The *Arabidopsis thaliana* E3 Ubiquitin Ligase BRIZ Functions in Abscisic Acid Response

Katrina J. Linden1,2†, Mon Mandy Hsia1,3†, Yi-Tze Chen1,4 and Judy Callis1,2,3,4*

1Department of Molecular and Cellular Biology, University of California, Davis, CA, United States, 2Integrated Genetics and Genomics Graduate Program, University of California, Davis, CA, United States, 3Biochemistry and Molecular Biology Graduate Program, University of California, Davis, CA, United States, 4Plant Biology Graduate Program, University of California, Davis, CA, United States

The ubiquitin system is essential for multiple hormone signaling pathways in plants. Here, we show that the *Arabidopsis thaliana* E3 ligase BRIZ, a heteromeric ligase that consists minimally of BRIZ1 and BRIZ2 proteins, functions in abscisic acid (ABA) signaling or response. *briz1* and *briz2* homozygous mutants either fail to germinate or emerge later than wild-type seedlings, with little cotyledon expansion or root elongation and no visible greening. Viability staining indicates that *briz1* and *briz2* embryos are alive but growth-arrested. Germination of *briz* mutants is improved by addition of the carotenoid biosynthetic inhibitor fluridone or gibberellic acid (GA3), and *briz* mutants have improved development in backgrounds deficient in ABA synthesis (*gin1-3/aba2*) or signaling (*abi5-7*). Endogenous ABA is not higher in *briz2* seeds compared to wild-type seeds, and exogenous ABA does not affect *BRIZ* mRNAs in imbibed seeds. These results indicate that *briz* embryos are hypersensitive to ABA and that under normal growth conditions, BRIZ acts to suppress ABA signaling or response. ABA signaling and sugar signaling are linked, and we found that *briz1* and *briz2* mutants excised from seed coats are hypersensitive to sucrose. Although *briz* single mutants do not grow to maturity, we were able to generate mature *briz2-3 abi5-7* double mutant plants that produced seeds. These seeds are more sensitive to exogenous sugar and are larger than seeds from sibling *abi5-7 BRIZ2/briz2-3* plants, suggesting that BRIZ has a parental effect on seed development. From these data, we propose a model in which the BRIZ E3 ligase suppresses ABA responses during seed maturation and germination and early seedling establishment.

Keywords: *Arabidopsis*, E3 ligase, abscisic acid, ubiquitin, hormone signaling, germination, ABI5, ABA2

INTRODUCTION

The ubiquitin system is a post-translational protein modification system in which E1, E2, and E3 enzymes catalyze the attachment of one or more ubiquitins to substrate proteins. E3s, or ubiquitin ligases, facilitate the transfer of activated ubiquitin from an E2 to the substrate, either directly or by forming a thioester bond with the ubiquitin prior to its transfer to the substrate. E3s are the key specificity components in ubiquitination of substrate proteins and therefore their presence and activity are important points of regulation. Additional proteins in the ubiquitin system modulate the activity, localization, or abundance of ubiquitinated...
proteins (Oh et al., 2018), remove ubiquitin, or modulate the above processes. In plants, the ubiquitin system affects multiple developmental and environmental responses and hormone signaling pathways (reviewed in Vierstra, 2011; Sadanandom et al., 2012; Gibbs et al., 2014; Kelley, 2018; Miricescu et al., 2018), including the abscisic acid (ABA) biosynthetic and signaling pathways (reviewed in Liu and Stone, 2011; Yu et al., 2016; Jurkiewicz and Batoko, 2018; Zhang et al., 2019).

Absciscic acid is a hormone that affects many aspects of plant development and stress responses (reviewed in Finkelstein, 2013; Yoshida et al., 2019). It influences embryo development, seed maturation, dormancy, germination, growth, senescence, and allows plants to respond appropriately to drought, salinity, and pathogens. The ABA biosynthetic gene GLUCOSE INSENSITIVE 1 (GIN1, also called ABA DEFICIENT 2, ABA2) encodes a short-chain dehydrogenase/reductase enzyme that converts xanthoxin to ABA aldehyde, and mutants have reduced ABA levels (Cheng et al., 2002; González-Guzmán et al., 2002). The canonical ABA signaling pathway (reviewed in Cutler et al., 2010) begins with ABA perception by PYRABACTIN/ PYRABACTIN-LIKE (PYR/PYL) proteins, followed by inhibition of clade A type 2C protein phosphatases (PP2Cs). Sucrose non-fermenting related-1 (SnRK) 2 type kinases previously held in check by PP2Cs are subsequently activated and can phosphorylate downstream targets including bZIP transcription factors such as ABI5 (Zhang et al., 2019) or ion channels in guard cells (Munemasa et al., 2015). The Raf-like kinase RAF10 interacts with PP2Cs and phosphorylates SnRK2s and several downstream transcription factors (Nguyen et al., 2019), and multiple other Raf-like kinases are important for SnRK2 phosphorylation and ABA responses (Lin et al., 2020). Additionally, the glyceron kinase-like protein BRASSINOSTEROID INSENSITIVE 2 (BIN2) phosphorylates PP2Cs (Cai et al., 2014), suggesting that Raf-like and BIN2 kinases modulate the core ABA signaling pathway.

Absciscic acid signaling is also regulated by the ubiquitin system, and dozens of E3 ligases have been identified (reviewed in Yu et al., 2016). The substrate binding subunit of a CULLIN4-type E3 ligase, DDA1 (for DET1-, DDB1-ASSOCIATED1), interacts with PYLs 4, 8, and 9 in vivo and facilitates the proteasomal degradation of PYL8 (Irigoyen et al., 2014). RING FINGER OF SEED LONGEVITY (RSL1) is a plasma membrane E3 that regulates intracellular trafficking of PYL4 (Bueso et al., 2014). The E3s PUB12 and PUB13 target the PP2C ABI1 for degradation (Kong et al., 2015), and the E3s RGLG1 and RGLG5 ubiquitinate the PP2Cs PP2CA, ABI2, and HAB2 in vitro and regulate their abundance in vivo (Wu et al., 2016). PHLOEM PROTEIN 2-B11 (PP2-B11) is an F-box protein in an SCF-type E3 that targets the kinase SnRK2.3 for degradation (Cheng et al., 2017).

Components downstream of SnRK2 kinases are also modulated by the ubiquitin system. The E3 RHA2b ubiquitinates MYB30, a transcription factor that negatively regulates ABA signaling, in vitro, interacts with it in vivo, and affects its accumulation in plants (Zheng et al., 2018). The E3 AIP2 (ABI3-interacting protein) ubiquitinates the transcription factor ABI3 in vitro and reduces its levels in vivo (Zhang et al., 2005). Loss of the E3 DESPIERTO results in decreased ABI3 and ABI4 expression during seed development, reduced ABA sensitivity during germination, and loss of dormancy (Barrero et al., 2010). LOSS OF GDU2 (LOG2, also referred to as AIRP3) binds to and monoubiquitinates the mature form of RD21, a drought-induced cysteine protease, in vitro, and LOG2 loss-of-function mutants are ABA hyposensitive (Kim and Kim, 2013).

Many E3s that affect ABA signaling do not have identified substrates to date. Reduced expression of the E3 RING-H2 FINGER A (RHA2a) or over-expression with the 35S promoter result in diminished or enhanced ABA responses, respectively (Bu et al., 2009). TUBBY9 encodes an F-box protein, a substrate-specificity subunit of the CUL1-based E3 ligases, and loss-of-function mutants have reduced ABA sensitivity, while TUBBY9 over-expression results in ABA hypersensitivity (Lai et al., 2004). RING DOMAIN AND DOMAIN-OF-UNKNOWN-FUNCTION 1 and 2 (RDUF1 and RDUF2) loss-of-function mutants are ABA-hyposensitive (Kim et al., 2012).

ABI5 is a bZIP transcription factor with a major role in seed germination (Lopez-Molina et al., 2001, and reviewed in Skubacz et al., 2016). The first loss-of-function allele, abi5-1, was recovered in a screen for ABA-resistant germination in Arabidopsis thaliana (Finkelstein, 1994). Characterization of multiple loss-of-function abi5 alleles indicates that plants lacking ABI5 are not phenotypically different from wild-type plants in the absence of exogenous ABA, including in their stomatal responses under low water potential conditions (Finkelstein, 1994; Nambara et al., 2002). ABI5 protein is highest in dry seeds (Piskurewicz et al., 2008) and its abundance is modulated by multiple E3 ligases. KEEP ON GOING (KEG) encodes an E3 whose loss-of-function mutants accumulate ABI5 protein and are hypersensitive to ABA (Stone et al., 2006). Recombinant KEG ubiquitinates ABI5 in vitro (Liu and Stone, 2010). The E3 CUL4-based substrate specificity factors DWA1 and 2 (DWD hypersensitive to ABI1 and 2) function in ABA signaling and negatively affect ABI5 levels in vivo (Lee et al., 2010a). HYPERSENSITIVE DCAF 1 (ABD1) is another substrate receptor of a CUL4-based ligase that interacts with ABI5 and plays a role in ABI5 degradation (Seo et al., 2014).

Loss-of-function mutants of many of the E3s described above have phenotypes that are modest or only visible under exogenous ABA treatment, with the exception of keg mutants, which grow slowly and have arrested growth after developing one set of true leaves (Stone et al., 2006). In addition to ABI5, KEG is implicated in degradation of other bZIP transcription factors (Chen et al., 2013) and is involved in intracellular trafficking and pathogen responses (Gu and Innes, 2012). These additional functions likely contribute to the keg loss-of-function phenotype.

Previously, we identified T-DNA insertion mutations in genes encoding two related E3 proteins called BRIZ1 (for BRAP2-RING-Znf Domain) and BRIZ2 (Hsia and Callis, 2010). Homozygous T-DNA insertion lines of either BRIZ gene have the same severe phenotype of post-germination growth arrest, suggesting that both proteins are required for the same processes. We reported that BRIZ1 and BRIZ2 proteins preferentially form heteromers in vitro and are found in the same complex in vivo (Hsia and Callis, 2010). Using a complementation assay,
we showed that wild-type RING domains of each protein and the BRIZ1-BRIZ2 interaction domain are required for in vivo function (Hsia and Callis, 2010). We proposed that BRIZ1 and BRIZ2 function as subunits of a heteromeric E3 ligase.

Here, we further characterize the phenotype of loss-of-function briz mutants and provide evidence that BRIZ1 and BRIZ2 suppress ABA signaling in germination and early seedling growth. briz mutants do not have elevated endogenous ABA levels and their germination is hypersensitive to exogenous ABA. Reduction of ABA with a biosynthetic inhibitor allows some post-germination growth of briz embryos. briz2 mutants exhibit improved germination and growth in abi5-7 or gin1-3 mutant backgrounds that have deficiencies in ABA signaling or synthesis, respectively. Similarly, briz1 mutants exhibit increased germination in the abi5-7 background. We also show that some briz2-3 abi5-7 double mutants are able to grow to maturity and set seed. Seeds from briz2-3 abi5-7 plants are hypersensitive to sucrose and glucose. These data support a model where the BRIZ E3 complex functions to suppress ABA responses during seed germination and post-germination seedling growth.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Col-0 (CS70000), briz1-1 (At2g42160, SALK_085207), briz2-1 (At2g26000, SALK_094761), briz2-2 (At2g26000, SALK_151060), briz2-3 (At2g26000, FLAG_122B09), and gin1-3 (At1g52340, CS6147) were obtained from the Arabidopsis Biological Resource Center in Columbus, Ohio1 and the briz lines were back-crossed at least four times to Col-0. The abi5-7 allele (At2g36270, E74-1) as described in Nambara et al. (2002) was first obtained from Eiji Nambara and later re-acquired from Ruth Finkelstein (UC Santa Barbara).

Seeds were surface-sterilized in a solution of 25% commercial bleach and 0.1% Triton-X100 (Sigma, 93443) for 10 min, rinsed with sterile H2O, and stratified at 4°C for at least 24 hours (b) before plating. For bacto-agar-grown seedlings, seeds were plated on solid growth media (GM) consisting of 4.3 g/L Murashige and Skoog basal salt mixture (Sigma, M 5524), 1% sucrose (Fisher Scientific), 0.5 g/L MES (Calbiochem 475893), 1X B vitamins (0.5 μg/ml nicotinic acid, 1 μg/ml thiamine, 0.5 μg/ml pyridoxine, and 0.1 μg/ml myo-inositol, all from Sigma), and 8 g/L BD Bacto Agar (Fisher Scientific), pH 5.7. After 2 weeks at room temperature under constant light, seedlings were transplanted from agar GM plates to soil and plants were grown at 20°C with 50% humidity and 16 h light/8 h dark.

Growth Media Modifications

A 100 mM fluridone (Chem Service, Inc. N-13217) stock in DMSO was diluted in GM to a final concentration of 10 μM. Plates with 0.1% DMSO were used as solvent controls for fluridone and BL plates. A 10 mM ABA (Sigma, A-1049) stock in ethanol was diluted in GM to a final concentration of 0.1 μM. A 100 mM gibberellic acid (GA3, Sigma, G-7645) stock in ethanol was diluted in GM to final concentrations of 10, 100, 200, or 1 mM. A 1 M aminocyclopropane carboxylic acid (ACC; Sigma, A-3903) stock in ethanol was diluted in GM to a final concentration of 50 μM. A 50 mM 2,4D (Sigma, D-8407) stock in ethanol was diluted in GM to a final concentration of 1 μM. Plates with 0.1% EtOH were used as solvent controls for ABA, GA3, 2,4-D, and ACC plates. GM plates with mannitol or glucose contained 1% mannitol or 1% glucose instead of 1% sucrose.

Genotyping

Primers sequences for PCR genotyping are listed in Supplementary Table S1. For briz1-1 lines, primer 9-097 was used with primer 9-098 to produce a WT gene-specific product, and primer 9-098 was used with the T-DNA left border primer 9-001 to produce a T-DNA junction product. For briz2-1 lines, primer 6-674 was used with primer 6-675 to produce a WT gene-specific product, and primer 6-675 used with the T-DNA left border primer 9-001 to produce a T-DNA junction product. For briz2-2 and briz2-3 lines, primer 6-981 was used with primer 6-982 to produce a WT gene-specific product. For briz2-2 lines, primer 6-982 was used with the T-DNA left border primer 9-001 to produce a T-DNA junction product. For briz2-3 lines, primer 6-982 was used with the T-DNA left border primer 8-133 to produce a T-DNA junction product. For abi5-7 lines, a dCAPs reaction was used. After amplification with primers abi5-7dCAPS-F and abi5-7dCAPS-R, products were digested with Hinfl and separated by 3% agarose gel electrophoresis. A second Hinfl site is present only in the abi5-7 allele. For gin1-3 lines, products generated with primers 6-745 and 6-746 were separated by 3% agarose gel electrophoresis. The gin1-3 allele has a 50 bp deletion and these primers span the deletion site, making a shorter PCR product. PCR genotyping was used to identify F2-F4 individuals of the specified genotype.

SCR/Germination Experiment

Age-matched seeds were used and were ~7 months post-harvest and the results were similar for seeds ~2 weeks post-harvest. Seed status was assessed at the designated times using forceps and a dissecting microscope in a sterile hood to be able to rotate each seed to see seed coat rupture (SCR) and radicle emergence (germination). The data represent the mean of three independent experiments, with three replicates of ~50 seeds per genotype per experiment.

Viability Staining

Seeds from BRIZ1/briz1-1, BRIZ2/briz2-1, and BRIZ2/briz2-2 plants were plated on GM plates and grown in a controlled environment (constant light, 22°C) for 15 or 30 days. Embryos that emerged from the seed coat were selected for the experiment. Sytox Orange nucleic acid stain in DMSO

1https://abrc.osu.edu/
(Invitrogen, S11368) was diluted in water to a working concentration of 250 nM. Fluorescein diacetate (FDA) powder (Invitrogen, F1303) was dissolved in acetone to make a 5 mg/ml stock, then diluted in water to a working concentration of 5 µg/ml. Embryos were either left untreated or were heat-treated at 98°C for 5 min. Embryos were stained for 10–15 min before imaging with an Olympus Confocal Laser Scanning Microscope (model FV5-LDPSSU). Sytox stained seedlings were imaged using the 10x lens and filter with laser excitation at 543 nm. FDA stained seedlings were imaged using the 10x lens and filter with laser excitation at 488 nm.

ABA Determination

The two silenced lines were the T2 generation from two independent briz2-1 lines initially complemented by expressing Myc-BRIZ2 protein (Hsia and Callis, 2010). Initially with 100% germination in a homozygous briz2-1 background, progeny from some individuals showed 0% germination. Seeds were soaked in water for 1 h at room temperature and water was removed before flash freezing. ABA levels were then determined by the Danforth Center, St. Louis, MO as described in Chen et al. (2009) with D6ABA used as an internal standard. A mix of D6ABA and ABA were used for a standard curve.

Embryo Excisions

Embryo excisions were performed as described in Lee et al. (2010b) and shown in Lee and Lopez-Molina (2013). Excised embryos were placed on sterilized 60 µM nylon net filters (Millipore #NY6002500) on top of GM solid media and photographed using a dissecting microscope. To identify briz1-1 and briz2-3 seeds, which are indistinguishable from wild-type at the dry seed stage, seeds from heterozygous BRIZ1/briz1-1 or BRIZ2/briz2-3 plants were plated on the appropriate growth media, and seeds that had not germinated after 48 h were selected for excision. Age-matched seeds were used.

SDS-PAGE and Western Blotting

For visualization of Myc-BRIZ2 protein, seeds were plated on GM plates and stratified at 4°C for 24 h, then ground in buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF, and one EDTA-free protease inhibitor tablet (Roche, 1836153)/10 ml. Bio-Rad Protein Assay was used to quantify total protein. About 50 µg total protein was loaded on a 9% polyacrylamide gel for SDS-PAGE separation, and then transferred to a PVDF-P membrane (Immobilon). Membranes were blocked in 5% nonfat powdered milk (Carnation or Sunny Select from local grocery stores) dissolved in TBS+ 0.1% Tween 20, as described above. ABI5 protein was detected with anti-ABI5 (gift from R. Vierstra, WU-STL) at a dilution of 1:5,000 followed by the secondary antibody peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L; Jackson ImmunoResearch) at a dilution of 1:10,000. ProSignal Dura (Prometheus # 20-301) was used as a chemiluminescent substrate. Chemiluminescence was digitally imaged in the linear range of detection using the ImageQuant LAS400 imaging system (GE).

Seed Size Measurements and Protein Comparison

For seed size measurements (Supplementary Figure S7), seeds were placed on double-sided tape on a microscope slide and photographed through a dissecting microscope. BRIZ2/briz2-3 abi5-7 and BRIZ2/briz2-3 AB15 were siblings from the cross used to generate the briz2-3 abi5-7 double mutant plants. Seed length was measured using Image J. For seed weight measurements, for each genotype, three groups of 100 seeds were weighed. For embryo size measurements, embryos were excised as described above and measured using Image J. Measurements were analyzed using GraphPad Prism.

RNA Extraction and Quantitative PCR

Seeds were grown in liquid GM under constant light for 6 days and treated with 50 µM ABA or 0.1% ethanol as mock treatment for 6 h. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, 74903) according to manufacturer’s instructions. About 2.4 µg of total RNA was used in a 20 µl reverse transcription reaction performed with Superscript III reverse transcriptase (Invitrogen, 18080-044). Real-time PCR amplification was performed with 50 µl of reaction solution containing 1 µl of first-strand cDNA, 10 pmol of each primer, and 1x SYBR Green Master Mix (Applied Biosystems, A46109). Relative transcript levels were obtained using the comparative Ct method. The experiment

https://www.danforthcenter.org/our-work/core-facilities/proteomics-mass-spectrometry/
RESULTS

briz Seeds Are Defective in Seed Coat Rupture and Germination, but briz Embryos Are Alive

Previously we briefly described the effects of loss-of-function T-DNA insertions in BRIZ1 and BRIZ2 in Arabidopsis (Hsia and Callis, 2010). Here, we more closely analyze the behavior of briz homozygous seeds over time and assess their ability to initiate SCR separately from radicle emergence. SCR typically occurs before radicle emergence, with the latter process generally acknowledged as germination (reviewed in Holdsworth et al., 2008). Examples of the three phenotypic classes quantified in Figures 1A–E are shown in Figure 1F: no SCR, no radicle; SCR; and SCR, radicle. Under our growth conditions, age-matched wild-type (Col) seeds rapidly transverse through SCR and complete germination by 48 h (Figure 1A, “SCR, Radicle” class). Seeds from plants heterozygous for a T-DNA insertion in the coding region of either BRIZ1 (briz1-1) or BRIZ2 (briz2-1, -2, and -3) segregate for two strikingly different phenotypes in a 3:1 ratio. Three-fourths of the seeds exhibit a wild-type phenotype, largely completing SCR and germination by 48 h at 20°C. However, one-quarter of the seeds fail to exhibit SCR even after 7 days (Figures 1B–E, “No SCR, No radicle” class). Between 7 and 14 days, a few seeds exhibit SCR (although it is often abnormal, with ruptures randomly across the surface rather than directly along the root axis), and some of these continue on to germinate. However, these germinated seedlings do not accumulate visible levels of chlorophyll, their cotyledons fail to expand and reflex, and root elongation is minimal (Figures 1G,H). The briz mutant phenotype referred to in this work includes both non-germinated seeds, seeds with SCR only, and the pale, undeveloped, late germinating embryos shown in Figures 1G,H. All briz1-1, briz2-1, briz2-2, or briz2-3 single mutants exhibit strongly
delayed SCR and when germination occurs, have phenotypically similar seedlings that fail to progress in development.

We hypothesized that the *briz* phenotype may result from embryo cell death and that passive absorbance of water could be sufficient for delayed SCR and late embryo protrusion from the seed coat. We used Sytox orange and FDA staining (Truernit and Haseloff, 2008) to test the viability of *briz* seedlings. Sytox orange penetrates non-viable cells and stains nuclei. FDA enters living cells and is converted to a fluorescent compound, fluorescein, by cytosolic esterases. Before staining and confocal microscopy, embryos were either left untreated or were killed with a brief 98°C heat treatment. As expected, control Col embryos did not stain with Sytox orange unless they were heat-treated (Figures 2T vs. S). Untreated Col embryos stained with FDA (Figure 2U), while heat-treated Col embryos did not (Figure 2V). These results show that cells in Col embryos are alive unless they are killed with heat treatment. Col seeds used in the experiment were soaked for a few hours in water because these seeds are morphologically similar to 15-day-old *briz* seeds.

Seeds from BRIZ1/*briz1-1*, BRIZ2/*briz2-1*, or BRIZ2/*briz2-2* plants were plated on GM for either 15 or 30 days before *briz* embryos were collected. Like Col embryos, 15-day-old *briz1* and *briz2* embryos did not stain with Sytox orange (Figures 2D–F) unless they were heat-treated prior to staining (Figures 2A–C). Untreated *briz1* and *briz2* embryos stained with FDA (Figures 2G–I). These results show that cells in 15-day-old *briz1* and *briz2* embryos are alive unless they are killed with heat treatment. The same tests were performed on 30-day-old *briz* embryos. Similar to 15-day-old embryos, 30-day-old *briz1* and *briz2* embryos were primarily Sytox orange negative (Figures 2M–O) unless heat-treated first (Figures 2J–L), and untreated embryos were FDA positive (Figures 2P–R). These results indicate that cells are alive in 30-day-old *briz* embryos even though the embryos are arrested at the germination/post-germination stage.

**The *briz* Phenotype Can Be Partially Rescued by the Carotenoid Biosynthetic Inhibitor Fluridone, and the Mutant Phenotype Returns With Addition of Low Levels of ABA**

Environmental conditions were sought that would promote germination and/or growth of *briz* embryos. Because single homozygous *briz* individuals cannot be grown to maturity for seed production, all studies were conducted on seeds from selfed heterozygous BRIZ/*briz* parents. When seeds from BRIZ1/*briz1-1* or BRIZ2/*briz2-2* plants were plated on GM alone or on GM containing the solvent controls DMSO or ethanol, ~0.25 of the seeds exhibited the *briz* phenotype (Figure 3; Supplementary Figure S1), as expected for the segregation of a single recessive trait (also see Figure 1). Chi-square goodness of fit tests confirmed that phenotypic segregation of seeds from heterozygous BRIZ1/*briz1-1* and BRIZ2/*briz2-2* plants fit an expectation of 3:1 wild-type:*briz*, with *p*-values of 0.18 and 0.35, respectively (*n* = ~220 seeds).

We tested the effects of light or various additions/modifications to GM plates and predicted that conditions which rescued the *briz* phenotype would reduce the fraction of seeds with a typical *briz* phenotype. This fraction was unaffected by incubating the plates at RT in the dark (Figures 3A,B). Neither the absence of sucrose nor the addition of mannitol had a significant effect on the fraction of *briz* seedlings (Figures 3A,B). Similarly, the fraction of *briz* seedlings was unaffected by addition of the synthetic auxin 2,4, dichlorophenoxyacetic acid (2,4 D), the ethylene precursor ACC, or BL (Figures 3A,B; see Supplementary Figure S1 for example of BL plates). The results were the same for seeds segregating for either *briz1-1* (Figure 3A) or *briz2-2* (Figure 3B).

By contrast, addition of fluridone, a carotenoid biosynthetic inhibitor that also blocks ABA biosynthesis (Bartels and Watson, 1978), resulted in a significant reduction of phenotypically *briz* embryos (Figures 3A,B). Similar results were observed for a second *briz2* allele, with seeds from a BRIZ2/*briz2-1* parent (Supplementary Figure S2; for examples of plates for all mutants, see Supplementary Figure S3A, which are non-green because of insufficient chlorophyll-protecting carotenoids, resulting in photo-oxidative damage to chlorophylls, Bartels and Watson, 1978). These results demonstrate that fluridone promotes further development of homozygous *briz* seedlings. However, few *briz* seedlings had expanded visible true leaves, and those that did were smaller than their wild-type siblings, indicating that suppression of the *briz* phenotype was incomplete.

To determine whether fluridone’s ability to suppress the *briz* phenotype is caused by a reduction in endogenous ABA and not a reduction in some other carotenoid-related metabolite, we tested the effect of including 0.1 μM ABA in the GM plates in addition to fluridone. While this concentration of ABA did not inhibit germination of wild-type Col seeds (Supplementary Figure S3B), it strongly affected germination of a subset of seeds from BRIZ1/*briz* parents. Around 25% of the seeds failed to germinate and looked identical to the *briz* seeds grown on GM plates (Figure 3). These results suggest that the growth-promoting effect of fluridone on *briz* seeds results from lowering endogenous ABA levels, not from affecting the levels of other products derived from the carotenoid biosynthetic pathway.

**briz** Seeds Do Not Hyper-Accumulate ABA

*briz* seeds phenotypically resemble wild-type seeds plated on a high concentration of exogenous ABA (Leung et al., 1994). One possible explanation for the strong growth-arrested phenotype (Figures 1, 2) and hypersensitivity to added ABA (Figure 3) seen in *briz* mutants could be an elevated level of endogenous ABA. To test whether *briz* seeds have higher (ABA) than wild-type seeds, we measured (ABA) in two lines whose progeny exhibited a 100% *briz* phenotype. These lines arose from two independent transgenic lines that initially expressed an epitope tagged form of BRIZ2 (Myc-BRIZ2) in the *briz2-1* background (Hsia and Callis, 2010). In subsequent generations, 100% of the seeds exhibited the *briz* phenotype, suggesting that both the endogenous BRIZ2 gene and the Myc-BRIZ2 transgene had silenced. Western blots of seed protein extracts verified that non-germinating seeds from one line (S, for silenced) did not express Myc-BRIZ2 protein encoded by the transgene,
while Myc-BRIZ2 protein was readily visible in an independent line that carried the same transgene and exhibited 100% germination (Figure 4A). Seeds from the two silenced lines and age-matched Col were hydrated for 1 h, after which ABA content was determined. briz1-1 seeds did not have a significantly higher level of ABA than the wild-type control, and in fact S1 had a slightly lower ABA level (Figure 4B). Analysis of ABA content in briz1-1 seeds was not possible because we could not obtain silenced lines in the briz1-1 mutant background that gave the 100% briz seeds needed for the analysis. Altogether these data suggest that briz embryos do not hyper-accumulate ABA, and that briz
embryos are hypersensitive to both endogenous and exogenous ABA.

**ABA Does Not Affect BRIZ mRNA in Seedlings**

To evaluate whether ABA affects BRIZ expression, we used qPCR to measure relative BRIZ mRNA levels in 6-day-old Col seedlings treated with for 6 h with ABA. Exogenous ABA induces robust transcriptional responses at this developmental stage (Lopez-Molina et al., 2001). Expression of RD29A, a well-known ABA-responsive gene (Nakashima et al., 2006), increased by approximately 100-fold after ABA treatment, indicating a strong response to ABA (Figure 4C). However, neither BRIZ1 nor BRIZ2 mRNA levels were affected by ABA treatment (Figure 4C).

**briz2-2 gin1-3 Double Mutants Have Increased Germination, Greening, and Growth**

To test whether the briz phenotype can be suppressed if endogenous ABA is reduced genetically, BRIZ2/briz2-2 plants were crossed with ABA-deficient gin1-3 plants. GIN1 (also called ABA2) encodes a short-chain dehydrogenase/reductase enzyme involved in ABA biosynthesis (Cheng et al., 2002). gin1-3 is a null allele with a deletion in the second exon which results in an early stop codon, and gin1-3 plants have low levels of endogenous ABA.

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**FIGURE 3** | Fluridone reduces the fraction of briz1-1 and briz2-2 mutants with a typical briz phenotype, which returns upon addition of abscisic acid (ABA).

**(A)** Seeds from heterozygous BRIZ1/briz1-1 plants were plated on agar GM plates or on GM plates lacking sucrose or containing 1% mannitol, 1 μM 2,4-D, 50 μM aminocyclopropane carboxylic acid (ACC), 10 μM brassinolide (BL), 100 μM fluridone (Flur.), or 100 μM fluridone + 0.1 μM ABA. Plates with 0.1% EtOH were used as solvent controls for the 2,4-D and ACC plates. Plates with 0.1% DMSO were used as solvent controls for the BL and fluridone plates. For the dark treatment, GM plates were wrapped in foil. Seedlings with a typical briz phenotype were scored after 12–15 days and represented as a fraction of the total seeds plated. Asterisks (****) represent p < 0.0001, ANOVA analysis (Graph Pad Prism). Bars represent ±SD. Data represent 3–4 independent experiments with 30–50 seeds each.

**(B)** Seeds from BRIZ2/briz2-2 plants, analyzed as described in (A).
(Cheng et al., 2002). Seeds from plants homozygous for gin1-3 and heterozygous for BRIZZ1/briz1-1 plants do not hyper-accumulate ABA, and ABA does not affect seedlings and support the hypothesis that briz2-2 seeds do not hyper-accumulate ABA, and ABA does not affect BRIZ1/briz1-1 were plated on GM. All 80 seeds germinated and briz1-1 gin1-3 mutants allowed the additional growth of briz2-2. gin1-3 seeds were identified by their slower germination and growth (circled in red, Figure 5A). After 2 weeks, an average of 92.3% of the briz2-2 gin1-3 mutants had grown (Figure 5B) and most had developed true leaves (Figures 5C,D). However, briz2-2 gin1-3 plants remained small and most eventually died on the GM plates or wilted quickly and died after they were transplanted to soil. The loss of GIN1 likely contributed to the poor survival of the double mutants because gin1-3 single mutants are smaller than wild-type plants and wilt quickly (Cheng et al., 2002) and were challenging to grow to maturity under our growth conditions. A few plants that were transplanted to soil were verified by PCR to be homozygous for briz2-2 (Supplementary Figure S4). These results show that a reduction in endogenous ABA allows further development of briz2-2 seedlings and support the hypothesis that briz2-2 seedlings are growth arrested due to ABA hypersensitivity.

In the gin1-3 Background, Heterozygous BRIZ1/briz1-1 Plants Produce More Underdeveloped Seeds Than Their WT BRIZ Sibs

BRIZ1/briz1-1 plants were also crossed with gin1-3 plants, and seeds from plants homozygous for gin1-3 and heterozygous for BRIZ1/briz1-1 were plated on GM. All 80 seeds germinated and looked wild-type. This was surprising, as we expected approximately 25% (the briz1-1 homozygotes) to have a slow growth phenotype similar to briz2-2 gin1-3 double mutants (see Figure 5). All seedlings were genotyped and only three possible BRIZ1/briz1-1 homozygotes were identified (Supplementary Figure S5A). These numbers do not fit the expected ratio of 1:2:1 WT:het:mutant for BRIZ1 (X² = 28.189, p < 0.0001). Instead, they fit a ratio of 1:2 WT:het (X² = 0.311, p = 0.577), with the briz1-1 gin1-3 double mutant seeds frequency much reduced. Both male and female homozygous briz1-1 gametophytes are still viable in the gin1-3 background (if either gametophyte is not viable, a 1:1 WT:het ratio would be expected, and these data do not fit that ratio, with X² = 8.165 and p = 0.004). In a larger plating of 300 seeds, only 12 seeds did not germinate (presumably homozygous for briz1-1), well below the expected number of 75. These results suggest that many homozygous briz1-1 seeds in the gin1-3 background do not develop to the mature seed stage.

To investigate why there were fewer than expected briz1-1 homozygotes, we examined siliques of heterozygous BRIZ1/briz1-1 plants in the gin1-3 background. Siliques of BRIZ1/briz1-1 gin1-3 plants contained more undeveloped or collapsed seeds (40.2%) than gin1-3 plants that were wild-type for BRIZ1 (24.6%; Supplementary Figure S5B). gin1-3 single mutants have defects in seed development and have many undeveloped seeds (Cheng et al., 2002), so our observation of 24.6% undeveloped seeds in gin1-3 plants that were wild-type for BRIZ1 was not surprising. These results suggest that in addition to seed loss due to gin1-3, many homozygous briz1-1 mutant seeds in the siliques of heterozygous BRIZ1/briz1-1 plants do not develop.

Despite the lower than expected number of homozygous briz1-1 seeds, we were able to identify one homozygous briz1-1 seedling (Supplementary Figures SSC,D). The seed took more than 1 week to germinate, and development was very slow...
FIGURE 5 | briz2-2 mutants have increased germination, greening, and growth in the gin1-3 background. Seeds from plants homozygous for gin1-3 and heterozygous for BRIZ2/briz2-2 were plated on plain GM. (A) Plate with seeds from a gin1-3 plant heterozygous for BRIZ2/briz2-2. Red circles are briz2-2 gin1-3 seedlings. (B) Fraction of homozygous briz2-2 mutants with green expanded cotyledons was recorded after 14 days. N = 3 plates with approximately 50 seeds per plate. Bars represent ±SD. Asterisks indicate a significant difference (** for p < 0.01) according to a t-test with Welch’s correction in GraphPad Prism. (C) Photos of homozygous briz2-2 seedlings at 18 days. Bars, 1 mm. (D) PCR genotyping of seedlings in panel (C) for BRIZ2, with Col as a WT control. GSP = PCR with gene-specific primers flanking the T-DNA insertion site. T-DNA = PCR with T-DNA primer and gene-specific primer. Primer sequences in Supplementary Table S1.

GA3 Increases Germination of briz Mutants

Abscisic acid and gibberellic acid (GA) act antagonistically during germination, so the ability of exogenous GA to affect the briz phenotype of delayed or no germination, or arrested seedling growth was tested. Seeds from heterozygous BRIZ1/briz1-1, BRIZ2/briz2-1, or BRIZ2/briz2-3 plants were plated on GM or GM containing GA3. Homozygous briz seeds were identified after 5 days by their slower germination and the percent of the briz class of seeds that germinated was scored at 1 and 2 weeks (if all briz seeds germinated, the percent is 100%). After 1 week on
GM, 0% of homozygous briz1-1 seeds, 3.3% of homozygous briz2-1 seeds, and 0.8% of homozygous briz2-3 seeds had germinated. On GM plates containing 10 μM GA3, these percentages increased to 19.0, 35.5, and 42.0%, respectively (Figures 6A–C). Increasing the concentration of GA3 to 100 μM, 200 μM (Figures 6A–C), or 1 mM (Figure 6D) did not greatly increase germination above that observed for 10 μM GA3.

**briz Embryos Are Hypersensitive to Sucrose and Glucose and Elongation Can Be Promoted by GA3**

The effects of exogenous hormones on the embryo can be blocked if the seed coat remains intact. Because briz seeds are slow to exhibit SCR, we excised embryos from their seed coats to better assess the effects of exogenous GA3 on embryo growth. To identify homozygous briz seeds for the experiment, which cannot be distinguished at the dry seed stage, seeds from heterozygous BRIZ/briz plants were plated for 48 h on the types of GM plates described in Figure 7, and non-germinated seeds were selected. PCR genotyping after the experiment confirmed that these were briz1-1 or briz2-3 mutants (Supplementary Figure S6). Embryos removed from their seed coats and placed on GM (plain GM contains 1% sucrose) for 5 days remained growth arrested, though an increase in size and anthocyanin was visible (Figure 7A), and the addition of GA3 had no effect (Figure 7B). Glucose had the same effect as sucrose, inhibiting greening and growth (Figures 7G,H). When placed on media without sugar (Figure 7C) or with 1% mannitol as an osmotic control (Figure 7E), cotyledon greening and expansion was visible after 5 days in both briz1-1 and briz2-3 embryos. On both medias, briz hypocotyls elongated with added GA3, while the roots remained short (Figures 7D,F). Wild-type Col embryos greened on all media types and had elongated hypocotyls on medias containing GA3 compared to the same medias without GA3 (Figure 7, bottom row). These results indicate that compared to Col, briz1-1 and briz2-3 single mutants are hypersensitive to the sucrose concentration present typically in GM (1%). A combination of excision from the seed coat and a lack of sucrose in growth media allows briz mutants to develop more than previously observed, and to better respond to GA3. Seed age likely affects briz responsiveness to lack of sucrose, as seeds stored at RT for >6 months showed reduced greening on media without sucrose (Supplementary Figure S7).

**ABI5 Levels Are Higher in briz Seeds**

ABI5 is a bZIP transcription factor that is important for ABA responses during germination (Lopez-Molina et al., 2002).

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**FIGURE 6 |** Gibberellic acid addition improves germination of briz1-1, briz2-1, and briz2-3 mutants. (A) Seeds from plants heterozygous for BRIZ1/briz1-1 were plated on GM with 0, 10, 100, or 200 μM GA3. Germination of briz seeds was scored after 1 week. Bars represent ±SD. Data from 2 to 4 independent experiments with 120–150 seeds per experiment. (B) Seeds from plants heterozygous for BRIZ2/briz2-1. (C) Seeds from plants heterozygous for BRIZ2/briz2-3. (D) Seeds were plated on GM with or without 1 mM GA3.
Given the strong growth arrest and ABA hypersensitivity without increased endogenous ABA in briz mutants, we asked whether ABI5 levels are higher in briz embryos. Seeds from BRIZ1/briz1 or BRIZ2/briz2-3 plants were plated on GM, stratified for 24 h at 4°C, and then incubated at RT for 72 h to identify briz seeds by their non-germination status. Protein extracts from these seeds were compared to protein extracts from wild-type seeds, either dry, stratified, or stratified and then incubated at RT for various timepoints (Figure 8; Supplementary Figure S8). In wild-type seeds, ABI5 levels fell after 24 h of stratification at 4°C, and continued to fall after the seeds were moved to RT. By 72 h, ABI5 levels were greatly reduced. By contrast, ABI5 protein was still visible in briz1-1 and briz2-3 seeds after 72 h at RT. These data indicate a correlation between lack of greening and growth and visible ABI5 protein levels in briz seeds.

In the abi5-7 Background, Both briz1 and briz2 Mutants Have Increased Germination, Greening, and Growth
To test whether ABI5 protein contributes to the growth arrest of briz embryos, we genetically eliminated ABI5. BRIZ1/briz1-1, BRIZ2/briz2-1, and BRIZ2/briz2-3 plants were crossed with abi5-7 plants. The abi5-7 null allele contains a point mutation resulting in an early stop codon, and abi5-7 seeds exhibit reduced sensitivity to ABA (Nambara et al., 2002). F3 seeds from F2 homozygous abi5-7 plants also heterozygous for BRIZ1/briz1-1, BRIZ2/briz2-1, or BRIZ2/briz2-3 were plated on GM. briz1-1, briz2-1, and briz2-3 mutants in the abi5-7 background had increased germination compared to the same alleles in wild-type ABI5 F3 siblings, though they grew more slowly than wild-type seedlings (Figures 9A, 10A). PCR genotyping confirmed that these small, slowly developing, green seedlings were double mutants (Figures 9, 10). We were able to recover two briz1-1 abi5-7 plants. Both grew to maturity on soil but produced few seeds, which failed to germinate (Figures 9D,E; Supplementary Figure S9).

F3 plants homozygous for abi5-7 and heterozygous for BRIZ2/briz2-3 were propagated to produce F4 seeds. Double mutant F4 seedlings with true leaves were transferred from plates to either soil or magenta boxes with GM. A subset survived to flower and produce seeds (Supplementary Figure S10). These plants produced leaves slowly and had small rosettes.
Seeds From briz2-3 abi5-7 Plants Are Hypersensitive to Sucrose and Glucose When Compared to Seeds From BRIZ2/briz2-3 abi5-7 Plants

Homozygous briz2-3 abi5-7 plants (Supplementary Figure S10) produced seeds. Interestingly, these seeds were longer and weighed more and their embryos were longer than seeds from wild type (Col) and single mutant abi5-7 plants (Supplementary Figure S11).

When seeds from the briz2-3 abi5-7 plants were plated on GM, germination was slow, as previously observed with briz2-3 abi5-7 seeds derived from heterozygous BRIZ2/briz2-3 abi5-7 plants (see Figure 10), but surprisingly, after 10 days, the germinated seedlings did not have green cotyledons on GM (with 1% sucrose; Figure 11A). To directly test whether the sucrose present in GM inhibits germination and growth of seeds from double mutant briz2-3 abi5-7 plants as observed previously with briz single mutant embryos, seeds were also plated on GM without sugar, GM with 1% mannitol instead as an osmotic control, or with 1% glucose instead of sucrose (Figure 11). Seeds from Col, abi5-7, and BRIZ2/briz2-3 plants were plated as controls. After 10 days, only 21.7% of briz2-3 abi5-7 seeds had germinated on GM containing sucrose, significantly lower than 88.3, 78.3, and 66.7% on GM lacking sugar or on GM containing 1% mannitol in place of sucrose (58.3% of total seeds plated for both; Figure 11D, right graphs). Some seeds from briz2-3 abi5-7 plants that germinated on GM (+1% sucrose) had green cotyledons initially (Supplementary Figure S12A), but they did not expand fully, the green faded after several days, and by 10 days the seedlings were noticeably purple (Figure 11C, leftmost image and Supplementary Figure S12B).

In addition to inhibited germination on GM containing sucrose or glucose, briz2-3 abi5-7 mutants had inhibited cotyledon greening and expansion (Figure 11). Ten days after plating, none of the briz2-3 abi5-7 seedlings on GM plates containing sucrose or glucose had expanded green cotyledons, a significant difference compared to ~70% of the germinated seedlings on GM lacking sugar or on GM containing 1% mannitol in place of sucrose (58.3% of total seeds plated for both; Figure 11D, right graphs). We also noticed that seedlings grown on GM without sucrose from briz2-3 abi5-7 plants, while green at 10 days, then yellowed/senesced earlier than seedlings from Col or abi5-7 plants when maintained on the same plates for 2.5 weeks (Supplementary Figure S13). To summarize these results, sucrose inhibited germination of briz2-3 abi57 mutants, while both sucrose and glucose inhibited cotyledon expansion and greening.
DISCUSSION

The two Arabidopsis BRIZ genes, BRIZ1 and BRIZ2, were originally identified in a reverse genetic screen for phenotypic effects resulting from T-DNA insertion in genes encoding RING-type E3 ligases (Hsia and Callis, 2010). Although BRIZ1 and 2 share the same domains, single loss-of-functions mutants in either briz1 or briz2 have similar phenotypes, indicating that they are not functionally redundant. BRIZ1 and BRIZ2 interact in vitro and when over-expressed in planta, and expression of truncated BRIZ proteins unable to form heteromers fail to complement the briz mutant phenotypes, leading us to propose that the two proteins normally function in a complex as a single E3 ligase activity (Hsia and Callis, 2010). However, the biological functions of the BRIZ heteromer remained unknown.

We show here multiple lines of evidence to support the hypothesis that briz1 and 2 embryos are hypersensitive to ABA. First, their macroscopic phenotypes resemble ABA-arrested seedlings: defective in germination, greening, true leaf formation and cotyledon expansion, while maintaining cell viability. briz1 and briz2 seeds are also defective in SCR, an ABA-modulated process. Although SCR was traditionally thought to be ABA-independent because SCR occurs on ABA-containing media that blocks subsequent radicle emergence, current evidence indicates that exogenous ABA does not penetrate through the seed coat or through the layer of cuticle outside the single cell layer of endosperm, which blocks ABA exposure to the embryo (De Giorgi et al., 2015). In nicking experiments, which expose embryos to the media, exogenous ABA is effective in blocking SCR in wild-type seeds (Barros-Galvão et al., 2019). In contrast, SCR in nicked abi5-7 seeds is almost completely insensitive to exogenous ABA, indicating a key role for ABI5 in this process (Barros-Galvão et al., 2019). Next, the carotenoid biosynthesis inhibitor fluridone, which reduces endogenous ABA levels, partially rescued the growth arrest of briz embryos. This effect was reversed on media containing fluridone plus 0.1 μM ABA, a concentration that does not inhibit germination of wild-type seeds. The partial rescue of briz2 growth in a genetic
background of reduced ABA content and the partial rescue of both briz loci in the abi5-7 mutant background additionally supports this model. Exogenous GA3, which functions antagonistically to ABA, promoted germination and cotyledon greening of briz mutants. Altogether, these results support the hypothesis that briz mutants are hypersensitive to ABA and that BRIZ plays a role in ABA signaling under non-stress conditions most predominantly during germination and early seedling growth.

Although briz1 and briz2 single mutants have the similar phenotypic differences from wild type, briz1 mutants were not phenotypically rescued to the same extent as briz2 mutants in either the abi5-7 or gin1-3 backgrounds. For example, while we were able to obtain mature briz2-2 gin1-3 double mutant plants, we could not obtain mature briz1-1 gin1-3 double mutants, likely as a result of increased double mutant seed loss observed in siliques of BRIZ1/briz1-1 gin1-3 plants. These results suggest that the in vivo functions of BRIZ1 and BRIZ2 are not completely redundant. We previously observed that their in vitro biochemical activities were not equivalent (Hsia and Callis, 2010), and these differences could result in loss of BRIZ1 having in a more severe effect in vivo, although we do not understand the mechanism.

The phenotype of briz mutants in the abi5-7 background supports a model in which BRIZ is important during germination...
but is less important for later vegetative growth. Our hypothesis is that BRIZ acts as a brake on ABA signaling, and loss of BRIZ leads to ABA hypersensitivity in briz seeds. Loss of ABI5, which promotes inhibition of germination by ABA (Finkelstein and Lynch, 2000), partially compensates for the hypersensitivity to ABA in briz abi5-7 double mutants and allows many seeds to germinate. However, their remaining ABA hypersensitivity is still strong enough to delay germination for an extended period of time. Once past germination and seedling establishment, the ability of briz2-3 abi5-7 double mutants to grow to maturity with some seed set suggests that loss of BRIZ2 does not play a major role during later vegetative and reproductive growth. Whether BRIZ2 affects ABI5 accumulation, localization, and/or phosphorylation is challenging to assess. 72-h-old non-germinated briz seeds do have detectable ABI5 protein, but whether this results from the arrested germinated state or is causal in arresting growth is not certain.

How exactly might BRIZ function in ABA signaling? The roles of BRIZ orthologs could provide clues. Human BRAP2/IMP (B RCA1-associated protein 2, also called IMP for Impedes mitogenic signal propagation) is an E3 ligase with the same BRAP2, RING, ZnF UBP, and coiled-coil domains as Arabidopsis BRIZ1 and BRIZ2. BRAP2/IMP acts as a
cytoplasmic retention factor for multiple proteins (Li et al., 1998; Asada et al., 2004; Davies et al., 2013) and reduces Ras/Raf/MEK/ERK signaling (Matheny et al., 2004). In Ras signaling, IMP sequesters the scaffold protein KSR1 and prevents KSR1 homo-oligomerization. In the presence of growth factor, IMP self-ubiquitinates and targets itself for proteasomal degradation. KSR1 is then free to homo-oligomerize and act as a scaffold for the kinase Raf and its substrate MEK. Like IMP, BRIZ could function as an adaptor protein between ABA signaling components.

Raf-like kinases and a MAPK scaffold protein have been identified in plants. There are 48 Raf-like kinases in *Arabidopsis* (Jonak et al., 2002), including CTR1 (also called SIS1 for SUGAR-INSENSITIVE1). Though mostly known for its role in ethylene signaling, CTR1 also plays a role in sensitivity to both ABA (Ghassemian et al., 2000) and sugar (Zhou et al., 1998; Gibson et al., 2001). RAF10 and RAF11 are *Arabidopsis* Raf-like kinases that promote seed dormancy and ABA response (Lee et al., 2015). It was recently reported that multiple other Raf-like kinases are also involved in ABA signaling (Lin et al., 2020). RECEPTOR FOR ACTIVATED C PROTEIN KINASE 1 A (RACK1A) is an Arabidopsis MAPK scaffold protein (Guo et al., 2007; Cheng et al., 2015). Arabidopsis RACK1 proteins are encoded by three genes (RACK1A, B, and C; Chen et al., 2006). *rack1a* mutants are hypersensitive to ABA (Chen et al., 2006), and the increased ABA hypersensitivity of *rack1a rack1b* and *rack1a rack1c* double mutants suggests that the RACK1 genes function redundantly to reduce ABA response (Guo et al., 2009). Whether BRIZ proteins have any relationship with Raf-like kinases or MAPK scaffold proteins is unknown. One hypothesis is that BRIZ might modulate the abundance or activity of a Raf-like kinase or a scaffold protein in an ABA-dependent manner.

We found that *briz1* and *briz2* embryos are hypersensitive to exogenous sucrose and glucose, which is not an osmotic effect. A number of ABA signaling mutants have altered sensitivities to sugar. For example, mutants that are insensitive to glucose include the PP2C phosphatase mutant *abi2-1* (Dekkers et al., 2008) and the transcription factor mutants *abi3-1* (Yuan and Wysocka-Diller, 2006; Dekkers et al., 2008), *abi4* (Arenas-Huerto et al., 2000), *abi5* (Arenas-Huerto et al., 2000), and *abi2* (Kim et al., 2004). Overexpression of *ABI3*, *ABI4*, or *ABI5* results in glucose hypersensitivity (Finkelstein et al., 2002). Sugar-related phenotypes are evident in alternative names for many ABA-related genes. *ABA2* is also called *GIN1* for GLUCOSE INSSENSITIVE 1, *ISI4* for IMPAIRED SUCROSE INDUCTION 4, and *SIS4* for SUGAR INSSENSITIVE 4. *ABI3* is also called *SIS10* for SUGAR INSSENSITIVE 10. *ABI4* is also called *GIN6* for GLUCOSE INSSENSITIVE 6 and has several other sucrose-based names including *SIS5* for SUGAR INSSENSITIVE 5. With mutant phenotypes related to both to both ABA and sugar, BRIZ joins the collection of genes involved in both signaling pathways.

While *abi5-1* mutants are somewhat resistant to high levels of glucose (Arenas-Huerto et al., 2000), *briz abi5-7* double mutants retain sensitivity to glucose and sucrose. This indicates that *briz* sugar sensitivity occurs independent of *ABI5*. Curiously, *abi5-7 briz2-3* double mutants derived from homozygous *abi5-7 briz2-3* parents are more sensitive to sucrose than double mutants derived from heterozygous *BRIZ2/briz2-3* parents, and seeds from *abi5-7 briz2-3* plants are larger than seeds from *abi5-7 BRIZ2/briz2-3* plants. It is possible that in ABA-hypersensitive *briz2-3* *abi5-7* plants, the transport of sugars or other nutrients to the developing embryo or seed is affected. The pigmentation visible in *briz* mutants plated on sucrose or glucose resembles descriptions of anthocyanins produced in response to high levels of exogenous sugar (Dekkers et al., 2008). The behavior of seeds from *briz2-3* *abi5-7* mutants, which are hypersensitive to the 1% sucrose in agar GM plates compared to *briz2-3* *abi5-7* mutants derived from *BRIZ2/briz2-3* *abi5-7* plants, suggests that the parental *BRIZ2* genotype affects sugar sensitivity.

The reasons for heterogeneity in *briz* germination phenotypes, and for the partial rescue of some but not all *briz* seeds with reductions in ABA synthesis, ABA signaling or with the addition of GA3, are unknown. These variable responses could reflect a phenomenon called bet-hedging, where differences in germination behavior are observed even when genetically identical seeds are grown under the same environmental conditions. These differences can act as a mechanism to ensure species survival under possible variable, stressful conditions (Bradford, 2002; Mitchell et al., 2017; Cortijo and Locke, 2020). GA and ABA can act as a bistable switch to control germination, and this switch can amplify variability in germination (Abley et al., 2020). Perhaps *briz* individuals with altered ABA responses have amplified differences in ABA-GA antagonism during germination, with individuals “flipping” either one way or the other.

Gibberellic acid opposes most ABA responses by repressing ABA synthesis and ABA signaling, and the ratio of GA:ABA regulates the developmental outcome (Liu and Hou, 2018; Shu et al., 2018; Vishal and Kumar, 2018). The initial GA signaling event is GA-induced degradation/inactivation of the DELLA family of proteins. DELLAs suppress germination, in part by repressing expansin gene expression to prevent endosperm cell wall weakening and expansion (Sánchez-Montesino et al., 2019; Xu et al., 2020), and promote anthocyanin biosynthesis by sequestering the negative regulators JAZ and MYBL2, freeing other TFs to promote transcription of anthocyanin biosynthetic genes (Xie et al., 2016). Although it remains possible that defective GA signaling could contribute to the observed ABA hypersensitivity, possibly in an organ-specific and/or developmentally specific manner, we observed that *briz* seed germination is enhanced and seedling hypocotyl elongate in response to exogenous GA. Therefore, we are framing further experiments to test the hypothesis that the BRIZ ubiquitin E3 ligase plays a role in the ABA and sugar response network.

In summary, BRIZ1 and BRIZ2 are two RING-type E3 ligases involved in ABA signaling or response, either directly or indirectly. BRIZ is important for germination and early seedling growth, and further analysis of BRIZ function will
provide insight into ABA’s role in regulating this critical developmental transition in the plant life cycle.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KL, MH, and JC designed the research and wrote the paper. KL, MH, Y-TG, and JC performed research and analyzed data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.641849/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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