxypeptidases need to be cleaved into A and B chains for activity. For example barley carboxypeptidase I is cleaved into two polypeptide chains for a heterodimer linked by disulfides, important for catalytic activity. The linker peptide containing 55 residues is endoproteolytically excised (22). There is one predicted cleavage linker in the BRS1 protein sequence (6). A Western assay suggested that BRS1 may be processed in vivo (Fig. 2A). To further confirm the in vivo processing of BRS1, large scale protein preparation and affinity purification were performed. Based on the immunological results of different protein fractions, total protein including soluble and membrane fractions was used for affinity purification. Homozygous bri1-5 35S-BRS1(H438A)-FLAG transgenic plants were used as a control. To ensure the quality of the affinity purification procedure, nontransgenic bri1-5 plants were included as a negative control. In BRS1(H438A)-FLAG, one of the catalytic triad, His, was mutated to Ala. Overexpression of BRS1(H438A)-FLAG in bri1-5 failed to suppress bri1-5 defective phenotypes, which implies that
BRS1(H438A)-FLAG is not biologically functional in planta (Fig. 4A). Both BRS1-FLAG and BRS1(H438A)-FLAG can be effectively purified from the transgenic plants. Typically, 9.37 μg of purified BRS1-FLAG can be recovered from 33.71 g of 2-week-old seedlings.

If BRS1-FLAG is cleaved as predicted, we expect to see two bands at 34 kDa (A chain) and 19 kDa (B chain), respectively. In Fig. 4B, the left section shows the Coomassie Blue-stained high percentage SDS-polyacrylamide gel (12.5%). About 300 ng of purified protein was loaded for each sample. In the lane for bri1-5 BRS1-FLAG, besides the intact BRS1-FLAG protein band there were two extra specific bands at ~36 kDa (F1) and 22 kDa (F2). The right section of Fig. 4B shows the Western analysis results with anti-FLAG M2 monoclonal antibody. Only the ~22-kDa band can be detected by the anti-FLAG antibody, because FLAG epitope was tagged only at the carboxyl terminus of BRS1. The molecular masses of these two bands are a little bit larger than predicted, possibly due to glycosylation. Trypsin digestion of the 36-kDa band shows that it is the N-terminal peptide of BRS1. Five tryptic peptides (ALPGQPK, TGSNLYLNKFAWNK, TAQDNLIFLIK, FPQYK, and AFSKPIINLK) were revealed using nano-electrospray quadrupole time of flight mass spectrometry (QTOF) (Fig. 4C). BRS1 protein is possibly cleaved within the first 30 amino acids to remove the signal peptide based on the peptide mass fingerprinting (DRIKALPGQPK peptide).

Fig. 4D shows the MS/MS spectrum of one of the tryptic peptides ALPGQPK in protein F1. Because all the detected peptides are within the predicted A chain from F1, we conclude that BRS1 is proteolytically processed to form A and B chains for activity, similar to that of wheat CPDW-II (23), barley carboxypeptidase I (22), and an Arabidopsis Ser-CP-like protein sinapoylgucose:choline sinapoyltransferase (SCT) (24).

To further demonstrate that BRS1 is processed in planta and was not cleaved by a contaminated endoprotease during a long affinity purification procedure, seedlings were quickly frozen in liquid nitrogen and total proteins were extracted with 2× SDS sample buffer in which all the proteins should be denatured. A Western blot analysis using this protein sample indicated that BRS1 was indeed cleaved to two chains in planta. The resulted A and B chains are apparently attached by disulfide bonds, because elimination of β-mercaptoethanol in the extraction buffer (sample buffer) greatly reduced the B chain signal (Fig. 4E).

To test whether BRS1 is processed by itself, the immunopurified BRS1 protein was incubated in a proteinase buffer (25 mM MES (pH 5.5) with 0.1% Triton X-100) used for BRS1 Ser-CP activity analysis. If BRS1 can process itself, we would expect to see the full-length BRS1 signal being gradually reduced, whereas on the other hand the signal of the processed product, the B chain signal, would be gradually increased. Half of the samples were treated with serine protease inhibitor PMSF. The fact that the full-length BRS1 signal and processed B chain signal remained unchanged during the incubation time period and that they showed no difference even with PMSF-treated samples clearly indicated that BRS1 cannot cleave itself. BRS1 is, therefore, processed by an in vivo unidentified protease. Interestingly, BRS1(H438A)-FLAG cannot be efficiently cleaved into A and B chains like its native counterpart (Fig. 4, 5D).
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B and E). It is possible that the point mutation in BRS1(H438A)-FLAG may have changed the tertiary structure of BRS1, which becomes inaccessible to the unknown endopeptidase responsible for the cleavage.

BRS1 Is an Active Serine Carboxypeptidase—To test whether BRS1 is an active serine carboxypeptidase and whether it has substrate preference, various dipeptides conjugated to the chromophore (FA), including a basic or a hydrophobic amino acid on either side of the scissile bonds, were used for enzyme activity assay. The enzyme activity was monitored by an absorbance decrease at 342 nm (1 mM substrate). Fig. 5 shows a time course of enzyme activity of the purified BRS1-FLAG protein toward the substrate FA-Arg-Leu. Almost no hydrolysis of FA-Arg-Leu by affinity-purified protein from bri1-5 35S-BRS1(H438A)-FLAG was detected; on the contrary, purified protein from bri1-5 35S-BRS1-FLAG consistently catalyzed the hydrolysis of FA-Arg-Leu.

When PMSF (2 mM), an irreversible inhibitor of enzymes with active serine residue, was added to the purified protein and incubated on ice for 2 h, the hydrolytic activity of BRS1-FLAG was inhibited by >90%. In contrast, leupeptin (100 μM), a reversible serine and cysteine protease inhibitor, can only inhibit the BRS1 hydrolytic activity by ∼50%. These data prove that BRS1 is an active serine carboxypeptidase with hydrolytic activity.

BRS1 Protein Has Broad Substrate Preference—Among the substrates tested, BRS1 can cleave basic (Lys or Arg) and hydrophobic (Leu, Phe or Ala) residues from the C terminus of the peptides, and FA-Arg-Leu is the optimal substrate. BRS1 cannot cleave acidic residues (Glu or Asp) (Fig. 6), similar to all other known Ser-CPs that do not remove acidic residues from the carboxyl terminus of the peptides (1).

Effect of pH and Temperature on the BRS1 Hydrolytic Activity—To obtain additional information about BRS1 biochemical characteristics, the effects of pH and temperature on the BRS1 hydrolytic reaction were analyzed. pH 5.5 appears to be the optimal pH for BRS1 hydrolytic activity (Fig. 7A). This result is consistent with other known Ser-CPs that have acidic optimum pH for activity toward peptide substrates (1). The optimal reaction temperature is ∼50–55 °C (Fig. 7B), which suggests that BRS1 is a heat stable enzyme, similar to the Ser-CP-like protein sinapoylglycerol:choline sinapoyltransferase, which was also heat stable (24).

DISCUSSION

Proteases are critical enzymes regulating many events in mammals, Drosophila, and yeast. In plants, both direct and indirect evidence suggest that proteases are important for many processing aspects during plant growth and development. For example, identification and characterization of several plant peptide hormones and their encoded genes suggest that many plant peptide hormones are initially synthesized as larger precursors. These precursors require proteases to process them into active peptide hormones. Systemin, an 18-amino acid defensive peptide signal, is synthesized in plants in response to herbivore attacks. The mature and active systemin peptide is derived from its 200-amino acid precursor called pro systemin by at least one endoprotease (25, 26). Phytosulfokine, a sulfated pentapeptide growth factor originally isolated from asparagus and later found in several other plant species, is also processed from a larger precursor (27). Interestingly, the precursors of phytosulfokine from a few plant species all contain dibasic processing sites flanking the mature phytosulfokine peptides, suggesting that the processing of these peptide signals may utilize similar mechanisms as the processing of animal and yeast prohormones (28). The receptors for systemin and phytosulfokine were recently identified biochemically, and both of them belong to the leucine rich repeat receptor-like protein kinase family (LRR-RLK) (29, 30). Given the fact that the Arabidopsis genome encodes 217 LRR-RLKs and that LRRs can usually perceive peptide signals (31–33), we would expect that processing peptide hormones by proteases may be a general paradigm in plant LRR-RLK signal transduction.

Direct involvement of proteases in plant growth, development, and disease resistance has also been substantiated on a few occasions. In tomato leaves, a 50-kDa protein, SBP50, was found to have specific binding affinity with systemin. SBP50 is a plasma membrane-associated cysteine protease sharing high homology with animal calpains, it was suggested that DEK1 could be involved in a proteolytic processing of a protein substrate important for CR4 receptor-like kinase signaling (35, 36). In tomato, a papain-like cysteine endoprotease, Rcr3, is required for CF2-dependent disease
resistance and suppression of autonecrosis. It was hypothesized that Rcr3 could process CF-2 or another protein that is required for plant pathogen defense (37). In an attempt to discover genes controlling stomatal development, Berger and Altman (38) identified an Arabidopsis sdd1 mutant that shows a 2–4-fold increase in stomatal density and clustered stomatal formation compared with that of wild type plants. Map-based cloning indicated that SDD1 encodes a cysteine protease-like protein, suggesting that SDD1 may be required for processing a peptide that mediates the signaling events in guard cell formation.

Using activation tagging, we identified bri1-1D as a dominant suppressor of a weak allele of bri1, bri1-5 (6). Transgenic and genetic experiments showed that overexpression of BRS1 or the dominant bri1-1D allele was able to suppress bri1-5 and bri1-9 mutants, both of which contain single amino acid substitutions in the extracellular domain of BRI1. Overexpression of BRS1, however, did not appear to suppress a kinase-dead bri1 mutant, bri1-1. Although overexpression of BRS1 can partially restore the dwarfed bri1-5 phenotype to wild type, it fails to make wild type plants taller. These results suggest that BRS1 can specifically regulate the BRI1 signaling pathway, possibly by adjusting a step involved in BR perception in the extracellular space. In bri1-5, BR perception seems to become a rate-limiting step for the BRI1 signaling pathway, and elevating BRS1 expression can partially compensate for the attenuated BR response caused by the single point mutation in the BRI1 extracellular domain. In this report, we experimentally demonstrate that BRS1 is likely to play a processing role in the extracellular space. First, BRS1 is expressed ubiquitously in a developing Arabidopsis plant, similarly to the expression patterns shown by both BRI1 and BAK1 (18, 20, 21, 39). The relative amount of expression in each tissue tested, however, is slightly different from that of BRI1 and BAK1. For example, BRS1 is expressed highly in young leaves, meristems, and unopened flowers, but at lower levels in inflorescence stems and roots. Interestingly, transgenic plants in bri1-5 harboring 35S-BRS1 usually grow taller than bri1-5 bri1-1D plants. RT-PCR analysis revealed that the BRS1 expression level in the inflorescence stems is positively correlated to the degree of bri1-5 suppression. Secondly, BRS1 is a secreted protein and, unlike several other Arabidopsis Ser-CPs previously studied, is an active serine carboxypeptidase. Our previous experiments indicated that a single mutation generated in one of the catalytic triad, H438A, disrupted the bri1-5 suppression capability of the BRS1. Now we have shown that BRS1(H438A) is an inactive Ser-CP. The Ser-CP activity of BRS1 is therefore essential for its bri1-5 suppression. Recent studies by Chory and co-workers suggested that the 70-amino acid island and the 22nd LRR in BRI1 are responsible for the direct binding of BRs (40), but this does not exclude the possibility that other peptides or Ser-CPs are involved in BR perception by blocking the BR binding site on BRI1. This hypothesis is supported by the fact that BRS1 has a relatively broad substrate preference compared with other peptide processing Ser-CPs such as yeast Kex1p. In the bri1-5 mutant where we believe the perception of BR becomes rate-limiting, overexpression of BRS1 may act by improving BR binding with BRI1 by specifically processing a BR-binding/enhancing peptide/protein or by removing a BR-binding/blocking peptide/protein. Recent discoveries of several plant proteases important for various signaling events have opened a new avenue of research. The data reported here will enhance our understanding of the involvement of proteases in regulating LRR-RLK signaling pathways. A full understanding of the molecular mechanisms of BRs in regulating BR signaling will depend on future identification of the substrate of BRS1.

Characterization of BRS1

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