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BR Signal Influences *Arabidopsis* Ovule and Seed Number through Regulating Related Genes Expression by BZR1

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ABSTRACT Ovule and seed developments are crucial processes during plant growth, which are affected by different signaling pathways. In this paper, we demonstrate that the brassinosteroid (BR) signal is involved in ovule initiation and development. Ovule and seed numbers are significantly different when comparing BR-related mutants to wild-type controls. Detailed observation indicates that BR regulates the expression level of genes related to ovule development, including *HLL*, *ANT*, and *AP2*, either directly by targeting the promoter sequences or indirectly via regulation by BR-induced transcription factor BZR1. Also, Western blot demonstrates that the dephosphorylation level of BZR1 is consistent with ovule and seed number. The intragenic *bzr1-1D* suppressors *bzs247* and *bzs248* have much fewer ovules and seeds than *bzr1-1D*, which are similar to wild-type, suggesting that the phenotype can be rescued. The molecular and genetic experiments confirm that BZR1 and AP2 probably affect *Arabidopsis* ovule number determination antagonistically.

Key words: ovule and seed number; BR; ovule development-related genes.

INTRODUCTION

The plant reproductive development aspires to seed. Seeds come from fertilized ovules. The ovule numbers decide the putative maximal number of seeds. Genes involved in ovule development have been identified and studied in species such as Petunia, rice, and *Arabidopsis*. It is reported that two MADS-box genes, *FBP7* and *FBP11* (floral binding protein), influence ovule development in Petunia (Angenent et al., 1995; Angenent and Colombo, 1999). Similarly, *OsMADS13* determines ovule identity in rice (Dreni et al., 2007).

In *Arabidopsis*, many genes have been identified in each phase of the ovule development process. *SUPERMAN* (*SUP*) and *INNER NO OUTER* (*INO*) suppress and promote integument, respectively, and also affect the asymmetric form of integuments (Gaiser et al., 1995; Villanueva et al., 1999). *SHORT INTEGUMENTS1* and *2* (*SIN1* and *SIN2*) are required for cell division during integument development (Robinson-Beers et al., 1992; Ray et al., 1996; Broadhvest et al., 2000). *TSO1* encodes a nuclear protein required for the orientation of cell elongation and cytokinesis in integument development (Hauser et al., 1998, 2000; Song et al., 2000). *BELL* (*BEL1*) positively regulates the identity of integuments (Robinson-Beers et al., 1992; Modrusan et al., 1994; Ray et al., 1994). *AGAMOUS* (*AG*) and *BEL1* both control ovule identification in a redundant manner (Western and Haughn, 1999). *AG* mRNA is present in the placenta before and during ovule initiation (Bowman et al., 1991; Reiser et al., 1995). The primordial number is decreased in *ag* mutants, which indicates that *AG* affects the ovule identification in *Arabidopsis* (Western and Haughn, 1999). *HUELLENLOS* (*HLL*) and *AINTEGUMENTA* (*ANT*) both promote ovule primordial growth redundantly and they also influence the development of integuments (Schneitz et al., 1998, 2004). *APETALA2* (*AP2*) affects both ovule and other floral organ pattern formation (Bowman et al., 1989; Kunst et al., 1989; Modrusan et al., 1994).

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The *Arabidopsis* gene *HLL* encodes a mitochondrial ribosomal protein and participates in patterning and growth of the *Arabidopsis* ovule. *hll* mutants exhibit defects in ovule growth and development, integuments are highly reduced or absent, and cells in the distal regions of some ovule primordia collapse (Schneitz et al., 1998; Skinner et al., 2001). The *ANT* gene encodes a putative transcription factor and shares homology with the floral homeotic gene *APETALA2* (*AP2*) (Jofuku et al., 1994; Weigel, 1995; Elliott et al., 1996; Klucher et al., 1996). *ANT* regulates primordia initiation and growth of all floral organs (Elliott et al., 1996; Klucher et al., 1996). Strong *ant* mutants have reduced floral organ numbers and altered floral organ formations. *ant* mutants have half the number of ovules per pistil and the gap between individual ovules is enlarged in *ant* mutants (Elliott et al., 1996). Very few ovule integuments grow in *ant* mutants (Elliott et al., 1996; Klucher et al., 1996; Baker et al., 1997; Schneitz et al., 1997). *AP2* encodes a transcription factor with two AP2 domains (Jofuku et al., 1994) and is involved in the regulation of floral meristem and organ identification (Modrusan et al., 1994). The *ap2-5* mutant allele (Kunst et al., 1989) has carpelloid sepals that have ectopic ovules and stigmatic tissue, smaller petals, and a reduction in the number of fertile stamens to four or two. No morphological abnormalities are observed in *ap2-5* pistils. The *ap2-6* (Kunst et al., 1989) is a strong allele where whorl-one organs are transformed into unfused carpels and petals are completely missing. The reproductive organs are fertile, although the number of stamens is dramatically reduced. There is an average increase in the number of ovules in *ap2-6* mutants, including all ovule types (12384 ovules in 172 gynoecia; Western and Haughn, 1999).

Ovule and seed developments are regulated by different signals, which are hypothesized to be multiple phenotype mutants of some signal pathways (Pagnussat et al., 2009; Olmedo-Monfil et al., 2010). Since ovule development-related genes *ANT* and *AP2* have been identified for BZR1 target genes via ChIP-chips experiments (Sun et al., 2010) and BR functions in *Arabidopsis* seed development (Li et al., 2001), we hypothesized that BR plays a crucial role in ovule and seed development through regulating ovule development-related genes. There are many reports of BR-deficient and insensitive mutants, such as BR-deficient mutant *det2* (Chory et al., 1991), having a mutation in the BR biosynthesis process and with lower BR levels. Also, the main components of the BR signal pathway have been studied extensively over the past decade (Kim and Wang, 2010; Li, 2010), including the membrane-localized receptor kinase BRI1 (Brassinosteroid Insensitive 1), which binds BR and activates the transduction (Li and Chory, 1997; Wang et al., 2001; Kinoshita et al., 2005), the BR signal negative regulator BIN2 (a GSK3-like kinase; Li and Nam, 2002; Mora-Garcia et al., 2004; Nam and Li, 2004; Ehsan et al., 2005; Tang et al., 2008; Kim et al., 2009), the transcription factors BZR1/BES1 (He et al., 2005; Yin et al., 2002; Wang et al., 2002; Zhao et al., 2002; Yin et al., 2005), and so on. A single proline-to-leucine substitution in the BZR1 PEST domain results in the accumulation of unphosphorylated BZR1 in the gain-of-function mutant *bzr1-1D*, which suppresses or partially rescues a majority of all phenotypes observed in the BR-insensitive mutants *bri1-5* and *bin2* (Wang et al., 2002). Through microarray studies, a number of BR response genes have been identified (Goda et al., 2004; Nemhauser et al., 2004; Vert et al., 2005; Nemhauser et al., 2006). BZR1 can directly target promoters or intragenic regions of a pair of transcription factor families (Zhang et al., 2009), suggesting that BZR1 can further influence downstream genes that are involved in plant physiological processes. A ChIP-chip experiment identified 953 genes, which are named BR-regulated and BZR1 target (BRBT) genes (Sun et al., 2010).

To date, there are a few systemic reports pertaining to the molecular mechanism of BR regulating seed number determination, and none on ovule number determination. We studied the BR-deficient and insensitive mutants and found they have lower seed numbers, smaller seed size, and abnormal seed morphogenesis, while BR-enhanced mutant has more seeds. In this paper, we describe the phenotypes of significantly decreased seed numbers in BR-deficient and insensitive mutants, as well as significantly increased seed numbers in BR-enhanced mutant. The ovule number of BR mutants also showed significant differences. Through molecular and genetic experiments, we demonstrated that BR can influence the ovule and seed number by BZR1 regulating ovule development-related genes directly or indirectly.

Table 1. Comparison of Seed/Ovule Ratio among Col, *bzr1-1D*, *bin2*, and *det2* and between WS and *bri1-5*.

	Col	*bzr1-1D*	*bin2*	*det2*	WS	*bri1-5*
Seed	50	64.5	20	39	42.3	28.1
Ovule	52.95	68.08	29.07	52	46.4	32.2
Seed/ovule	0.94	0.95	0.69	0.75	0.91	0.87

RESULTS

BR Signal Affects Seed and Ovule Number in *Arabidopsis*

It has been shown previously that seed development is affected in BR mutants (Li et al., 2001). We analyzed the seed number of the BR-deficient mutant *det2*, the BR-insensitive mutants *bri1-5* and *bin2-1* (*bin2* for short, which is a gain-of-function mutant. Here, *bin2* means *bin2* +/–, since *bin2* homozygous mutant has no seed), the BR signal-enhanced mutant *bzr1-1D*, and Col-0 control. From this analysis, the average seed number of Col is 50, with

individual counts ranging from 44 to 56, 39 for *det2* (37–43), 20 for *bin2* (12–25), and 64.5 for *bzr1-1D* (55–72). In order to remove the ecotype inference, we also confirmed this result by comparing the seed numbers of *bri1-5* (the average number 28.1 (22–36)) and the wild-type control WS (the average number 42.3 (37–49)).

The morphological analysis and anatomic pictures of siliques of BR-related mutant and transgenic lines show that BR signal positively regulates silique growth and seed development (Figure 1A–1L; red arrows point out developmental seeds in *det2* and *bri1-5*). In BR-deficient and insensitive mutants, the siliques are shorter and thinner (Figure 1A and 1C–1F), while, in the BR signal-enhanced mutant *bzr1-1D*, the siliques are longer and thicker (Figure 1A, 1B, and Supplemental Figure 1B). The siliques of the *bzr1-1D* mutants appear to be more crowded with seeds compared to Col (Figure 1G, 1H, and Supplemental Figure 1A). The detailed average seed number per millimeter silique is 3.95 for Col and 4.46 for *bzr1-1D*, respectively. All the seed numbers suggest BR is a positive regulator in seed number determination (Supplemental Figure 1).

We further investigated the ovule numbers of BR-related mutants. It is reported that the duration of ovule development corresponds to the 9–12 phases of flower development (Smyth et al., 1990; Schneitz et al., 1995). Flowers representing the above stages from each of the mutants and control plants were analyzed for ovule pattern and number in the pistils. The average ovule number for *bzr1-1D* is 67.5; individual counts range from 58 to 78, compared to 55.4 (50–62) for wild-type control. *bin2* has an average ovule number of 30.2 (27–31) and *det2* has an average number of 44.1 (40–48; Figure 1M–1P and 1T). The ovule number is increased greatly in *bzr1-1D*, while the ovule number is reduced in *det2* and *bin2* compared to wild-type Col. The ovule number of *bri1-5* was also counted, giving an average of 32.2 (29–35), which is significantly less than WS, with an average of 46.4 (41–51) (Figure 1Q, 1R, and 1T).

The ratios of seed/ovule number of BR-deficient and insensitive mutants are lower than Col and BR signal-enhanced mutant *bzr1-1D*, indicating BR might also contribute to other seed development-related processes, such as male fertility, pollen grain germination, pollen tube growth, and fertilization (Table 1).

Previous results demonstrate that *DET2* is a key gene in BR biosynthesis and BRI1 is the receptor of BR, as well as a positive regulator of BR signal. BIN2 negatively regulates BR signal while BZR1 positively regulates BR signal. The results from the statistical analysis of the numbers of seeds and ovules from the loss-of-function mutants *det2* and *bri1-5* (with decreased seed and ovule counts) and gain-of-function mutants *bin2* (with decreased seed and ovule counts) and *bzr1-1D* (with increased seed and ovule counts) indicate that BR signaling positively regulates ovule and seed number.

Arabidopsis Ovule and Seed Number Determinations Are Regulated by BZR1

The ovule and seed increase observed in the *bzr1-1D* mutant suggests that BZR1 might be involved in ovule and seed number determination/regulation. Using qRT–PCR, we detected the BZR1 activity in Col, *bzr1-1D*, and two *bzr1-1D* suppressors *bzs247* and *bzs248* (Tang et al., 2011) by the expression of *CPD* (Figure 2A), which confirmed to be down-regulated by BZR1 (He et al., 2005). BZR1 repressed the *CPD* expression level *in vivo* (He et al., 2005); the recovered transcription levels of *CPD* in *bzs247* and *bzs248* confirm the rescue. Statistical analysis of ovule numbers was performed in the lines Col, *bzr1-1D*, *bzs247*, and *bzs248*, giving numbers of 53.6 (ranging from 50 to 59), 66 (range from 60 to 71), 48.1 (range from 45 to 51), and 48.5 (ranging from 46 to 52), respectively (Figure 2D). The average seed numbers of these four plants are 51 (45–58), 60.5 (58–62), 33.3 (27–42), and 40.8 (32–46), respectively. Observation of the seeds arrays in siliques indicated that *bzs247* and *bzs248* seeds are not as crowded as *bzr1-1D*, in contrast to *bzr1-1D* (Figure 2B and Supplemental Figure 1A). The ovule and seed numbers are all lower than *bzr1-1D* and similar to Col (Figure 2C–2E). These results demonstrate that BZR1 plays an important role in ovule and seed number determination.

Dephosphorylated BZR1 Increases Ovule and Seed Number

The dephosphorylation level of BZR1 determines BZR1 activity. To identify whether the dephosphorylation status of BZR1 affects ovule and seed numbers, we compared the ovule and seed numbers of several BR signal-related transgenic lines. The control line named W2C contained the pBZR1:: BZR1::CFP vector (the *BZR1* promoter drives the open reading frame of the BZR1 gene and CFP fusion protein). The dominant line named MX3 and M4C contain the same pBZR1::BZR1*::CFP vector but are two different alleles (the *BZR1* promoter drives the open reading frames of the mutated BZR1 gene and CFP fusion protein, which can enrich the level of dephosphorylated BZR1). The seeds in MX3 and M4C siliques line more tightly than W2C (Figure 3A–3C and Supplemental Figure 1C) and ovule numbers in MX3 and M4C are higher than in W2C (Figure 3D–3F). The statistical results also show that the two mutant lines, MX3 and M4C, both have higher ovule (Figure 3G) and seed numbers (Figure 3H) when compared to the control line. The transgenic lines confirm the observation that BZR1 plays a role in ovule and seed number determination as well as mutants. It has been shown that BZR1 activity is regulated by the level of dephosphorylation (Wang et al., 2002). We identified the dephosphorylation level of BZR1 by Western blot analysis in both W2C and MX3. There is a greater proportion of dephosphorylated BZR1 in MX3 compared to W2C (Figure 3I and 3J), indicating more active BZR1 increase ovule and seeds number. This further implicates BZR1 functions in ovule and seed number determination.

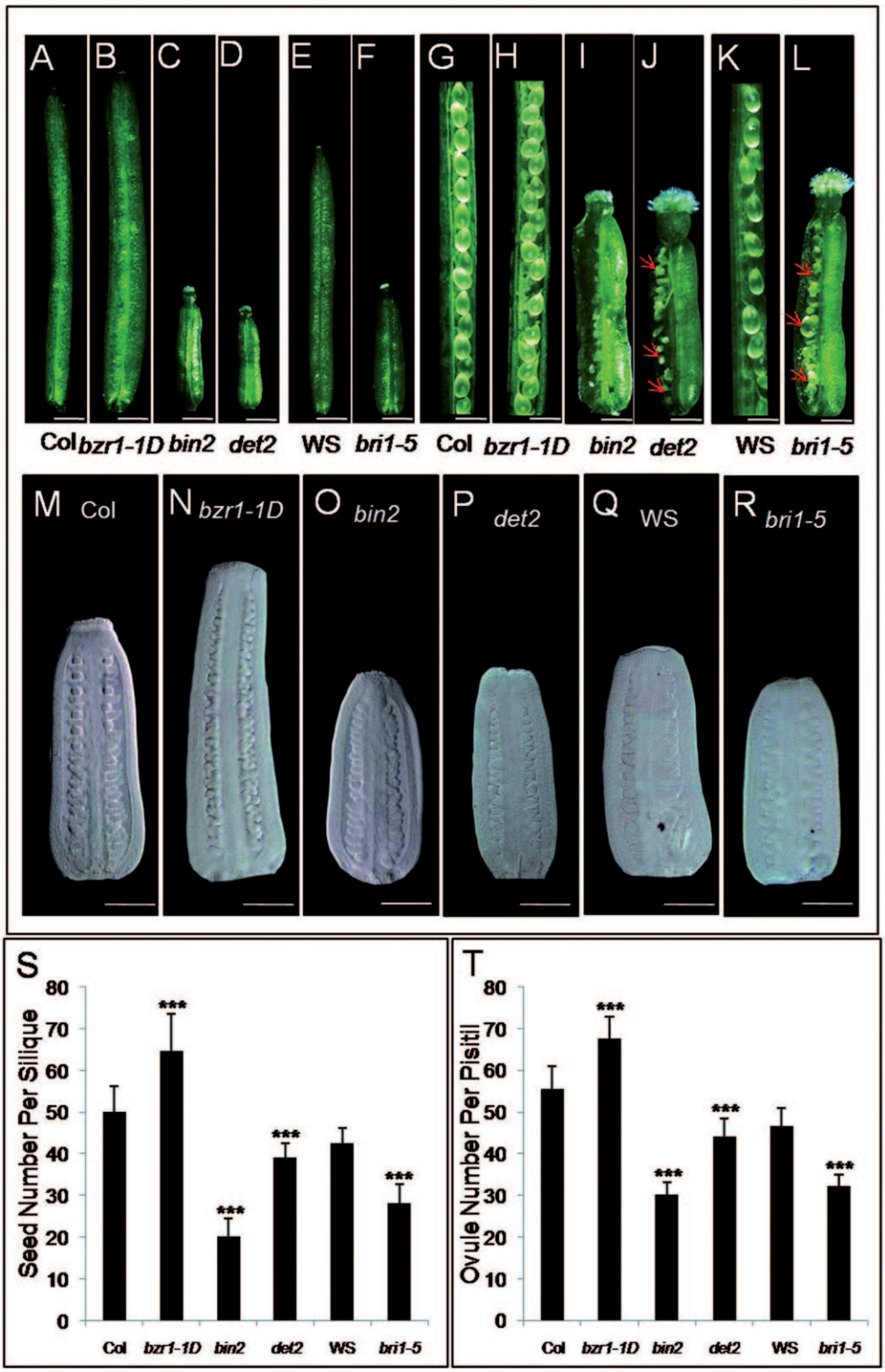

Figure 1. BR Signal Affects the Seed and Ovule Number of *Arabidopsis*.

(A–F) Comparison of BR-related mutant siliques, bar = 1 mm.

(G–L) Seeds line in BR signal mutants. Pictures were captured by anatomical lens, bar = 0.5 mm.

(M–R) Ovules line in BR signal mutants captured by DIC, bar = 0.1 mm.

(S) Statistical results of seed number of BR signal mutants. *bin2* means *bin2*+/–.

(T) Statistical results of ovule number of BR signal mutants. Error bars represent SD (standard deviation, $n = 30$). We performed statistical analysis through *t*-tests between every two sets of data. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Red arrows point out some developed seeds in *det2* and *bri1-5* mutants, for example.

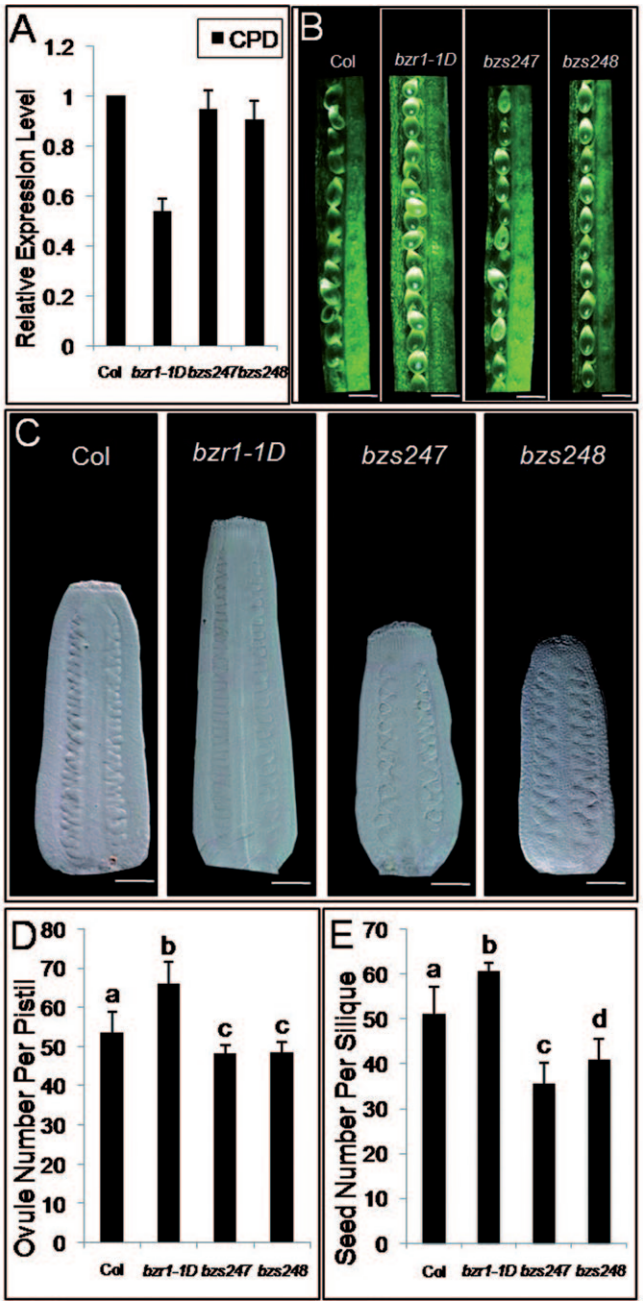

Figure 2. BZR1 Is Involved in Ovule and Seed Number Determination.

(A) Detection of the expression level of *CPD* through qRT–PCR; gene expression was normalized against constitutively expressed *PP2A*. Error bars represent SD (n = 3).

(B) Comparison of seed arrangements. Pictures were captured by anatomical lens, bar = 0.5 mm.

(C) Ovule alignment pattern; the pictures were captured by DIC, bar = 0.1 mm.

(D) Statistical results of ovule number.

(E) Statistical results of seed number. Error bars represent SD (n = 30). We performed statistical analysis through t-tests between every two sets of data. Values that differ at the 0.05 significance level are labeled with different letters 'a', 'b', 'c', and 'd'.

The other evidence is provided by the gain-of-function mutant *bin2* (for seed number comparison, we use *bin2*+/–). Lithium (LiCl) is a well-studied GSK3 kinase inhibitor (Klein and Melton, 1996; Stambolic et al., 1996), which can inhibit the process of BIN2 phosphorylating BZR1 (Zhao et al., 2002; Peng et al., 2008). There are more seeds in LiCl-treated *bin2* than in the control (*bin2* without LiCl treatment). This suggests BIN2 functions in decreasing the seed number by phosphorylating BZR1 and inhibiting BZR1 function (Supplemental Figure 2A–2F).

BR Regulates the Expression Level of Ovule Development-Related Genes through Direct and Indirect Ways

The control line Col was treated with both BR and BRZ (brassinazole, BR biosynthesis inhibitor) and the expression levels of ovule and seed developmental genes (*HLL*, *ANT*, and *AP2*) were analyzed by qRT–PCR. Both *HLL* and *ANT* are up-regulated by BR treatment and down-regulated by BRZ treatment, while *AP2* is down-regulated by BR treatment and up-regulated by BRZ treatment (Figure 4A and 4B). The *AP2* expression does not show much difference between mock and BR treatment. We performed several independent repeats and statistical analysis by t-test. The result shows that there is significant difference between mock and BR treatment, confirming that *AP2* can be down-regulated by BR. The expression levels of *HLL*, *ANT*, and *AP2* were also analyzed in *bzr1-1D*, *bzs247*, and *bzs248* by qRT–PCR. The transcriptions of *HLL* and *ANT* are induced in *bzr1-1D* while *AP2* is reduced. *AP2* expression decreased in BR signal-enhanced mutant, further confirming that *AP2* is down-regulated by BR. The expression levels of these genes have a slight change in *bzs247* and *bzs248*, which are similar to Col (Figure 4C). These results indicate that BR influences the ovule development through regulating related gene transcriptions.

Treatment for 60 min with the reported concentration (100 mM) of LiCl leads to a decrease in the expression level of the *CPD* gene (Supplemental Figure 2G). *CPD* is a marker gene of BZR1 activity and is up-regulated greatly in the *bin2* mutant (Yan et al., 2009). Further to this, *HLL* and *ANT* are up-regulated and *AP2* is down-regulated, demonstrating that BZR1 dephosphorylation indeed influences the expression level of ovule-related genes.

A ChIP (Chromatin Immunoprecipitation) experiment was performed to identify whether *HLL*, *ANT*, and *AP2* are direct targets of BZR1 in W2C and MX3 (Figure 4D). The results show that *AP2* and *ANT* are BR-regulated BZR1 target genes (BRBT). The target regions are in the promoter sequences of these two genes. *DWF4* is the positive control of this experiment. The significant enrichment of *DWF4* has been checked by t-test. The putative BRRE domains (BR Response Element) are in the upstream sequences of *AP2*, *ANT*, and *DWF4*, the ChIP–PCR primers designed in promoter sequences depending on the binding information from Sun's paper (Sun et al., 2010), while *HLL* is not a direct target gene of BZR1 but can be regulated by BR, indicating that BZR1 positively regulates *HLL* in an indirect way. The *ANT* and *AP2* accumulation levels in MX3 are much

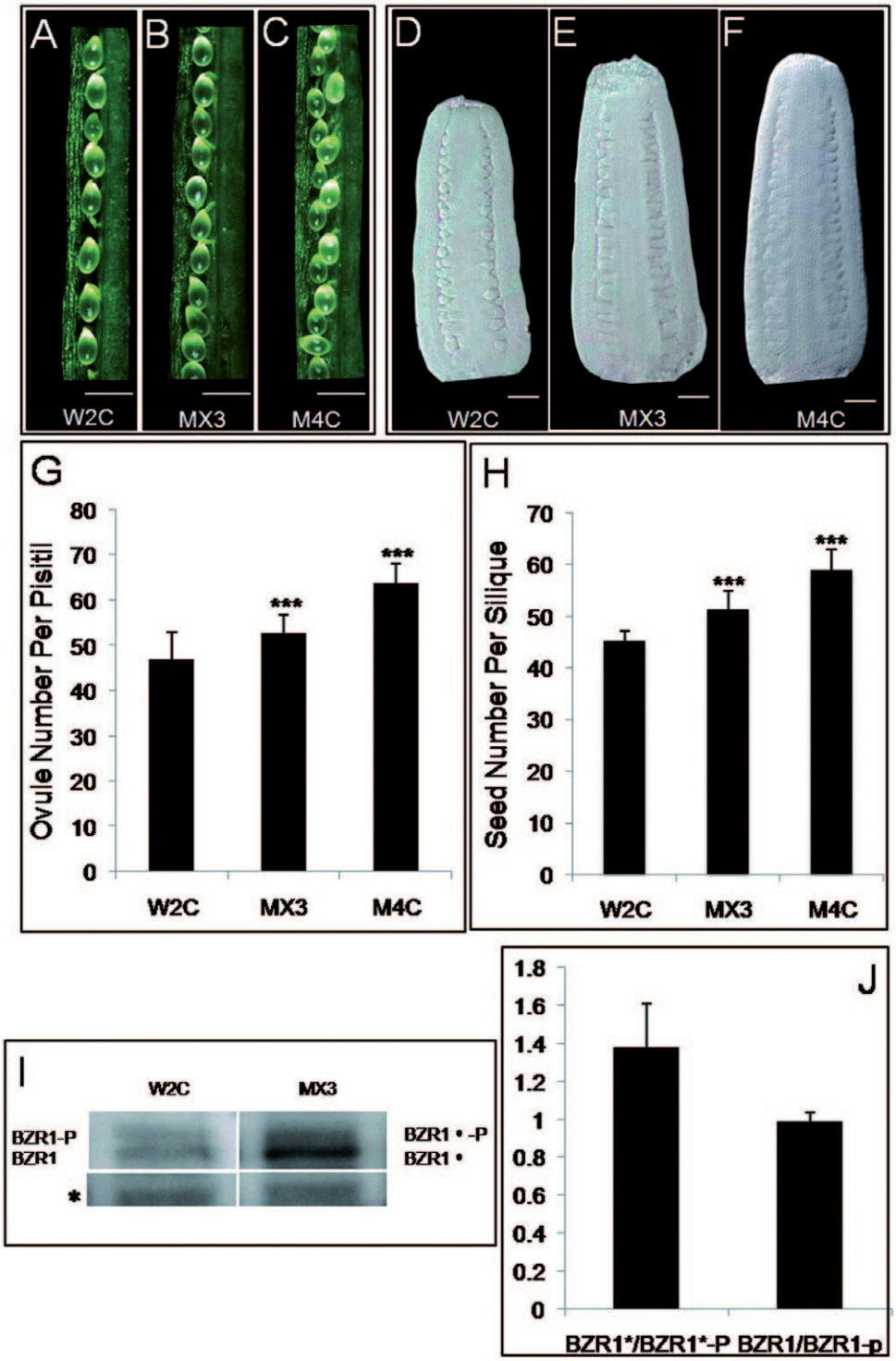

Figure 3. The Phenotype Analysis and Dephosphorylation Detection of BZR1 in Dominant Transgenic Plants.

(A–C) Comparison of seed alignment; pictures were captured by anatomical lens, bar = 0.75 mm.

(D–F) Comparison of ovule arrangement; pictures were captured by DIC, bar = 0.1 mm.

(G, H) Statistic results of ovule and seed number in W2C, MX3, and M4C. Error bars represent SD (n = 30). We performed statistical analysis through t-tests between every two sets of data. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(I) Detection of phosphorylation and dephosphorylation levels of BZR1 by Western blot in W2C and MX3. BZR1 and BZR1-p indicate dephosphorylated and phosphorylated forms of BZR1. BZR1* and BZR1*-p indicate dephosphorylated and phosphorylated forms of mutated BZR1. And the star denotes non-specific bands used for loading controls.

(J) The dephosphorylation level of mutated BZR1 in MX3 and wild-type BZR1 in W2C shown by BZR1*/BZR1*-p and BZR1/BZR1-p separately. Western blot data were measured by Image J. Error bars represent SD (n = 3).

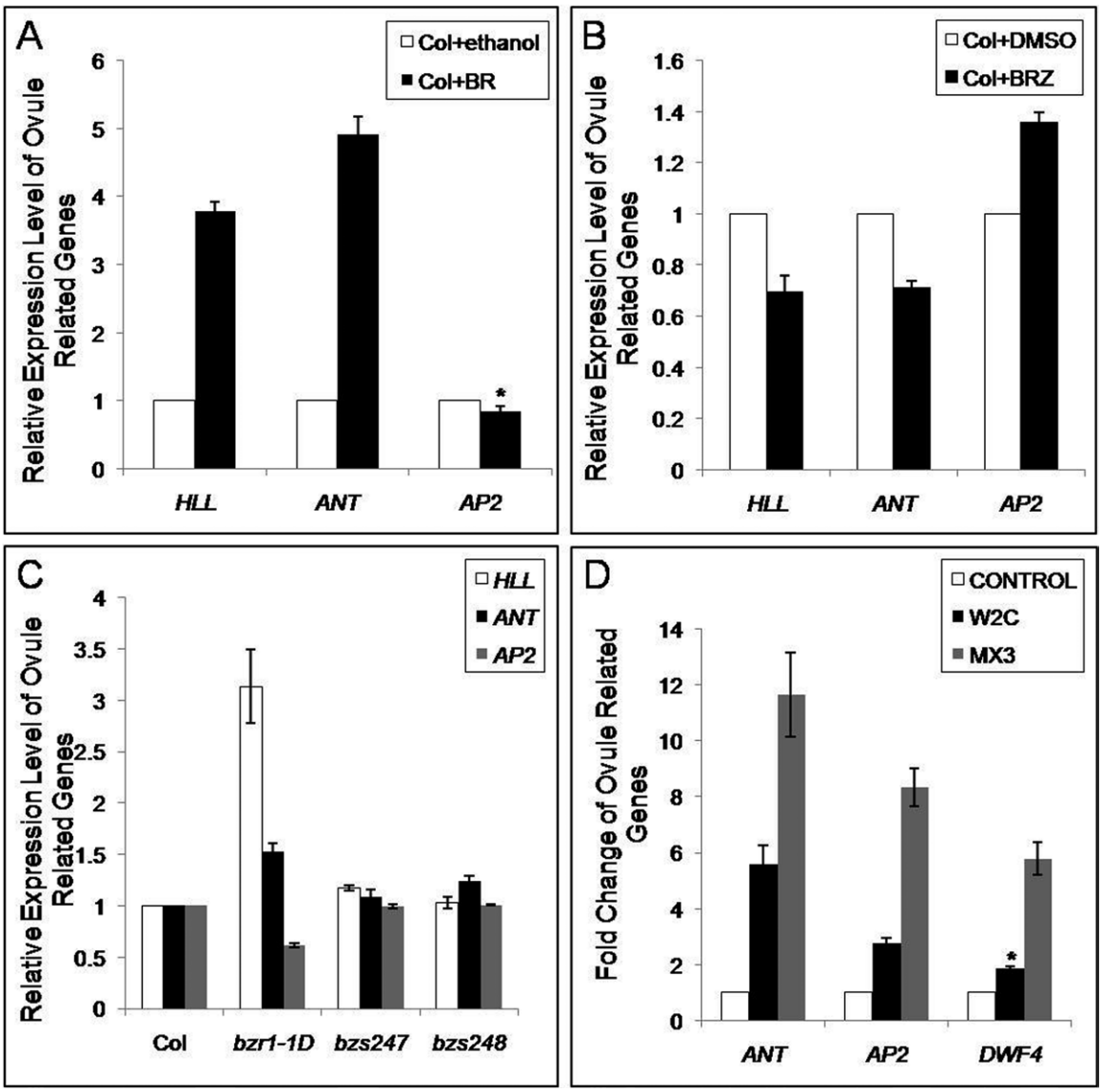

Figure 4. BR Regulates Ovule Development-Related Genes at Transcription Level.

(A) qRT–PCR detection of ovule development-related genes by BR treatment.

(B) qRT–PCR detection of ovule development-related genes by BRZ treatment.

(C) The relative expression level of ovule development-related genes in Col, *bzr1-1D*, *bzs247*, and *bzs248*.

(D) Detection of BZR1 targeting *ANT* and *AP2* DNA sequences by ChIP. *DWF4* is used for positive control. Gene expressions were normalized against constitutively expressed *PP2A*. Error bars represent SD (n = 3). We performed statistical analysis through t-tests between every two sets of data.* $P < 0.05$.

higher than in W2C, providing further evidence that *ANT* and *AP2* genes are direct targets of BZR1. These results confirm the data in the ChIP-chip experiment performed by Sun et al. (2010).

BZR1 and AP2 Affect Each Other and Are Involved in Ovule Development

We crossed *bzr1-1D* and *ap2-5* mutant lines and identified the homozygous posterity plants. Through counting the ovule and seed number, the average ovule numbers of Col, *bzr1-1D*, *ap2-5*, and the double mutant are 52.9 (ranging from 45 to 65), 68.1 (54–87), 60.4 (47–74), and 74.8 (53–86), respectively. The seed numbers of those four plants are 52.4 (49–55), 60.9 (57–65), 46 (35–55), and 45.6 (24–64). The ovule numbers of the four plants demonstrate that there is no obvious epistatic effect between AP2 and BZR1 in the regulation network of ovule number determination. They might not be in the same pathway. qRT–PCR analysis shows the expression level of *AP2* is down-regulated in *bzr1-1D*, *ap2-5*, and *bzr1-1D/ap2-5* double mutant. The marker gene *CPD* is also down-regulated in *bzr1-1D*, *ap2-5*, and *bzr1-1D/ap2-5* double mutant, indicating enhanced BZR1 activity in these three plants. The molecular

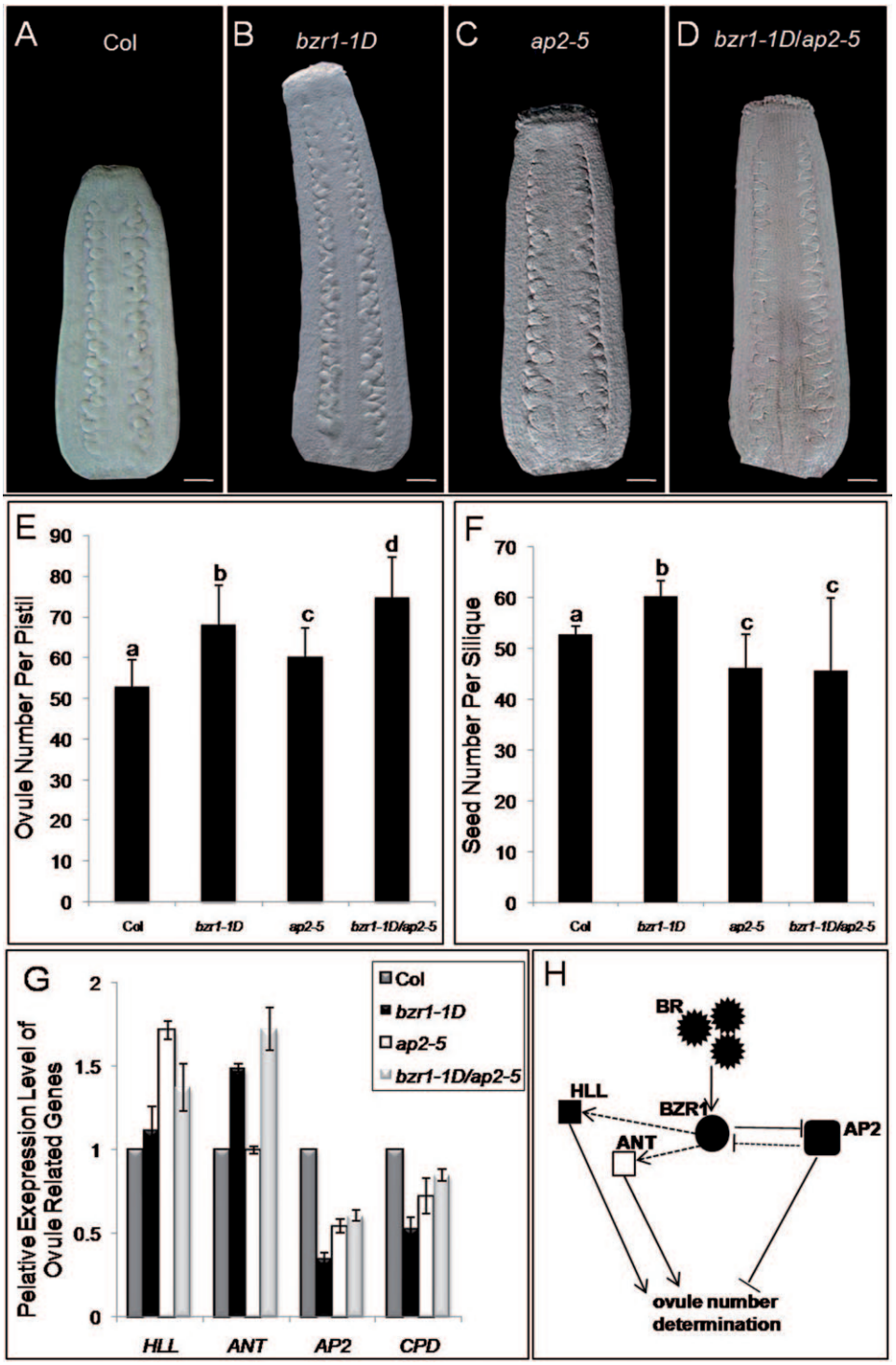

Figure 5. BZR1 and AP2 Antagonism Functions in Ovule Development.

(A–D) Ovule pictures of Col, *bzr1-1D*, *ap2-5*, and *bzr1-1D/ap2-5* double mutant, bar = 0.05 mm.

(E, F) The statistic results of ovule and seed number of Col, *bzr1-1D*, *ap2-5*, and *bzr1-1D/ap2-5*. Error bars represent SD (n = 30). We performed statistical analysis through t-tests between every two sets of data. Values that differ at the 0.05 significance level are labeled with different letters 'a', 'b', 'c', and 'd'; * $P < 0.05$.

(G) Detection of ovule development-related genes by qRT–PCR; gene expressions were normalized against constitutively expressed *PP2A*. Error bars represent SD (n = 3).

(H) Model of the BR signal that regulates ovule number. The BR signal actives BZR1, which is involved in the ovule number determination process. The activated BZR1 stimulates those ovule development-related genes like *HLL*, *ANT*, and *AP2* indirectly or directly. Besides, AP2 and BZR1 possibly antagonize each other to participate in the ovule number determination process.

experiments suggest BZR1 activity has been repressed by AP2 and *AP2* expression has been repressed by BZR1; both cases lead to more ovules, which are consistent with the number of ovules of double mutant (Figure 5A–5G). Furthermore, we detected the expression level of *HLL* and *ANT* in these plants. All three mutants tend to have an increase in gene expression for both *HLL* and *ANT*, and a decrease in *AP2* (Figure 5G). The seed number is not in the same trend as the ovule number because that *AP2* gene also affects male fertility and zygote development (Ohto et al., 2005). Here, we focus on the ovule number to deduce that *BZR1* and *AP2* probably antagonistically influence *Arabidopsis* ovule number.

Above all, BR induces BZR1 activity, which functions in ovule and seed number determination. Dephosphorylated BZR1 stimulates ovule development genes like *HLL* and *ANT* through either indirect or direct mechanisms. On the other hand, *AP2* and *BZR1* affect each other and regulate ovule number determination through inhibiting the activity of BZR1 and repressing *AP2* expression, respectively (Figure 5H).

DISCUSSION

BR Signal Affects *Arabidopsis* Ovule and Seed Number to Varying Extents

The reproductive development of *Arabidopsis* has been well studied over the past few years and many signaling pathways involved have been identified (Irish, 2010; Sun et al., 2010; Shi and Yang, 2011). BR signaling has been confirmed to affect this process, as there are several phenotypes in reproductive organ initiation and formation, which leads to total or partial sterility in BR-deficient and insensitive mutants (Szekeres et al., 1996; Kim et al., 2005). Statistical analysis of seed numbers in BR-deficient mutants indicates a significant decrease in most of them, whereas the BR signal-dominant mutant *bzr1-1D* has more seeds than control. During ovule and seed development, some ovules fail to develop seeds, especially in the mutants that have defects in fertilization and zygote development processes. In order to remove the effect of BR and other signals in fertilization and seed development processes, ovule numbers were compared. The seed/ovule number ratio of WT and *bzr1-1D* is close to 100% (94% and 95%, respectively; Table 1). However, the ratio of *det2* and *bin2* lines is closer to 70% (69% and 75%, respectively; Table 1), indicating there would be defects in fertilization and zygote development in BR-deficient mutants compared to WT and BR-dominant mutant, which indicates that BR also affects fertilization and zygote development.

Confirmed again, the seed/ovule number ratio of WS is 91%, while *bri1-5* is 87%. This ratio of *bri1-5* is not as low as *bin2* and *det2*, which might be because *bri1-5* mutant has inosculated carpel in low presentation, which is due to abnormal organ boundary formation by BR deficiency (data not published), and we did not count this kind of ovule.

As mentioned above, the seed/ovule number ratio means that fertilization and zygote development are also influenced by BR signal deficiency. It would be very difficult to distinguish which is the main reason for BR function in seed development—maternal tissue or paternal tissue? It has been hypothesized that BR mutants have fewer seeds because of shorter pollen tubes and lower fertilization efficiency and the sterility could be partially rescued by crossing WT pollen grains (Ye et al., 2010). With our current results, we can conclude that the ovule number has been reduced in BR-deficient mutant and crossing WT pollen does not completely rescue the phenotype. BR signal deficiency not only represses pollen tube elongation, but also affects ovule initiation and development. BR signal represses seed development through both maternal and paternal pathways.

BZR1 Is A Key Gene Function in BR-Mediated Ovule and Seed Number Determination

bzr1-1D ovule and seed number are significantly higher than Col control. The ovule and seed numbers are also significantly higher in the MX3 line (enhanced BZR1 activity by expressing a dominant mutant form of BZR1) than in the W2C line (expressing wild-type BZR1 protein in plant, the control line of MX3). The dominant mutant form of BZR1 has a higher dephosphorylation level due to a mutation in the BIN2 phosphorylation site. This indicates the level of BZR1 dephosphorylation and BZR1 activity can positively regulate ovule and seed numbers. We detected the BZR1 dephosphorylation level *in vivo*, which confirmed the hypothesis. The difference between *bzr1-1D* and Col is more obvious than MX3 and W2C, and the average ovule number of MX3 is only slightly higher than Col (60 versus 55); this difference may be a result of the transgenic process. The strict comparison should be MX3 and W2C, but not MX3 and Col. The M4C line is another transgenic plant with higher BZR1 activity. The statistical analysis of ovule and seed number showed that M4C had significantly higher ovule and seed numbers than W2C, again confirming that BZR1 functions in the determination of ovule and seed numbers.

bzs247 and *bzs248* are intragenic suppressors of *bzr1-1D*. The basis line for producing this revertant population is a mutant of *bzr1-1D* without trichome. These *bzr1-1D* were processed by introducing a *gl* mutation for easy observation. The phenotypes of this *bzr1-1D* have a few differences to the original *bzr1-1D*, including no trichome and smaller flowers, but no significant difference in ovule numbers (Supplemental Figure 3). *bzs247* and *bzs248* are shown to rescue the phenotype of *bzr1-1D*, resulting in no kink, normal adult plant size, decreased ovule number close to WT, and so on, which provides further evidence that BZR1 positively regulates ovule number determination effectively. The physiological experiment of *bin2*+/– treatment with LiCl confirms the conclusion, too.

qRT–PCR and ChIP–PCR experiments show that BZR1 regulates ovule development gene expression levels. There is a little change in the *AP2* transcription level between mock and

BR treatment *in vitro*. It might be because it is hard to do the perfect treatment in parallel. We performed several independent experiments to repeat the results. And we performed the statistical analysis and found that there is a significant difference between mock and BR treatment (Figure 4A). The *AP2* expression level has also decreased in *bzr1-1D* mutant and increased under BRZ treatment. The three independent results show the same orientation, confirming our conclusion. The logical results suggest that the *AP2* transcription level could be regulated by BR treatment. The change degree is not severe, indicating that there might have been other regulation methods besides transcription level regulation.

The Interaction between BR and Other Signals in Ovule Number Determination

Many genes have been reported to play roles in ovule development, however, most of them do not determine ovule number directly, such as *INO*, *SUP*, *TSO*, and *SUP* (Gaiser et al., 1995; Sakai et al., 1995; Schneitz et al., 1997; Hauser et al., 1998). The ovule phenotypes of their loss-of-function mutants include abnormal formation and inactivity in fertilization, among others. In our study, we focus on the phenotype of low ovule number with normal ovule function. *HLL*, *ANT*, and *AP2* were published as ovule number variation-related genes (average number of *ap2-6* is 72 calculated using data in Western and Haughn, 1999). *HLL* and *ANT* promote while *AP2* restrains ovule numbers. Even though some ovules in *ap2* mutants are ectopic (Kunst et al., 1989; Bowman et al., 1991), the number of normal ovules is still higher than WT. We investigated the expression level of these genes by qRT–PCR in WT plants treated with BR and BRZ, as well as in the BR signal dominant mutant *bzr1-1D* and the suppressors *bzs247* and *bzs248*. The expression level of *AP2* shows a slight change, suggesting that other regulation levels may be involved in AP2 function in ovule number determination, too. The reported *hll* and *ant* mutants are both in *Landsberg erect* ecotype. We gained double mutations of *bzr1-1D* and *ap2-5* and observed the phenotypes. The ovule number and seed number of *bzr1-1D/ap2-5* double mutants are significantly higher than Col, *bzr1-1D*, and *ap2-5*, while *bzr1-1D*, *ap2-5*, and the double mutant all have higher ovule numbers. The similar result of seed number is not observed, as AP2 also functions in fertilization and zygote development. qRT–PCR results demonstrate that repressing *AP2* expression and enhancing BZR1 activity both lead to higher ovule number. The molecular data and genetic results lead us to logically conclude that *BZR*1 positively and *AP2* negatively regulate ovule number. BZR1 is more active in the *ap2-5* mutant while *AP2* is repressed in *bzr1-1D* mutants. BZR1 and AP2 may have antagonistic effects in ovule number determination.

Although expression levels of *AP2*, *ANT*, and *HLL* are all affected by BZR1, *AP2* and *ANT* are shown to be direct targets of BZR1, while *HLL* is not. We deduce that HLL may function with interacting proteins that could be directly regulated by BZR1. Or there are other transcription factors downstream of BZR1 that directly regulate *HLL* gene expression.

As mentioned above, BR signal affects ovule and seed development through regulating gene expression through either directly targeting or indirectly regulating BZR1. BZR1 and AP2 possibly regulate ovule number antagonistically.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* mutant plants are all in Colombia-0 ecotype background. The *ap2-5* mutant was obtained from John J. Harada (University of California, Davis). All the plants are surface-sterilized, plated on solid medium (1/2 Murashige and Skoog (MS) Medium, and 1% agaer (Invitrogen), pH 6.5), and vernalized at 4°C for 3 d prior to germination. The plants were grown in a greenhouse supplemented with 24 h light or 16/8 h light and dark at 22°C.

Counting Ovule Number by Differential Interference Contrast Microscopy (DIC) and Anatomical Lens

It is reported that the ovule developmental phase division before fertilization corresponds to flower development stages 9–12 (Schneitz et al., 1995). To observe ovule initiation in the pistils in the mutant plants, flowers at stage 10 were cleared in a chloralhydrate/glycerol/water solution (8 g: 1 ml: 3 ml) overnight. Pistils were dissected under a stereomicroscope and subsequently photographed by using a Zeiss Axiophot D1 microscope with DIC imaging. Images were recorded with an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1). In order to count the ovule number, flowers of stage 12 that had not been fertilized were prepared and dissected under the Leica S8APO anatomical lens and the ovule number was counted directly.

BR and BRZ Treatment

100 μM mother solution of BR and BRZ were prepared. BR was dissolved in 80% ethanol and BRZ was dissolved in DMSO (dimethylsulfoxide). Inflorescences with young floral buds (flower stages 1–12) were immerged in 100 nM BR and 1 μM BRZ for 3 h. As control, floral buds also were treated by 1000 times diluted 80% ethanol and 100 times diluted DMSO for 3 h.

LiCl Treatment

For silique analysis, *bin2* with the 12th-stage flower was irrigated by water with 10 mM LiCl, while, for qPT–PCR, *bin2* inflorescences with young floral buds (until stage 12) were treated with 100 mM LiCl and H_2O for 60 min, respectively.

Gene Expression Analysis by qRT–PCR

Total RNA isolation and reverse transcription were performed using inflorescences with young floral buds (flower stages

1–12). Oligonucleotide primers for qRT–PCR were preformed according to the published cDNA sequence of *ANT*, *HLL*, *AP2*, *CPD*, and *PP2A* (control). The following sequences are the primers used in this experiment: *ANT* (5′-GCGTTACAAGACATAGATGGA-3′ and 5′-TGCAACATATTCTTGTCTAGTC), *HLL* (5′-GCTCTCCAAAGGACGTTCATT-3′ and 5′-CAACAATGATATCGCCAAGTCTT-3′), *AP2* (5′-AGCAGCACAACCCTCAACAG-3′ and 5′-CCAGATGTGCTAAAGACGGAG-3′), *CPD* (5′-GACGCTACGAGTGGCTAA-3′ and 5′-GAACCGCTCTAAACGATG-3′), and *PP2A* (5′-AGCAGCACAACCCTCAACAG-3′ and 5′-CCAGATGTGCTAAAGACGGAG-3′).

Western Blot and Data Analysis

Freshly collected or frozen samples (stage 9–12 flowers of W2C and MX3) were grounded thoroughly in 2 SDS sample buffer using a plastic pestle and boiled at 100°C for 5 min. After 5 min of centrifugation, insoluble pellet was removed. Soluble proteins were separated on a 10% SDS–PAGE gel, transferred to an Immobilon-P polyvinylidene difluoridemembrane (Millipore), and analyzed by Western blot with an anti-YFP antibody (Mora-Garcia et al., 2004). The signal on the Immobilon-P membrane was detected using the horseradish peroxidase-conjugated goat anti-rat-IgG secondary antibody (Abcam) and recorded by the SuperSignal West Pico chemiluminescent substrate system (Pierce). We used Image J to measure the gray value of these bends (BZR1, BZR1-P, BZR1*, and BZR1-P*, which were obtained from Western blot), and then we calculated the gray value ratio of BZR1/BZR1-P and BZR1*/BZR1-P*.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) experiment was performed following the procedure described by He et al. (2005) with some modifications. In brief, 3 g stage 9–12 flower tissues of W2C and MX3 lines were cross-linked for 10 min in 1% formaldehyde by vacuum filtration. Nuclei were isolated from the tissues and re-suspended in 2× lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 200 mM NaCl, 0.5% Triton X-100, 1 mM PMSF). The chromatin was released by sonicating and DNA fragments of an average size of 0.5–1 Kb was achieved using super-centrifuge. The extract DNA was diluted with an equal volume of lysis buffer and pre-cleared with salmon sperm DNA/protein-A agarose beads at 4°C for 30 min. The supernatants were then incubated with affinity-purified polyclonal anti-GFP antibodies (Invitrogen A11122) at 4°C for 1 h. The chromatin–antibody complex was precipitated with salmon sperm DNA/protein-A agarose beads at 4°C overnight. The beads were then washed twice with 0.5× lysis buffer, once with high salt wash buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 500 mM NaCl, 0.5% Triton X-100), once with LiCl buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.25 M LiCl, 0.5% NP-40, 0.5% deoxycholate), and twice with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer. The immunoprecipitated protein and DNA were eluted with 1% SDS, 0.1 M $NaHCO_3$, and the crosslink was reversed by incubation at 65°C overnight in the presence of 250 mM NaCl. DNA was extracted by phenol/chloroform and precipitated with LiCl/ethanol after adding 10 μg of glycogen. The precipitated DNA was re-suspended with TE buffer and analyzed by qRT–PCR. All primers were designed to amplify a promoter region within 500 bp upstream of the translational start codon. The following sequences are the primers used in this experiment: *UBC30* (5′-CAAATCCAAAACCCTAGAAACCGAA-3′ and 5′-AACGACGAAGATCAAGAACTGGGAA-3′), *CNX5* (5′-TGACATCGTCTTCTTTGCTGCTTCT-3′ and 5′-TGCCTTCTTGAGCTTTAACTCTTCC-3′), *CPD* (5′-CCATTGAAGAAGAAGATGATGATGA-3′ and 5′-CCCCCCGTGTGCCCACTC-3′), *DWF4* (5′-GGGTTTGACTGTCCAGTTCGGTAAT-3′ and 5′-ACCCTTAGGATATGGGAAAAGGGTG-3′), *ANT* (5′-CAATCGAGATGAGACCTG-3′ and 5′-ATTCAACTGCCAAATACA-3′), *AP2* (5′-TGAATATTGTCAACAGTTGTT-3′ and 5′-ACTTTGGTTATTTGACTATTT-3′), and *PP2A* (5′-AGCAGCACAACCCTCAACAG-3′ and 5′-CCAGATGTGCTAAAGACGGAG-3′).

PCR reactions were performed in 25 μl reaction with the following protocol: 94°C for 3 min, then 50 cycles of 94°C for 10 s, 58°C for 10 s, and 72°C for 15 s.

Accession Numbers

HLL At1g17560; *ANT* At4g37750; *AP2* At436920; *PP2A* At1g69960; *DWF4* At3g50660; *CNX5* At5g55130; *CPD* At5g05690; *UBC30* At5g56160.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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