BRIZ1 and BRIZ2 Proteins Form a Heteromeric E3 Ligase Complex Required for Seed Germination and Post-germination Growth in Arabidopsis thaliana*

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Ubiquitin pathway E3 ligases are an important component conferring specificity and regulation in ubiquitin attachment to substrate proteins. The Arabidopsis thaliana RING (Really Interesting New Gene) domain-containing proteins BRIZ1 and BRIZ2 are essential for normal seed germination and post-germination growth. Loss of either BRIZ1 (At2g42160) or BRIZ2 (At2g26000) results in a severe phenotype. Heterozygous parents produce progeny that segregate 3:1 for wild-type:growth-arrested seedlings. Homozygous T-DNA insertion lines are recovered for BRIZ1 and BRIZ2 after introduction of a transgene containing the respective coding sequence, demonstrating that disruption of BRIZ1 or BRIZ2 in the T-DNA insertion lines is responsible for the observed phenotype. Both proteins have multiple predicted domains in addition to the RING domain as follows: a BRAP2 (BRCA1-Associated Protein 2), a ZnF UBP (Zinc Finger Ubiquitin Binding protein), and a coiled-coil domain. In vitro, both BRIZ1 and BRIZ2 are active as E3 ligases but only BRIZ2 binds ubiquitin. In vitro synthesized and purified recombinant BRIZ1 and BRIZ2 preferentially form heterooligomers rather than homo-oligomers, and the coiled-coil domain is necessary and sufficient for this interaction. BRIZ1 and BRIZ2 co-purify after expression in tobacco leaves, which also requires the coiled-coil domain. BRIZ1 and BRIZ2 coding regions with substitutions in the RING domain are inactive in vitro and, after introduction, fail to complement their respective mutant lines. In our current model, BRIZ1 and BRIZ2 together are required for formation of a functional ubiquitin E3 ligase in vivo, and this complex is required for germination and early seedling growth.

The ubiquitin pathway is a protein modification pathway that regulates many processes such as cell cycle, signal transduction, translational regulation, and chromatin remodeling (1–4). This pathway utilizes three enzymes for attachment of ubiquitin to its target. An E1 (ubiquitin-activating enzyme) forms a thioester bond with ubiquitin in an ATP-dependent manner. E1 then passes the attached ubiquitin to an E2 (ubiquitin-conjugating enzyme), which also forms a thioester bond with ubiquitin. Subsequently, the E3, also called a ubiquitin ligase, either accepts this activated ubiquitin before transfer to the substrate or serves as a scaffold to bring the substrate and E2-ubiquitin together. After ubiquitin is linked to the substrate, additional ubiquitin moieties can be conjugated to the previously attached ubiquitin, forming polyubiquitin chains. Ubiquitinated proteins have a number of fates, with the most characterized being degradation by the proteasome (5–7). Additionally, nonproteosomal fates have been described (8–10).

In the ubiquitin pathway, the E3 is an important contributor to substrate specificity. Currently, there are ~1300 E3 ligases predicted in the annotated proteome of Arabidopsis thaliana, which includes ~480 RING-type E3 ligases (11–13). The RING domain, consisting of eight conserved cysteine or histidine residues (referred to as metal-binding residues), which coordinates two zinc ions in a cross-brace motif, was identified in animal and yeast proteins in the early 1990s as a conserved domain of unknown function (14). The RING domain was first hypothesized to be involved in ubiquitin pathway in 1998 (15) and subsequently demonstrated to be a ubiquitin ligase domain in 1999, capable of mediating ubiquitination in vitro (16–18). To date, only a small number of RING-type E3 ligases in A. thaliana have been functionally characterized (19) where they are involved in diverse processes, including cell cycle, light perception, hormone signaling, embryo development, pathogen response, and DNA repair (1, 20–27). Despite these advances, the in vivo roles for the majority of the A. thaliana RING-type E3 ligases have not yet been characterized.

Here, we characterize two A. thaliana E3 ligases named BRIZ1 and BRIZ2 (BRAP2 RING ZnF UBP domain-containing protein) that have the same predicted domains as the mammalian and fungal BRAP2/IMP family of proteins. The BRCA1-associated protein 2 (BRAP2), a human RING-type E3 ligase (28), was first identified as a cytosolic interactor of breast cancer type 1 susceptibility protein (BRCA1) via the BRCA1 nuclear localization signal (28). BRAP2 (also known as IMP2 for

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1 and 2.

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2 The abbreviations used are: IMP, impedes mitogenic signal propagation; KSR, kinase suppressor of Ras; ZnF UBP, zinc finger ubiquitin-binding protein; GM, growth media; ORF, open reading frame; GST, glutathione S-transferase; HA, hemagglutinin; UBQ, ubiquitin; FL, full length.

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impedes mitogenic signal propagation) was later shown to be a negative regulator of the Raf-MEK-ERK signaling pathway (29). BAP2/IMP expression results in phosphorylation of the scaffold protein Kinase Suppressor of Ras (KSR) and KSR inactivation in the absence of Ras signal transduction. Binding of GTP-Ras to BAP2/IMP initiates autoubiquitination and degradation, releasing KSR (29).

The same predicted domains shared by Arabidopsis BRIZ and BAP2/IMP proteins are a BAP2, a RING, a zinc finger ubiquitin-binding protein (ZnF UBP) domain, and a coiled-coil region. In this study, the biochemical functions of the RING and ZnF UBP domains (30) and the coiled-coil region of these proteins were investigated. The RING domain is required for in vitro ligase activity. The ZnF UBP domain of BRIZ2, but not BRIZ1, binds ubiquitin though the C-terminal Gly-Gly residues of ubiquitin (30), and the coiled-coil domain mediates heterooligomeric interactions. The biological function of these two proteins was evident from their loss of function phenotype. T-DNA insertions disrupting the coding region of either BRIZ1 or BRIZ2 lead to abnormal germination and post-germination arrest that is rescued by expression of the respective protein from an introduced transgene but not their ligase-inactive versions. In addition, a truncated version of BRIZ1 that is active as an E3 but does not interact with BRIZ2 is unable to complement briz1 loss of function mutant. Thus, both BRIZ proteins are essential for normal seed germination and post-germination growth in A. thaliana. This stage of growth is tightly controlled by environmental conditions. Extrapolating from the functions of mammalian and yeast BAP2/IMP, the BRIZ1/2 E3 complex might play a role in embryo and seedling perception of environmental conditions.

**EXPERIMENTAL PROCEDURES**

**Plant Material, Growth, and Transformation—**A. thaliana seeds, ecotype Col-0, segregating for briz1-1 (SALK_085207), briz2-1 (Salk_094761), or briz2-2 (SALK_151060) were obtained from Arabidopsis Biological Resource Center, Columbus, OH (31), for briz1-2 (GK-653C09, N462625), a GABI-KAT line, from Nottingham Arabidopsis Stock Centre, Leicestershire, UK (32). BC2, F2 seeds segregating for briz1-1 mutant were used for experiments and PCR in Fig. 1. Seeds were sown on soil and grown under 24 h of light at 20 °C with 50% humidity or surface-sterilized with 30% (w/v) bleach and 0.1% (v/v) Triton X-100, plated on growth media (GM; 1× Murashige and Skoog salts (Sigma) with 1% (w/v) sucrose 2.5 mm MES, 0.1 μg/ml pyridoxine-Cl, 0.1 μg/ml myoinositol, 8g/liter BactoAgar (BD Biosciences), pH 5.7), and grown under continuous light at room temperature. Plants were transformed using the Agrobacteria-mediated floral dip method (33).

Cloning—The Qiagen RNasy plant RNA kit (Qiagen) was used to isolate RNA from 10-day-old Col-0 seedlings following the manufacturer’s instruction and used for reverse transcription reaction followed by PCR to amplify open reading frames of BRIZ1 and BRIZ2. pDONR201 (Invitrogen) was used as a subcloning vector for full-length BRIZ1 and BRIZ2 open reading frames (ORFs) and its derivatives. PCR was used to amplify portions of the BRIZ coding regions using pDONR full-length cDNA clones as templates and were recombined back into pDONR. Primer sequences used are in supplemental Table 1. BRIZ1 truncations used are listed as follows: BRIZ1-N, BRIZ1-C, BRIZ1-BRF, and BRIZ1-CC contain amino acids 1–229, 231–488, 1–364, and 277–488, respectively. The BRIZ2-truncated proteins, BRIZ2-N and BRIZ2-C, contain amino acids 1–241 and 207–479, respectively. All clones were sequence-verified. BRIZ sequences were identical to the predicted ORFs available in TAIR version 8 (Arabidopsis Information Resource). There is only one gene model for BRIZ1 but two for BRIZ2. Gene model At2g26000.2 likely encodes the in vivo synthesized protein because all BRIZ2 sequences isolated from cDNA in this study correspond to gene model At2g26000.2; 9 out of the 10 cDNA sequences available correspond to this model; and finally, gene model At2g26000.2 complements the briz2 mutant phenotype (see under “Results”). For expression in bacteria, BRIZ ORFs were introduced into pDEST15 (Invitrogen) for expression as a fusion with glutathione S-transferase (GST) or into a modified destination vector, pQLINKHD (34) for expression as a His6-tagged protein. To express BRIZ sequences in an in vitro transcription and translation system, cDNAs were recombined into pEXP1–9×Myc, which was created from pEXP1–DEST (Invitrogen) by replacing the His6 sequence between the Ndel and Nhel sites with the 9×Myc sequence from pHBI-9×Myc (35). His6-HA (hemagglutinin)-ubiquitin (UBQ-WT) was cloned by recombining a pDONR201 plasmid containing HA-ubiquitin (36) with pDEST17 (Invitrogen) to add the His6 tag. For production of the His6-HA-tagged form of UBQ missing the last two amino acids, which are glycine residues (UBQ-ΔGG), the HA-ubiquitin coding region was amplified with primers 5′-GGGCAAGTTTTGCAA-AAAAGCAGCTGTGATAGACTACCTTATAGCAGTGGTTCGAGTACCG-3′ and 5′-GGGGACCATTTGTGCAAAGAAGCCTGTTCTGAGAAGGAGACCC-3′ following by cloning into pDONR201 and recombination into pDEST17. Two rounds of PCR-mediated mutagenesis on pDONR cDNAs were performed (QuikChange kit, Stratagene) to generate full-length BRIZ proteins with cysteine to alanine substitutions in the metal ligand binding residues 3 and 4 in the RING domain (for primers, see supplemental Table 1), designated BRIZ1-mRING and BRIZ2-mRING.

Genomic DNA isolated from 10-day-old Col-0 seedlings was used as template for generating a DNA fragment containing 1.8 kbp of BRIZ1 5′ using primers 5′-CTCTGAGATGACATCGCCAGAAGC-3′ and 5′-GGGCAAGTTTTGCAA-AAAAGCAGCTGGTACCCCGCCAGATCCCGGTGGTGGG-3′. Col-0 cDNA was used to amplify BRIZ1 ORF without a stop codon using primers 5′-GCTTCTGGCATGTTCTCATCTTCAGAGAAG-3′ and 5′-GGGACCATTTGTGCAAAGAAGCTGGTACCCCGCCAGATCCCGGTGGTGGG-3′. A DNA fragment containing the BRIZ1 1.8-kbp 5′ sequence with BRIZ1 cDNA was generated by overlapping PCR using the DNA fragments described above as templates, cloned into pDONR201, and recombined into pEARLY302 (37) for expression of a C-terminally FLAG-tagged protein. BRIZ2, BRIZ1, BRIZ1-mRING, BRIZ2-mRING, and BRIZ1-BRF sequences were recombined into pGW2B21 (38) to express stably in Arabidopsis N-terminally tagged 10×Myc proteins. For constructs used in tobacco transient expression assays, pDONR201 con-
taining BRIZ1, BRIZ1-BRF, BRIZ1-CC, or BRIZ2 sequences were recombined into pEarleyGate 203 to introduce the Myc epitope tag and into pEarleyGate 201 to introduce the HA epitope tag, respectively (37).

**Genotyping**—For genotyping the briz1-1 allele, primers 5′-GGATACAGGAATTCGGACG-3′ and 5′-TAATGGA-TAGCACCCTGATGCA-3′ were used. For genotyping the briz2-1 allele, primers 5′-AGGCTAAGCAGGACAGGT-3′ and 5′-GCTTGGCAATCTCTGTGCAG-3′ were used. For genotyping the briz2-2 allele, primers 5′-GGCTGC-GGAAGATGTAGCTGC-3′ and 5′-CCATTAGGTTTCCAA-GCTGAGAGTGTG-3′ were used. Primer 5′-TGTTTCAGC- TAGTGCGGGCCATCG-3′ that anneals at left border of T-DNA was used to produce T-DNA gene junction-specific bands.

**Complementation Experiment**—6–8-Week-old plants with BRIZ1/briz1-1 or BRIZ2/briz2-1 genotypes were transformed by the Agrobacterium-mediated floral dip method (33), strain AGL1, containing binary vectors with the respective open reading frames in pEarleyGate 302 or pGWB21. T1 seeds for the BRIZ1 complementation experiment with the full-length wild-type BRIZ1 ORF were sown on soil and sprayed with 0.006% (v/v) glufosinate/ammonium every 2 days for total of 12 days. T1 seeds from all other complementation experiments were plated on agar containing 50 μg/ml hygromycin. DNA from T1 plants was isolated followed by genotyping to identify briz homoyzogotes or BRIZ2/briz heterozygotes. These were propagated to generate plant lines homozygous for the expression construct and then tested for the ability to produce briz homozygous plants with a wild-type phenotype.

**Protein Purification**—GST-BRIZ1 full-length protein was expressed in Escherichia coli strain BL21 (DE3) pLysS in 500-ml cultures. Transformed cells were grown at 37 °C for 2–3 h until reaching an A600 nm of 0.4–0.5 before induction with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside at 25 °C for 4 h. Cells were harvested by centrifugation and frozen overnight at −80 °C. Cell pellets were thawed briefly on ice and resuspended in 100 ml of lysis buffer containing 25 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100. For purification, 200 μl of 50% slurry glutathione-Sepharose high performance beads (GE Healthcare) were added to the cleared lysate and incubated for 1 h at 4 °C followed by two washes with 25 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% Triton X-100 and three washes with 0.01% Triton X-100. GST-BRIZ2 full-length protein was expressed in E. coli strain BL21AI (Invitrogn) and induced with 0.2% (v/v) L-arabinose. Cells were grown, induced, harvested, lysed, and protein-purified using the same methods as described above.

**In Vitro Activity Assays**—GST fusion proteins were eluted from 100 μl of glutathione-Sepharose beads with 100 μl of buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% Triton X-100 supplemented with 50 mM reduced glutathione. 40 μl of glycerol was added to eluted GST proteins for storage at −80 °C. Remaining glutathione-Sepharose beads were stored with buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% Triton X-100, and 40% glycerol. His6-tagged proteins were purified in a similar manner using nickel-Sepharose high performance beads (GE Healthcare) in the presence of 20 mM imidazole in the lysis and wash buffer and eluted with 500 μl imidazole. SDS-PAGE followed by Coomassie Blue staining or protein assay (Bio-Rad) was used to quantitify purified protein preparations. Western blot analysis using polyclonal anti-GST (Santa Cruz Biotechnology, Inc.) or monoclonal anti-His antibody (GE Healthcare) was used to verify identity of fusion proteins. Other antibodies used for GST fusion or His6 fusion protein detection were goat anti-rabbit IgG horseradish peroxidase secondary antibodies (Jackson Immuno Research) and goat anti-mouse IgG-horseradish peroxidase secondary antibodies (Kirkegaard & Perry Laboratories), respectively.

**In Vitro Transcription and Translation and Pulldown Assay**—Plasmids for expression of Myc-BRIZ full-length (FL) and BRIZ truncations were added to a TnT T7 coupled reticulocyte lysate system following the manufacturer’s protocol (Promega). Reactions were incubated at 30 °C for 2 h. For pulldown experiments, 5–10 μl of bead-bound GST-BRIZ protein was incubated with TnT reactions supplemented with 50 μl of binding buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, and 1 EDTA-free protease inhibitor tablet (Roche Applied Science)/10 ml. After rocking at 4 °C for 2 h, beads were collected by centrifugation and washed five times for 20 min each in 1 ml of binding buffer as indicated above. 20 μl of 5× Laemmli sample buffer (5× LSB, 125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, and 10% (v/v) β-mercaptoethanol) were added to the washed bead fractions and boiled for 5 min followed by separation by 10% SDS-PAGE and anti-GST or anti-Myc Western blot analysis.

**In Vitro BRIZ Binding Assay**—2 μg of GST-BRIZ proteins on glutathione-Sepharose beads were incubated with 1 μg of purified His6 BRIZ2 for 2 h at 4 °C in 100 μl of binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, and 1 EDTA-free protease inhibitor tablet/10 ml) (Roche Applied Science). Beads were collected by centrifugation and washed three times with 1 ml of binding buffer. 20 μl of 5× LSB was added to bead fractions and boiled followed by 10% SDS-PAGE separation and anti-His or anti-GST Western blotting as described above.

**In Vitro Ubiquitination Assay**—In vitro ubiquitination assays were performed as described previously (12) with minor modifications, such as excluding phosphocreatine and creatine kinase and including 1.64 μg of His6-HA-ubiquitin and 500 ng of eluted GST-BRIZ proteins. 30–μl reactions contained 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM ATP, 0.2 mM dithiothreitol, 50 ng of recombinant yeast E1, and 250 ng of His6-AtUBC10 (39). Immediately after mixing, an aliquot was removed (pre-reaction). Reactions were incubated at 30 °C for 2 h and stopped with 6 μl of 5× LSB and placed at 95 °C for 5 min. Proteins were separated by 10% SDS-PAGE followed by anti-HA or anti-GST Western analysis.
separated by 12% SDS-PAGE followed by anti-HA or anti-GST Western blotting. For polyubiquitin chain binding, 50 μg of poly-1–7 Lys-48 linked ubiquitin chains (Boston Biochem) were incubated with GST fusion proteins bound to glutathione-Sepharose beads for 2 h at 4 °C in binding buffer with 60 mM imidazole. Beads were collected and washed as above. For Western analysis, glutathione beads were boiled, and the supernatant was separated by 10% SDS-PAGE followed by anti-ubiquitin (12) or anti-GST Western blotting.

BRIZ Phylogenic Analysis and Sequence Alignment—Searches for BRIZ orthologs were done using Phytodrome version 3.1.1 data base and BLAST search at NCBI data base (//blast.ncbi.nlm.nih.gov) using both BRIZ amino acid sequences. Sequence alignment was done using the ClustalX program to generate initial alignment. Se-Al sequence editor (Evolution Biology Group, University of Oxford, UK) was used for manual editing of the alignment. A rooted phylogenic tree was generated by PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Sinauer Associates) with heuristic method and 100 bootstrap replicates.

**RESULTS**

Both BRIZ1 and BRIZ2 Are Essential for Normal Seed Germination—Multiple *A. thaliana* RING domain-containing proteins have been previously shown to function as ubiquitin E3 ligases, catalyzing ubiquitination in vitro (12). However, the in vivo functions of many of these proteins are unknown. To gain further insight into the physiological processes regulated by RING-type ubiquitin E3 ligases, the phenotypes of *A. thaliana* lines with a T-DNA insertion in a RING-domain-containing gene (31) were evaluated. For one such insertion line (SALK_085207), we were unable to obtain adult plants homozygous for the T-DNA insertion. This insertion is in an uncharacterized RING protein-encoding gene (At2g42160) that we named BRIZ1 (for BRAP2-RING-ZnUBP, see below) and designated this specific insertion allele briz1-1 (Figs. 1A and 2A). In progeny from heterozygous BRIZ1/briz1-1 plants, the embryos from one-fourth of the seed were slow to emerge from the seed coat. After emergence, the embryos remained white with unexpanded cotyledons even after 12 days on GM (Fig. 1B, middle and right panels). DNA was isolated from a pool of these white embryos, and PCR-based genotyping failed to yield a PCR product corresponding to the wild-type locus but...
generated a T-DNA gene PCR product, indicating that the small white embryos were homozygous for the T-DNA briz1-1 insertion (Fig. 1E). The other three-fourths of the seedlings looked identical to Columbia (Col) seedlings of the same age, producing expanded green cotyledons and four true leaves after 12 days of growth on GM (Fig. 1B). PCR-based genotyping of these phenotypically wild-type plants, either as individuals (data not shown) or as small pools (∼5), revealed them to be either BRIZ1/briz1-1 heterozygotes or wild-type BRIZ1 homozygotes (Fig. 1E). A second T-DNA insertion in the 3′-UTR named briz1-2 (GK-653C09) was also analyzed, and it yielded adult plants homozygous for the insertion, suggesting that BRIZ1 expression was unaffected as a result of this insertion (Fig. 1A and data not shown). Thus, the briz1-1 mutant phenotype is recessive, requiring T-DNA insertion in both BRIZ1 alleles, suggesting that loss of BRIZ1 function yields seeds that fail to germinate normally.

The predicted protein encoded by BRIZ1 contains, in addition to the RING domain, significant identity to two other described domains as well as a predicted coiled-coil region at the C terminus (Fig. 2A). Near the N terminus is a BRAP2 domain (28) and C-terminal to the RING domain is a ZnF-UBP domain (see below). BLAST searches identified a second uncharacterized protein containing these same four domains in the predicted Arabidopsis proteome encoded by locus At2g26000, which we named BRIZ2. Two T-DNA insertional alleles of BRIZ2 were analyzed (briz2-1 and briz2-2, SALK_094761 and SALK_151060, respectively). Surprisingly, the phenotype of progeny from briz2-1 and briz2-2 heterozygotes also segregated one-fourth white unexpanded embryos identical to those arising from briz1-1 heterozygotes (Fig. 1, C and D, respectively, middle and right panels). Genotyping using DNA isolated from small embryos failed to produce a gene-specific product, indicating that these white embryos were homozygous for the T-DNA insertion (Fig. 1, F and G, for briz2-1 and briz2-2, respectively). These observations indicate that despite the similarity between the two proteins, BRIZ1 and BRIZ2 are not functionally redundant, and loss of either results in the identical phenotype, i.e. slow germination and arrested seedling growth.

BRIZ1 and BRIZ2 Are Found in Multiple Vascular Plant Species, although Nonvascular Plants, Fungi, and Animals Have Only One BRIZ-like Protein—There are BRIZ-like proteins in animals and fungi, called BRAP2/IMP, which share all four domains with AtBRIZ1 and -2, but there appears to be only one such protein present in these nonplant proteomes (29). For this reason, we investigated whether plant-predicted proteomes resembled Arabidopsis in containing multiple BRIZ-like proteins. Other annotated plant-predicted proteomes were analyzed for the presence of BRIZ-like proteins. Two different proteins with the same four domains as BRIZ1 and BRIZ2 were identified in both monocot and dicot angiosperm species as follows: grape (Vitis vinifera), poplar (Populus trichocarpa) Brachypodium (Brachypodium distachyon), and rice (Oryza sativa) in addition to A. thaliana and the closely related species Arabidopsis lyrata (Fig. 2B and data not shown). Arabidopsis BRIZ2 has a canonical RING-H2 domain, although AtBRIZ1 has a noncanonical RING domain with glutamine instead histidine at metal-binding residue five (see supplemental Fig. 1 for alignment). Among all these species, one BRIZ-like protein clades with Arabidopsis BRIZ1, whereas the other clades with Arabidopsis BRIZ2 based on alignments of the full-length pro-

FIGURE 2. Two BRIZ-like proteins are present in vascular plants. A, SMART domain prediction of BRIZ and BRIZ-like proteins. BRAP2 (hexagon), RING (circle), ZnF-UBP (trapezoid), and coiled-coil (oval) domains. B, cladogram with bootstrap values of BRIZ-like proteins from different organisms. Ar, A. thaliana; Bd, B. distachyon; Cr, C. reinhardtii; Hs, Homo sapiens; Mm, Mus musculus; Os, O. sativa; Pp, P. patens; Pt, P. trichocarpa; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe; Sm, S. moellendorffii; Vv, V. vinifera; Xl, Xenopus laevis. Protein sequence alignment is shown in supplemental Fig. 1. GenBank accession numbers are as follows: AtBRIZ1, NP_181746; AtBRIZ2, NP_180170; OsHg55480, CAD41704; Os02g09060, NP_00146102; Vv00035537001, C1B20546; Vv00016936001, XP_002285333; Pt018s12820, XP_002325207; Pp0007s08600, XP_002310111; Pp1s40_290V2.2, XP_001760023; Cr10g454400, XP_001698355; HsBRAP2/Imp, AAP93638; MmBRAP2/Imp, AAK28079; XlBRAP2/Imp, AAS20335; ScETP1/YHL010C, AAS56441; SpAC16E8.13, NP_594226. Sequences for Brachypodium and Selaginella are not in GenBank, and the numbers used on the tree are listed on Phytozome database.

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tein (Fig. 2B), suggesting that angiosperms have orthologs of both proteins. There are also two different BRIZ-like proteins in the sorghum (*Sorghum bicolor*) genome. Although both BRIZ1- and BRIZ2-like proteins can be identified in sorghum (data not shown), amino acid assignments are uncertain outside of the conserved regions, so these proteins were not included in the phylogeny. Two BRIZ proteins are predicted from the *Selaginella moellendorfii* genome, a seedless vascular plant. One, Sm227454, contains a RING-H2 domain similar to BRIZ2 (supplemental Fig. 1). The second predicted protein in *Selaginella*, Sm403400, does not have canonical metal-binding residues at positions 3–6, and codons for these amino acids could not be found after manual inspection of the genomic sequence (supplemental Fig. 1). Only one BRIZ-like protein was found in the translated genomes of the moss *Physcomitrella patens* and the green algal species *Chlamydomonas reinhardtii* (Fig. 2B and supplemental Fig. 1).

**Full-length BRIZ ORFs Rescue the Mutant Phenotype**—To confirm that the mutant phenotype is due to T-DNA disruption of the BRIZ coding region and does not result from another spurious mutation or from another closely linked T-DNA insertion, BRIZ ORFs were introduced into their respective heterozygous briz plants, and the ability to obtain phenotypically wild-type plants genetically homozygous for *briz* at the respective BRIZ locus (referred to here as complemented) was tested. DNA containing 1.8 kbp of genomic DNA 5′ of the BRIZ1 translation start site followed by BRIZ1 ORF with an in-frame C-terminal FLAG epitope tag was introduced into heterozygous *BRIZ1/briz1-1* plants (supplemental Fig. 2A). Five independent T1 plant lines with wild-type growth (supplemental Fig. 2B and data not shown) were homozygous for the *briz1-1* T-DNA allele by PCR (supplemental Fig. 2C), indicating that the mutant phenotype associated with this T-DNA allele was due to the loss of BRIZ1. Similarly, after introduction of DNA containing *BRIZ2* cDNA sequences in-frame with an N-terminal 10×Myc epitope tag downstream of a widely active plant viral cauliflower mosaic virus 35S promoter (supplemental Fig. 2D), eight independent T1 plant lines with wild-type growth were homozygous for the *briz2-1* allele as determined by genotyping (supplemental Fig. 2F and data not shown), indicating that the mutant phenotype resulted from lack of BRIZ2 protein. Protein extracts of the same lines also were analyzed by Western blotting. Although FLAG-tagged BRIZ1 was not detectable in complemented plants (data not shown) probably because it was expressed from its own 5′-flanking region, Myc-tagged BRIZ2 protein expressed from the 35S promoter was detectable in homozygous *briz2-1* complemented plants (supplemental Fig. 2G). Thus, the post-germination arrest phenotype results from the lack of expression of *BRIZ1* or *BRIZ2* because wild-type plants can be recovered by expression of a BRIZ-encoding transgene in a homozygous *briz* mutant background.

**Both BRIZ1 and BRIZ2 Have Ubiquitination Activity in Vitro**—The ability of recombinant GST-BRIZ1 and GST-BRIZ2 to catalyze ubiquitin transfer *in vitro* was tested to determine whether they each have E3 activity (Fig. 3, A and B). Based on the amino acid alignment (supplemental Fig. 1), BRIZ1 has a glutamine instead of the typical histidine or cysteine at predicted metal-binding residue five within the RING domain, suggesting that it might be inactive as an E3. However, both GST-BRIZ1 and GST-BRIZ2 are capable of catalyzing ubiquitination in an *in vitro* assay with the E2 AtUBC10 (Fig. 3, A and B, respectively, lanes 1–3). This activity required both E1 and E2
as described previously for RING E3s (12). The fact that GST-BRIZ1 could still catalyze in vitro ubiquitination indicates that glutamine can replace histidine or cysteine to preserve secondary structure and maintain catalytic activity, at least in vitro with this E2. Full-length GST-BRIZ1-mRING and GST-BRIZ1-mRING with two substitutions in conserved metal-binding residues (C3A and C4A) had no in vitro activity above the negative controls, demonstrating that RING domain is required for catalyzing in vitro ubiquitination (Fig. 3, A and B, compare lanes 3 and 6). A truncated form of BRIZ1, lacking the coiled-coil domain, was also active as a GST fusion (Fig. 3C), indicating that the coiled-coil domain is not required for in vitro ligase activity.

BRIZ2 Binds Ubiquitin and the C Terminus of Ubiquitin Is Important for Interaction—Proteins with a predicted ZnF UBP domain such as hIsoT and the BRIZ-like protein from yeast, ScIMP, have been shown previously to bind ubiquitin and require the ubiquitin C-terminal Gly-Gly residues for binding (30). Both Arabidopsis BRIZ1 and BRIZ2 have a predicted ZnF-UBP domain, and the residues important for ubiquitin recognition in IsoT are present in both BRIZ1 and BRIZ2 (Fig. 4A for sequence alignment). Therefore, we hypothesized that both BRIZ proteins bind ubiquitin. To test this hypothesis, we used an in vitro ubiquitin binding assay incubating monomeric His6-HA-ubiquitin (UBQ-WT) immobilized on nickel-Sepharose with soluble GST-BRIZ proteins. In this assay, GST-BRIZ2 interacted with ubiquitin, although GST-BRIZ1 did not have a detectable ubiquitin interaction (Fig. 4B). This BRIZ2 interaction with UBQ-WT required the two C-terminal residues of ubiquitin as GST-BRIZ2 failed to interact with ubiquitin lacking these amino acids (UBQ-ΔGG, Fig. 4B).

We also verified the GST-BRIZ2 and ubiquitin interaction with a different binding assay (Fig. 4C). In this assay, GST-BRIZ proteins were immobilized on glutathione-Sepharose beads and incubated with a mixture of soluble monoubiquitin and Lys-48-linked ubiquitin chains containing 2–7 ubiquitin residues. GST-BRIZ2 pulled down polyubiquitin Lys-48 chains, but incubation with GST-BRIZ1 did not recover any ubiquitin chains. Despite the presence of conserved residues in both proteins, BRIZ2, but not BRIZ1, interaction with ubiquitin was detected in our assays. The same protein preparations used in these assays were verified to be active in in vitro ubiquitination assays, indicating that at least some portion of the protein was properly folded (data not shown).

BRIZ Proteins Preferentially Form Hetero-oligomers in Vitro—Because single homozygous BRIZ1 and BRIZ2 T-DNA insertion lines have identical phenotypes, BRIZ1 and BRIZ2 are likely to be required in the same pathway. As other RING proteins such as mammalian BRCA1 and BARD1 (41, 42) form hetero-oligomers, we tested whether BRIZ1 and BRIZ2 form a complex. Pulldown experiments using recombinant BRIZ1 or BRIZ2 full-length (FL) or truncated BRIZ proteins with a subset of their domains were performed (Fig. 5). In vitro synthesized Myc-tagged BRIZ1-FL proteins were added to bacterially expressed and purified GST-BRIZ1-FL or GST-BRIZ2-FL bound to glutathione-Sepharose beads. Bead-bound GST-BRIZ2-FL preferentially pulled down the other BRIZ protein, Myc-BRIZ1-FL, but in sharp contrast, bead-bound GST-BRIZ1-FL interacted very weakly with the Myc-tagged version of itself, Myc-BRIZ1 (Fig. 5B).

To determine the region of BRIZ1 required for interaction with BRIZ2, Myc-BRIZ1 was expressed without either its N- or...
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BRIZ1 and BRIZ2 preferentially form hetero-oligomers and interact through their predicted coiled-coil domains. A, diagram of full-length (FL) or truncations of BRIZ1 used in pulldown reactions. Myc epitope tag (rectangular), BRAP2 (hexagon), RING (circle), ZnF-UBP (trapezoid), coiled-coil (oval) are shown. B, Myc-BRIZ1-FL, Myc-BRIZ-N, and Myc-BRIZ-C proteins were synthesized using rabbit reticulocyte lysates as evidenced by Coomassie Blue staining (Fig. 5A). Bound proteins were analyzed using anti-Myc antibody (INPUT) or anti-GST to verify presence of a GST fusion protein in each sample. C, Myc-BRIZ1-FL, Myc-BRIZ1-BRF, and Myc-BRIZ1-CC were synthesized as in B. 5% input is shown on the left lanes of top panel. In vitro synthesis reactions and translation reactions. 5% input is shown on the left lanes of top panel. Myc-tagged proteins were incubated with GST-BRIZ1 FL protein bound to glutathione-Sepharose beads. Bound proteins were analyzed using anti-Myc antibody (top panel) or anti-GST to verify presence of a GST fusion protein in each sample. D, diagram of full-length or truncations of BRIZ2 used in pulldown experiments. E, Myc-BRIZ1 FL, Myc-BRIZ2-N, and Myc-BRIZ2-C were synthesized as in B. 5% input, left panel. Myc-tagged proteins were incubated with GST-BRIZ1 FL protein bound to glutathione-Sepharose beads. Bound proteins were analyzed using anti-Myc antibody (top panel) or anti-GST (bottom panel). Input samples were prepared at the same time and run on a separate gel and immunoblotted at the same time. F, top panel, GST-BRIZ1-FL, GST-BRIZ2-FL, and His6-BRIZ2-FL expressed in E. coli were purified from extracts using either glutathione or nickel-Sepharose beads and separated by 10% SDS-PAGE and stained with Coomassie Blue (CB). Input for in vitro binding assay analyzed by anti-His antibody is shown on right. Bottom panel, GST-BRIZ1-FL or GST-BRIZ2-FL bound to glutathione-Sepharose beads were incubated with soluble His6-BRIZ2-FL. Bound proteins were detected using anti-His antibody (left panel) or anti-GST-antibody (right panel). Asterisks in C and E denote a nonspecific anti-Myc immunoreactive band. WB, Western blot.

C-terminal halves and mixed with bead-bound GST-BRIZ2. Only Myc-BRIZ1-C, with the C-terminal ZnF-UBP and coiled-coil domains, interacted (Fig. 5B). The N-terminal half of BRIZ1 with the BRAP and RING domains did not bind GST-BRIZ2. To further delineate the region required, Myc-BRIZ1 protein containing the BRAP, RING, and ZnF-UBP domains (BRIZ1-BRF) or only the coiled-coil region (BRIZ1-CC) (Fig. 5A) were incubated with GST-BRIZ2. GST-BRIZ2-FL pulled down only those BRIZ1 proteins containing a coiled-coil region, Myc-BRIZ1-FL and Myc-BRIZ1-CC (Fig. 5C). The truncated protein Myc-BRIZ1-BRF lacking the coiled-coil domain, although active as an E3 ligase in vitro (Fig. 3C), did not interact with GST-BRIZ2-FL (Fig. 5C).

The reciprocal experiment yielded the same result (Fig. 5D). In vitro synthesized Myc-BRIZ2 proteins containing either the N- or C-terminal halves were incubated with bead-bound GST-BRIZ1. The Myc-BRIZ2-N protein did not interact with GST-BRIZ1, whereas the Myc-BRIZ2-C containing its coiled-coil region did interact (Fig. 5E). Myc-BRIZ1-FL binding to GST-BRIZ2 served as a positive control (Fig. 5E). In conclusion, all results demonstrate that BRIZ1 and BRIZ2 preferentially form hetero-oligomeric complexes as opposed to homo-oligomeric complexes, and the C-terminal coiled-coil domain is necessary and sufficient for the interaction.

BRIZ1 Interacts with BRIZ2 Directly in Vitro—To test if BRIZ1 and BRIZ2 can interact directly, GST-BRIZ1, GST-BRIZ2, and His6-BRIZ2 were expressed in E. coli and purified to near homogeneity from lysates as evidenced by Coomassie Blue staining (Fig. 5F, top left two panels). As observed previously, GST-BRIZ1 but not GST-BRIZ2 pulled down soluble purified His6-BRIZ2 (Fig. 5F, lower panels). This result strongly suggests that BRIZ1 and BRIZ2 interact directly to preferentially form hetero-oligomeric complexes via their coiled-coil domains.

BRIZ1 and BRIZ2 Interact in Vivo—To test if BRIZ1 and BRIZ2 interact in planta, N. benthamiana leaves were infiltrated alone or together with two different Agrobacteria, each containing a plasmid for expression of Myc-BRIZ1 or HA-BRIZ2 in plants and the infiltrated leaves harvested 3 days later. Expression could be detected in total protein extracts after infiltration (Fig. 6A, input fraction lanes). Immunoprecipitation experiments were performed on leaf extracts using anti-Myc-agarose beads. Anti-Myc beads pulled down HA-BRIZ2 only from leaf extracts co-expressing Myc-BRIZ1 and HA-BRIZ2 (Fig. 6A, lanes 5 and 12). Anti-Myc beads did not pulldown detectable HA-BRIZ2 from extracts expressing Myc-BRIZ1 alone (Fig. 6A, lanes 4 and 11), HA-BRIZ2 alone (lanes 6 and 13), or from mock infiltration extracts (Fig. 6A, lanes 7 and 14).
These results demonstrate the BRIZ1 and BRIZ2 are found in the same complex in planta.

To test if the coiled-coil domain is sufficient for interaction in planta, Agrobacteria containing a plasmid for plant expression of Myc-BRIZ1-BRF, Myc-BRIZ1-CC, or HA-BRIZ2, the same proteins tested for in vitro interactions (see Fig. 5), were infiltrated alone or together as described above. Expression of these truncated proteins could be detected in total protein extracts after infiltration (Fig. 6B). Immunoprecipitation experiments were performed as described above. Anti-Myc beads pulled down HA-BRIZ2 only from leaf extracts co-expressing Myc-BRIZ1-CC and HA-BRIZ2 (Fig. 6C, lanes 4 and 10). Anti-Myc beads did not pull down detectable HA-BRIZ2 from extracts expressing HA-BRIZ2 alone, when co-expressing Myc-BRIZ1-BRF or from mock infiltration extracts (Fig. 6C, lanes 5 and 6 and 11 and 12). These in vivo interaction assays verified our in vitro pulldown experiments using the same BRIZ1 truncations (BRIZ1-BRF and BRIZ1-CC, Fig. 5A). After in vivo expression, we observed higher molecular weight forms that are immunoreactive with both anti-Myc and anti-HA antibodies (Fig. 6), but the cause for this altered migration of a subset of the proteins is not known. Interestingly, higher molecular weight forms are visualized after expression of BRIZ-CC but not BRIZ1-BRF (Fig. 6, B and C). The presence of slower migrating forms of Myc-BRIZ1-CC are independent of introduction of an interacting BRIZ2 protein (compare Fig. 6B, lanes 2 and 4).

BRIZ1 Protein That Lacks the Coiled-coil Region Fails to Complement the Briz1 Mutant—The similar phenotypes between briz1 and briz2 mutant lines and their ability to interact in vitro and in vivo suggest that the active ligase is a complex of the two proteins. To test the requirement for interaction in vivo, we used the BRIZ1 opening reading frame lacking the coiled-coil domain, called BRIZ1-BRF. This protein is active as an E3 ligase in vitro (Fig. 3C), but it fails to interact with BRIZ2 in vitro and in vivo (Figs. 5C and 6B). After introduction of the BRIZ1-BRF expression cassette into BRIZ1/briz1 plants, two independent lines homozygous for the transgene and heterozygous for the briz1-1 allele were characterized in detail. In these lines, BRIZ1-BRF protein was detectable at levels equivalent to or greater than that of a briz1 homozygous line expressing wild-type full-length BRIZ1. Despite the presence of the transgene expressing BRIZ1-BRF, progeny from a BRIZ1/briz1-1 heterozygote still segregated 3:1 for the briz phenotype (Fig. 7A and Table 1), and no complementation was observed. These results demonstrate that a BRIZ1 protein unable to interact with BRIZ2 does not complement BRIZ1 function suggesting that interaction is required in vivo.

RING Domain of BRIZ Proteins Is Required for in Vivo Function—To determine whether both BRIZ1 and BRIZ2 RING domains are required for in vivo function, we performed an analogous experiment as described above for in vivo expression of BRIZ1-BRF. The same BRIZ1 and BRIZ2 ORFs with amino acid substitutions in the RING domain that render these proteins inactive in vitro (Fig. 3, A and B) were introduced into their respective briz heterozygous plants. After several generations, two plant lines for each BRIZ protein were characterized in detail. The expression levels for the BRIZ-mRING proteins...
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were comparable with or greater than the control lines, which express tagged wild-type full-length BRIZ in the homozygous T-DNA mutant background (Fig. 7, B and C). However, these BRIZ-mRING proteins were unable to complement the briz mutant phenotype (Table 1). Only BRIZ/briz plant lines were obtained, and these segregated as expected for BRIZ/briz seedlings in the next generation. In conclusion, the RING domains of both BRIZ1 and BRIZ2 are required for in vivo function.

**DISCUSSION**

*A. thaliana* BRIZ proteins characterized in this work are essential players in seed germination and the initiation of post-germination development under control conditions. T-DNA insertional alleles disrupting the coding region of either *BRIZ1* or *BRIZ2* alone have nearly identical mutant phenotypes, demonstrating that their functions are not redundant and suggesting that they work in the same pathway. Combined with their direct interaction in vitro and presence in the same complex in planta, we hypothesize that a BRIZ1-BRIZ2 hetero-oligomeric complex is the active E3 ligase required for seed germination and progression past a seedling growth checkpoint. Two BRIZ-like proteins can be identified in all the well annotated vascular plant genomes available, suggesting conservation of this complex and its function. The lack of sequenced genomes from gymnosperms prevents us from determining whether two BRIZ proteins are present in this group. Clearly, the green algal species *Chlamydomonas* and the moss *Physcomitrella* lack two BRIZ-like proteins, but whether the early branching vascular plant *Selaginella* has both proteins is currently uncertain. Although two different BRIZ-like proteins appear to be encoded in the *Selaginella* genome and one is BRIZ2-like, we cannot conclude whether the other is orthologous to BRIZ1 because the current predicted BRIZ-like protein has diverged from both AtBRIZ1 and AtBRIZ2, and there are no mRNA sequences available to verify expression and to identify the splice sites to confirm the predicted open reading frame.

The biochemical activities of several regions of BRIZ proteins were characterized in this study. The RING domain of BRIZ2 has all eight predicted metal-binding residues and is active in vitro and required in vivo. In contrast, BRIZ1 has a noncanonical RING domain with the conserved histidine at metal-binding position 5 replaced with a glutamine. Despite this substitution, BRIZ1 is also active as an E3 ligase by itself in vitro, and an intact RING domain is required in vivo. Histidine or cysteine is typically the metal-binding residue that interacts with one of the bound zinc ions and when substituted activity is lost (12). However, BRIZ1 is not unique in being active with

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**TABLE 1**

Complementation analysis using plant lines expressing 10×Myc-BRIZ FL, truncated, and modified forms

| Transgene          | Seedling phenotype | 3:1 x test |
|--------------------|--------------------|------------|
|                    |                    | WT | briz | value | p value |
| 10×Myc-BRIZ1-BRF   |                   | 1  | 22  | 0.476 | 0.42    |
| 10×Myc-BRIZ1-FL    |                   | 1  | 23  | 0.278 | 0.60    |
| 10×Myc-BRIZ1-mRING|                   | 2  | 0   | 0.086 | 0.77    |
| 10×Myc-BRIZ2-FL    |                   | 1  | 0   | 0.333 | 0.0001  |
| 10×Myc-BRIZ2-mRING|                   | 2  | 15  | 0.476 | 0.49    |
metal ligand substitutions because active RING proteins have been identified with residues other than histidine or cysteine at metal-binding position 5. The RING-G (43) and RING-D (12) types of RING proteins are active in vitro and have glycine and aspartate, respectively, at metal-binding position 5 instead of cysteine or histidine.

Another characteristic of BRIZ proteins is a predicted ZnF UB domain. Like the ZnF UB domain of the yeast BRIZ-like protein, ScBRAP2/IMP, BRIZ2 binds monoubiquitin, requiring the ubiquitin C-terminal two residues (30). Curiously, BRIZ1 does not appear to bind ubiquitin in vitro. The crystal structure of a ZnF UB domain of human isopeptidase T identified three residues essential for ubiquitin binding (Trp-209, R221A and Y261A-isoT numbers). The identical residues are found in both BRIZ1 and BRIZ2. Thus, the molecular basis for the difference in ubiquitin binding between BRIZ1 and BRIZ2 remains unclear. In addition, the biological significance of ubiquitin binding independent of ubiquitin protease activity is unknown. It is possible that ubiquitin binding to BRIZ affects ubiquitin transfer to its substrate or to itself.

BRIZ proteins also contain a BRAP2 domain. Originally identified in human BRAP2/IMP, it is required for binding to a nuclear localization signal in BRCA1 (28). The physiological significance of this interaction is not known. In addition, yeast BRAP2/IMP also has similar affinity to the nuclear localization signal in BRCA1 (28). The physiological significance of this interaction is not known. In addition, yeast BRAP2/IMP also has similar affinity to the nuclear localization signal of BRCA1, suggesting the function is conserved (28).

The BRIZ proteins contain an extensive predicted C-terminal α-helical region within which is found a predicted coiled-coil region. We demonstrated that this region is necessary and sufficient for hetero-oligomeric interactions in vitro and in planta and that BRIZ proteins strongly prefer to interact with each other rather than with themselves. In addition, plants stably expressing 10×Myc-BRIZ1-BRF did not complement the briz1 mutant phenotype, suggesting the BRIZ hetero-oligomer is essential for seed germination. The oligomeric nature of mammalian and yeast BRAP2/IMPs are unknown. Given that only one BRIZ-like protein is present in these organisms (29), whether BRAP2/IMP forms homooligomers is of interest. Our results, from a combination of genetic and in vitro biochemical studies, support a model that Arabidopsis BRIZ hetero-oligomers are the required functional unit in vivo.

As expected, substitutions in the RING domain of BRIZ proteins (BRIZ-mRING) resulted in no detectable in vitro activity compared with wild-type BRIZ protein. Interestingly, stable plant lines expressing BRIZ-mRING did not complement their respective briz mutant phenotypes suggesting that both E3 RING domains are required in vivo. This observation joins the example of yeast RING Slx8p-Slx8p/Hex3p heterodimer where mutations in either RING domain abolish in vivo resistance to DNA damage stress (44).

The Arabidopsis BRIZ1/2 hetero-oligomer joins a growing number of RING/U-box E3 ligases that have been demonstrated to be oligomeric, and some use the same structural element. The U-box proteins hCHIP (45) and yeast Prp19 (46) form a dimer and tetramer, respectively, via coiled-coil regions. Other RING/U-box proteins utilize a different secondary element for homo- and hetero-oligomerization, such as α-helices for the RING BRCA1/BARD heterodimer (47, 48) and the RAG1 (49) and AtPUB14 homodimers (50).

In several hetero-oligomer RING/U-box E3s, each subunit is not equivalent biochemically. The yeast RING Slx5p/Hex3p has no detectable activity by itself in vitro compared with the related RING Slx8p. However, Slx5p/Hex3p stimulates the in vitro ubiquitination of RAD52 by Slx8p. The mammalian RING protein MdmX in in vitro assays appears to stimulate the ubiquitination activity of Mdm2, and both are required for regulating p53 in vivo (51). The RING heterodimers, mammalian BRCA1/BARD1 (48, 52) and RING1b/Bmi (53), are more active when co-expressed, suggesting that heterodimer formation regulates the activity. However, inclusion of both BRIZ proteins together in an in vitro ubiquitination assay had no effect on nonsubstrate ubiquitination (data not shown). Thus, the biochemical difference between the homomeric forms and the heteromeric form of BRIZ proteins remains to be determined and may require identification of a substrate. Altogether, this study and previously published studies suggest that the oligomeric organization of RING E3 ligases is a critical aspect of their function.

The mammalian BRIZ-like protein BRAP2/IMP modulates Ras-regulated Raf-MEK-ERK signaling pathways by binding to and preventing the dimerization of a scaffolding protein called KSR (54). Dimerization as well as relocation of KSR to the cell surface result in ERK activation. BRAP2/IMP also binds selectively to GTP-bound Ras (29). The current model proposes that after signal perception, BRAP2/IMP association with activated Ras stimulates the E3 ligase activity of BRAP2/IMP, targeting itself for degradation. Loss of BRAP2/IMP releases KSR for oligomeric interactions and subsequent ERK activation.

The biological function of mammalian and yeast BRAP2/IMP provides little information on the in vivo function of plant BRIZ proteins. In contrast to other organisms, no Ras proteins are predicted in the annotated genomes of Arabidopsis and rice (55, 56), making direct analogies to BRAP2/IMP function difficult. In addition, no predicted protein with significant sequence identity to KSR has been identified in Arabidopsis, and there is limited information on signaling scaffold proteins in plants in general. Although BRIZ proteins appear to regulate a key step in the plant life cycle that is sensitive to environmental conditions, the cellular response pathway regulated by BRIZ proteins might differ considerably from that described for the IMP proteins. Further elucidation of BRIZ function will provide insight into the unique processes that plants have utilized with conserved proteins.

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