Brassinosteroid Regulates Seed Size and Shape in *Arabidopsis*

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ABSTRACT

Seed development is important for agriculture productivity. We here demonstrate that brassinosteroid (BR) plays crucial roles in determining the size, mass and shape of Arabidopsis seeds. The seeds of the BR-deficient mutant de-etiolated-2 (det2) are smaller and less elongated than those of wild type plants, due to a decreased seed cavity, reduced endosperm volume and integument cell length. The det2 mutant also showed delay in embryo development, with reduction in both the size and number of embryo cells. Pollination of det2 flowers with wild type pollen yielded seeds of normal size but still shortened shape, indicating that the BR produced by the zygotic embryo and endosperm is sufficient for increasing seed volume but not for seed elongation, which apparently requires BR produced from maternal tissues. BR activates expression of SHORT HYPOCOTYL UNDER BLUE1 (SHB1), MINISEED3 (MINI3) and HAiku2 (IKU2), which are known positive regulators of seed size, but represses APETALA2 (AP2) and AUXIN RESPONSE FACTOR 2 (ARF2), which are negatively regulators of seed size. These genes are bound in vivo by the BR-activated transcription factor BZR1, and they are known to influence specific processes of integument, endosperm and embryo development. Our results demonstrate that BR regulates seed size and seed shape by transcriptionally modulating specific seed developmental pathways.

RUNNING TITLE BR regulates seed size and shape

KEY WORDS brassinosteroid, seed size, seed shape, endosperm, embryo, integument
INTRODUCTION

Seed development in flowering plants is a complicated process controlled by a complex network. The double fertilization in the embryo sac generates two zygotic products, the embryo and endosperm. The embryo gives rise to the daughter plant while the endosperm provides nutrients for embryo during embryogenesis and germination (Lopes and Larkins, 1993; Faure et al., 2002). Seed coats, which enclose the embryo and endosperm, are differentiated from maternally derived integuments (Haughn and Chaudhury, 2005) and the final seed size and weight are coordinately determined by the growth of the endosperm, embryo and integument (Berger et al., 2006; Zhou et al., 2009).

The molecular mechanisms of seed development have been studied extensively and many genes involved in seed development have been identified. These include SHORT HYPOCOTYL UNDER BLUE1 (SHB1), HAIKU1 (IKU1), MINISEED3 (MINI3) and HAIKU2 (IKU2), which promote the development of endosperm and embryo, and positively regulate the seed size (Garcia et al., 2003; Luo et al., 2005; Zhou et al., 2009; Wang et al., 2010). IKU1, MINI3, and IKU2 successively act in the same pathway that is required to expand the seed cavity and to induce the endosperm growth in the early phase of seed development (Luo et al., 2005). IKU1 directly interacts with MINI3 (Wang et al., 2010). SHB1 is a positive regulator of seed development and may activate MINI3 and IKU2. SHB1 has also been shown to enhance the embryo cell proliferation and expansion through an unknown IKU2-independent pathway (Zhou et al., 2009). In contrast, APETALA2 (AP2) inhibits integument growth and the development of embryo and endosperm, and reduces the seed size. AP2 determines seed weight and seed size by coordinating the growth of endosperm, embryo and maternal integuments (Jofuku et al., 2005; Ohto et al., 2005; Ohto et al., 2009). In addition, AUXIN RESPONSE FACTOR 2 (ARF2) determines seed mass and seed size through regulating integument growth. Deficiency of ARF2 results in the dramatically increased seed size and weight due to the extra
cell division in the integuments, which leads to the formation of enlarged seed coats (Schruff et al., 2006). The cytochrome P450 CYP78A5 (KLUH or KLU) has been shown to act as a maternal regulator of seed size by stimulating the integument cell proliferation (Ito and Meyerowitz, 2000; Adamski et al., 2009). Additional factors, including FERTILIZATION INDEPENDENT SEED 2 (FIS2), AINTEGUMENTA (ANT), RETARDED GROWTH OF EMBRYO 1 (RGE1), ARABIDOPSIS HISTIDINE KINASE (AHK2, AHK3 and AHK4) and CYTOKININ INDEPENDENT 1 (CKI1), regulate the seed weight and size through epigenetic regulation of endosperm development and paternal imprinting, regulation of embryo proliferation, endosperm and integument development (Sun et al., 2010).

Brassinosteroid (BR), a kind of steroid hormone, plays important roles in plant growth and development, such as stem elongation, leaf expansion, vascular differentiation, stress tolerance, senescence and stomatal development (Clouse and Sasse, 1998; Gudesblat et al., 2012; Kim et al., 2012; Wang et al., 2012). An important role for BR in plant reproductive growth and seed development has been suggested by the studies of BR deficient and insensitive mutants of Arabidopsis, Pisum sativum, Solanum lycopersicum, and Orzya sativa (Fujiocka and Yokota, 2003; Nomura et al., 2007; Ye et al., 2010). In Arabidopsis the weak BR-deficient mutant de-etiolated-2 (det2) (Fujiocka et al., 1997) has lower fertility in comparison to the wild type (Ye et al., 2010), and the strong BR-deficient mutants dwarf4 (dwf4) and constitutive photomorphogenesis and dwarfism (cpd) are completely male sterile (Choe et al., 1998; Ye et al., 2010). Rice mutants with defects in BR biosynthesis or signaling also show reduced seed length (Hong et al., 2005; Tanabe et al., 2005; Morinaka et al., 2006). Overexpression of a BR-biosynthetic gene in rice increase seed filling and seed size (Wu et al., 2008). dwarf mutant lk (BR deficient mutant) of pea has irregularly shaped seeds (Nomura et al., 2007). BR acts through the cell surface receptor BRASSINOSTEROID INSENSITIVE1 (BR1) (Kim et al., 2009) and transcription factor BZR1 and BZR2/BES1 (Wang et al., 2002; Yin et al., 2002; He et al., 2005) to control a large number of BR-responsive genes (Sun et al., 2010; Yu et al., 2011).
Although it has been reported that BR affects seed size and shape (Hong et al., 2005; Takahashi et al., 2005; Tanabe et al., 2005; Morinaka et al., 2006), the molecular mechanism of BR regulation of seed development has remained unclear.

To understand the mechanisms by which BR controls seed size and shape, we analyzed the seed development in BR-deficient and -insensitive mutants and the effects of BR on genes known to control various aspect of seed development. Our studies indicated that BR increased seed size by affecting the integument, endosperm and embryo development, and BR-activated BZR1 directly regulated several genes known to control the seed size. We further showed that the seed shape would be determined largely by BR signal of maternal tissues, whereas BR produced by the embryo and endosperm seemed sufficient to increase seed size, providing evidence for a mode of localized actions of BR in seed development.
RESULTS

BR-Deficient and -Insensitive Mutants Have Small Seeds

To evaluate the effects of BR on seed growth, we analyzed the size and weight of seeds of the BR-deficient mutant det2 and the BR receptor mutant bri1-5 (a weak allele of bri1 mutant) (Fujioka et al., 1997; Wang et al., 2001). The mature dry seeds of det2 and bri1-5 were smaller than wild type seeds (Figure 1A-E), and the embryos from mature det2 seeds were significantly smaller than those of wild type (Figure 1F and 1G). The det2 seeds were 17% lighter than wild type (Columbia, Col) (Figure 1H), and the seeds of bri1-5 were about 10% lighter than the wild type seeds (Wassilewskija, Ws) (Figure 1H). The seeds of det2 and bri1-5 had shorter length but slightly bigger width compared to the wild type controls (Figure 1I-J). The seeds of brassinazole-resistant 1-ID (bzr1-ID), a gain-of-function mutant of the BZR1 gene, were of not bigger size to wild type (Figure 1A, 1C, 1H, 1I and 1K), also the bzr1-ID mutant also showed a short-hypocotyl phenotype, which are thought to be due to feedback inhibition of BR biosynthesis (Wang et al., 2002; He et al., 2005). These results suggested that BR positively regulated seed size and was involved in seed shape determination.

To investigate the cellular basis for the altered seed size and shape of det2, we examined the cell size and cell number of the embryonic shoot or hypocotyl and embryonic root apex in mature det2 and wild type seeds using scanning electron microscopy (SEM). The results showed that the cell length of det2 hypocotyls was reduced by 23% in comparison to wild type, and cell width was not obviously altered (Figure 1L). In addition, the numbers of cells in the epidermal cell files from the base of cotyledon junction to the embryonic root apex, and along the axis of cotyledon, were smaller in det2 mutant than in wild type (Figure 1M). Microscopic analysis further showed that the integument cells of det2 mutants were significantly shorter than wild type (Supplementary information, Figure S1). These results indicated that
the decrease in seed size and seed weight in det2 was correlated with reduced embryo cell size and cell number, and decreased integument cell length.

The sections of ovaries also showed that the endosperm cellularization of det2 was delayed. The endosperm cellularization of wild type began at 5 days after pollination (DAP) when embryos were at the early-heart stage, whereas det2 endosperm cellularization was initiated at late 6DAP to 7DAP when embryos were close to the middle-heart stage. Endosperm cellularization of det2 and wild type seeds was finished at 9 and 7 DAP, respectively (Supplementary information, Figure S1), indicating that the reduced seed cavity and endosperm volume, delayed embryo development, decreased embryo cell size and number and integument cell length possibly contributed to the reduced seed size of det2.

**Tissue-specific Effects of BR on Seed Size and Shape**

Seed is a complex organ that contains both maternal tissues (integument) and zygotic tissues (embryo and endosperm). Control of seed size involves complex interactions among the zygotic embryo and endosperm, the maternally derived seed coat; and the parent plant (Schruff et al., 2006). To dissect the effects of BR synthesis and signaling in specific tissues on seed size and shape, we performed reciprocal crosses of wild type with BR-deficient and BR-insensitive mutants to create seeds that contain wild type embryo and endosperm but mutant integuments. In order to avoid the volume effect of siliques to seed shape, we compared the ovaries shape in the very early stages after fertilization. After hand-pollination, the ovaries in early, middle and mature-heart stages were cleared and analyzed respectively under differential contrast microscopy. The heart shape embryos indicated the same angles of the photography, especially in middle-heart stage. As shown in Figure 2 and Supplementary information Figure S3, the wild type, det2/-, dwf4/-, and bri1/- ovaries produced by hand-pollinating the corresponding pollens to self pistils for hand-pollinating control. det2/-, dwf4/-, and bri1/- ovaries were smaller and shorter than wild type. On
average, the det2-1 seeds were 16% lighter than those of wild type, and the dwf4 (-/-) and the bri1-116 (-/-) seeds were 17% and 18% lighter than those of wild type, respectively. The ovaries from the det2, dwf4, and bri1-116 mutant plants pollinated with wild type pollen, resulting in wild type embryos and endosperm but mutant integuments, were bigger but of same round shape compared to those from self-pollinated mutant plants (Figure 2A, Supplementary information, Figure S3A-G). Consistent with these observations of developing ovaries, the det2 (+/-), dwf4 (+/-), bri1-116 (+/-) mature seeds produced from det2, dwf4 or bri1-116 pistils pollinated with wild type pollens had similar seed weight and seed area as wild type seeds, which showed rescue of the seed size but not the seed shape by the wild type alleles from the pollens (Figure 2B-E). In contrast, the ovaries and seeds produced by wild type plants pollinated by the mutant pollens are almost as the same size and shape of wild type, consistent with the recessive nature of these mutations (Figure 2A-E). These results indicated that BR produced by the embryo and endosperm increased the seed size but had little effect on seed shape, whereas BR signaling in the integument contributed to the elongated shape of Arabidopsis seeds. The inability of BR produced in the embryo and endosperm to rescue the seed shape suggested that BR had limited mobility and BR synthesized in these internal seed organs had little effect on the integument cells.

**BR Treatment Increases Seed Size and Affect Seed Shape**

Application of BR to the inflorescence of wild type and det2 mutants resulted in enhanced seed weight in both wild type and det2 mutant (Figure 3A). But the seed weight of det2 mutant under BR treatment was lower than wild type seeds without BR treatment, indicating the exogenetic BR could not fully rescued BR deficiency in det2 mutant. The seed area of det2 mutant was recovered to wild type level under BR treatment; and there was no significant difference of seed area between wild type and det2 mutant (Figure 3A). For seed shape, ratio of seed length to width of wild type plant was not changed by BR treatment, and that of det2 mutant was enhanced (Figure
The length/width ratio of det2 mutant under BR treatment was lower than wild type, suggesting that BR treatment could not fully rescued seed shape. The explanation of partially rescue would be the efficiency of extrinsic BR treatment. Above all, we still could conclude that BR directly regulated seed size and affected seed shape.

BR Regulates the Expression of Seed Size-Related Genes

Previous studies indicated that seed size is coordinately determined by the growth of the endosperm, embryo and integument (Berger et al., 2006; Zhou et al., 2009), and several genes involved in these processes have been identified. We thus analyzed whether any of the genes known to regulate seed size or shape were regulated by BR. The expression levels of these genes in wild type plant and det2 mutant under BR treatment or Brassinazole (BRZ), a triazole compound that specifically blocks brassinolide biosynthesis by inhibiting DWF4 (DWARF4) gene (Asami et al., 2001), were first analyzed. As shown in Figure 3, the expression levels of the SHORT HYPOCOTYL UNDER BLUE1 (SHB1), HAIKU1 (IKU1), MINISEED3 (MINI3), HAIKU2 (IKU2), HEAT SHOCK FACTOR 15 (HSF15) and KLUH (KLU) genes, which were positive regulators of seed size, were significantly increased after three hours of BR treatment, and obviously reduced by three hours of treatment with the BR biosynthetic inhibitor brassinazole (BRZ) in both the wild type and det2 siliques (Figure 3B-C). Then the transcription level of these genes were analyzed in det2 and bri1-5 siliques at 4 to 5 days after hand-pollination. As shown in Figure 3D, the expression levels of positive regulators of seed size, were significantly reduced in the det2 and bri1-5 mutants, but slightly increased or unaffected in thebzr1-1D mutant. The transcript levels of the APETALA2 (AP2) and AUXIN RESPONSE FACTOR 2 (ARF2) genes, which were negative regulators of seed size, were decreased by BR treatment and were increased by BRZ treatment in wild type and det2 siliques (Figure 3B-C); as well as enhanced in the det2 and bri1-5 mutants but slightly decreased in thebzr1-1D mutant (Figure 3D). In addition, the expressions of FERTILIZATION
INDEPENDENT SEED 2 (FIS2), AINTEGUMENTA (ANT), RETARDED GROWTH OF EMBRYO 1 (RGE1), ARABIDOPSIS HISTIDINE KINASE (AHK1, AHK3 and AHK4) and CYTOKININ INDEPENDENT 1 (CKI1), which are all related to seed size, were changed under altered BR levels (Supplementary information, Figure S4). These results suggested that BR increased seed size by activating the expressions of many genes that possibly increase seed size and repressing the genes that inhibit seed growth.

Previous genome-wide studies have identified direct target genes of the BR-signaling transcription factors BZR1 and BZR2/BES1 (Wang et al., 2002; Yin et al., 2002); the data suggest that those important seed development-regulating genes mentioned above (SHB1-MINI3-IKU2, AP2 and ARF2) are direct targets of BZR1, but not BES1 (Sun et al., 2010; Yu et al., 2011). So we further performed BZR1 chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) analysis to confirm the direct involvement of BZR1 in the regulation of seed development-related genes. The results showed that in vivo BZR1 binds to the promoters of SHB1, IKU1, IKU2, AP2 and ARF2 (Figure 3E). Although MINI3 was not direct target of BZR1, its interacting protein IKU1 (Wang et al., 2010), was a direct target of BZR1 (Figure 3E). It has been reported that SHB1 regulates the expression of MINI3 (Zhou et al., 2009). So it was deduced that BZR1 indirectly regulated MINI3 through IKU1 or SHB1. Additional genes involved in regulating seed size, including KLU, HSF15, FIS2, RGE1, ANT, AHK1, AHK3, AHK4 and CKI1 (Sun et al., 2010), were all confirmed to be direct targets of BZR1 by ChIP-qPCR detection (Figure S4). These results suggested that BZR1 directly mediated BR regulation of genes involved in seed development.

MINI3 and SHB1 act downstream of BR in Regulating Seed Size

To further investigate whether the reduced expression of SHB1, IKU1, MINI3 and IKU2 in det2 mutant were responsible for the small-seed phenotype, we analyzed the
genetic interactions of these genes with det2-1. Overexpression of MINI3 in det2 mutant resulted in obviously heavier seeds, although the seed length, width, the ratio of length to width, and seed area were not significantly affected (Figure 4A-4E). Overexpression of MINI3 in wild type plant also resulted in heavier seeds, while other seed traits were unaffected (Supplementary information, Figure S5 and Table S1). These results suggested that MINI3 possibly acted downstream of BR and mediates BR-regulation of seed weight but not seed shape.

The short hypocotyl under blue 1-Dominant (shb1-D) mutant produces heavier seeds (Zhou et al., 2009). We crossed shb1-D with bri1-5, which are both in the Ws ecotype background. Analysis of more than ten bri1-5×shb1-D double mutant individuals showed increased seed mass and seed area compared to those of bri1-5 or wild type, and no obvious difference when compared to shb1-D (Figure 4F-I). The seed length of bri1-5 shb1-D were larger than that of bri1-5 and shorter than shb1-D, while the seed width of bri1-5 shb1-D was larger than either bri1-5 or shb1-D (Figure 4G). The ratio of length to width of bri1-5 shb1-D was not obviously different from that of bri1-5, and was significantly smaller than that of Ws or shb1-D (Figure 4H). These results showed that shb1-D can partially restore the seed weight and seed size of bri1-5 mutant, consistent with SHB1 acting downstream of BR in promoting seed size. However, the BR’s effect on seed shape was independent of SHB1.

Genetic studies by crossing the shb1, mini3 and iku2 mutants, with bzr1-1D mutants were performed to further test whether SHB1, MINI3 and IKU2 acted downstream of BZR1 and BR signal. The mass and area of seeds produced from shb1 bzr1-1D, mini3 bzr1-1D and iku2 bzr1-1D were all significantly lower than those of bzr1-1D mutants, and had no obvious difference from those of shb1, mini3 and iku2 mutants (Figure 5A-B, and 5E). The other seed traits including the ratio of length to width in shb1 bzr1-1D, mini3 bzr1-1D and iku2 bzr1-1D were also similar to that of shb1, mini3, and iku2 mutants, respectively (Figure 5C and 5D). These genetic results were consistent with SHB1, MINI3 and IKU2 acting downstream of BZR1.
AP2 and ARF2 Are Negative Regulators of Seed Size Acting Downstream of BR

It is reported that AP2 negatively regulate seed size and mass. The weak (ap2-5) and strong (ap2-6) allele of AP2 mutants produced larger seeds (Jofuku et al., 2005; Ohto et al., 2005). Double mutants of det2 with ap2-5 and ap2-6 were generated and analyzed to investigate whether the increased expression of AP2 in det2 was responsible for the small-seed phenotype of det2. As shown in Figure 6, mutations in AP2 suppressed the small-seed phenotype of det2. Seed mass and seed area of ap2-5 det2 was significantly larger than that of the det2 mutant, and similar to the ap2-5 mutant (Figure 6B and 6E). The ap2-5 det2 seeds had notably increased length and width, but unchanged ratio of length to width, compared to det2 (Figure 6C and 6D). Seeds from ap2-6 det2 were dramatically heavier and larger than det2, and were not obviously different from those of ap2-6 (Figure 6A, 6B and 6E). In contrast, the seed shape of ap2-6 det2 was similar to det2 but different from ap2-6. The seed length and width of ap2-6 det2 were notably increased but their ratio remained the same compared to det2. The seed length of ap2-6 det2 was shorter than those of ap2-6, but the seed width was increased compared to those of ap2-6. There was no significant difference in seed shape between ap2-6 det2 and det2 (Figure 5C and 5D). These results indicated that seed weight and seed size of ap2 det2 double mutant were similar to the single ap2 mutants, whereas the seed shape of double mutants was similar to det2 but notably different from ap2, suggesting that AP2 acted downstream of DET2 and played a negative role in BR-regulation of seed size. On the other hand, BR mediated seed shape regulation was independent of AP2.

To investigate whether AP2 acted downstream of BZR1 and BR signal, we performed double mutation analysis combining ap2-6 with bzl1-ID mutants. Seeds produced from ap2-5 bzl1-ID (Huang et al., 2012) and ap2-6 bzl1-ID double mutants were significantly heavier and larger than those of bzl1-ID mutants, and were not obviously different to those of ap2-5 and ap2-6 mutants, respectively (Figure 5A, 5B
and 5E). Other seed traits such as seed length and width, and the ratio of length to width in $ap2-5$ $bzr1-1D$ and $ap2-6$ $bzr1-1D$ double mutants resembled those of $ap2-5$ and $ap2-6$ mutants (Figure 5C and 5D). These data was consistent with $AP2$ acting downstream of $BZR1$.

It has been shown previously that the embryos of $ap2$ mutants have larger cell size and a greater number of cells (Jofuku et al., 2005; Ohto et al., 2005). Analysis showed that the embryo cell length of $ap2-6$ $det2$ was significantly larger than that of $det2$ mutant and similar to $ap2-6$ mutant. But there was no obvious difference in cell width between $ap2-6$ $det2$ double mutants and $ap2-6$ mutants (Supplementary information, Figure S1 and S6). The embryo shoot and embryo root apex or cotyledon of $ap2-6$ $det2$ double mutants produced more cells than $det2$ mutants, and the cell number of $ap2-6$ $det2$ double mutants was similar to that of the $ap2-6$ mutants (Supplementary information, Figure S1 and S6). These results indicated that $AP2$ mutation rescued the phenotypes of smaller cell size and fewer cell numbers of the $det2$ mutant, suggesting that the increased expression of $AP2$ in $det2$ contributed to the small-seed phenotype; and that $AP2$ was involved in BR regulation of embryo cell size and number.

$AP2$ affects seed cavity, endosperm size and endosperm cellularization (Ohto et al., 2009). Our observation showed that the seed cavity and endosperm size of $ap2-6$ $det2$ was larger than those of $det2$ at the same stage, and was similar to that of $ap2-6$. The seed development of $ap2-6$ and $ap2-6$ $det2$ started more slowly than wild type in the first five days, but the growth speed increased soon after giving rise to a cavity size similar to wild type after 7DAP. The final embryo sizes of $ap2-6$ and $ap2-6$ $det2$ were bigger than those of wild type (Supplementary information, Figure S2). Similar to $ap2-6$, $ap2-6$ $det2$ initiated endosperm cellularization when embryos were at the middle-heart stage at ~6DAP, and completed cellularization when embryos were at the late-heart stage at ~7DAP (Supplementary information, Figure S2). These results indicated that $AP2$ was epistatic to $det2$, and played an important role in BR regulation of seed cavity, endosperm and embryo development.
ARF2 is a negative regulator of seed size and mass. Loss-of-function mutant arf2-8 has increased seed size (Schruff et al., 2006). We crossed arf2-8 with det2 to test whether the increased expression of ARF2 in det2 was responsible for the smaller seed size of det2. As shown in Figure 7, the seed weight and seed area of arf2-8 det2 were notably larger than those of det2 or wild type, and not obviously different to arf2-8. The seed length of arf2-8 det2 was larger than that of det2, but shorter than that of arf2-8; and the seed width was increased compared to det2 or arf2-8 (Figure 7C). There was no significant difference in the ratio of length to width compared with det2, however, the ratio was significantly lower than wild type or arf2-8 (Figure 7D). These results indicated that the seed weight and seed size of arf2-8 det2 double mutants resemble arf2-8 phenotypes, and ARF2 acted downstream of DET2 as a negative regulator of seed size. On the other hand, BR regulation of seed shape was independent of ARF2.
DISCUSSION

Seed size is a major agricultural trait, and our study demonstrated an important role of BR in promoting seed development. Our studies showed that deficiency of BR biosynthesis and signal transduction led to smaller seed size and abnormal seed shape in Arabidopsis. The effects of BR on seed size and seed shape were attributed to BR actions in the embryo/endosperm and maternal tissues, respectively. While BR was known to increased cell size and cell division in general, our molecular analysis provided evidence that BR modulated specific developmental regulators of seed development. Thus, our study linked BR signaling to developmental pathways that controlled seed size and shape. This knowledge not only provided insight into the molecular mechanism of hormonal regulation of seed development but also potential mean for improving grain yield.

BR Plays Crucial Roles in Regulating Seed Size and Seed Mass

The positive effect of BR on seed growth has been observed in BR-deficient mutant and BR-overproducing rice plants (Wu et al., 2008). Our analysis of Arabidopsis BR mutant showed similar requirement of BR for normal seed development. The BR-deficient mutant det2 and BR-insensitive mutant bri1 both had reduced seed size, and BR treatment obviously increased the det2 seed weight, area and ratio of seed length to width, although the enhanced seed weight and length/width ratio are still not as normal as wild type (Figure 3A). We further showed that the reduced seed size of det2 was mainly due to reduced embryo cell size and cell number, and smaller integument cell size (Figure 1). In flowering plants, the embryo and the endosperm, which are enclosed by maternal seed integument, are derived from double fertilization, and the development of these three components are finely coordinated (Garcia et al., 2003). Our studies suggested that BR regulated seed size by coordinating the growth of the embryo and integument through independent mechanisms that depend on BR produced in each tissue. Pollinating BR-deficient or –insensitive mutants with wild
type pollen, which resulted in functionally wild type embryo in the mutant integument, rescued the size but not shape of the seed, suggesting that BR produced in the embryo and endosperm were sufficient for increasing seed size, but the elongated seed shape required BR production and signaling in the integument. This observation also suggested that BR could not move far from the source cells that synthesize it. Consistent with such independent action of BR in embryo/endosperm on seed size and BR action in the integument determining seed shape, altering several BR regulated genes suppressed the small-seed phenotype of det2 but not the seed shape. Our results suggested that BR regulated seed size and seed shape through independent actions of distinct downstream genes acting separately in the embryo/endosperm and the integument.

BR Activates SHB1-MINI3-IKU2 Pathway and Repress AP2 and ARF2 to Increase Seed Size though directly binding their promoters by BZR1

BZR1 is a very important transcription factor in BR signal pathway. And bzr1-1D is a BR signal enhanced mutant. The seed weight, area and shape of bzr1-1D has not significant difference with wild type. We statistic analyzed bzr1-1D seed phenotypes in different grow places and found that the bzr1-1D seeds area and weight might be lower than wild type under poor condition in some time, but the differences are still quite little; which could be explained for feedback inhibition to BR biosynthesis genes (He et al., 2005), or too many seeds per siliqque (Huang et al., 2013); or the putative function of BZR1/BES1 in seed development (Yu et al., 2011). We did not analyze bes1-D in this work because that the important seed development-regulating genes, like SHB1-MINI3-IKU2, AP2 and ARF2, are not the direct target genes of BES1. BES1 possibly regulates seed development through targeting other seed development-regulating genes (like KLU and AHK4, which are the target genes of both BZR1 and BES1. Sun et al., 2010; Yu et al., 2011) and regulates their expressions, which might be worse to investigate in the future.
Our results showed that the expression of many genes involved positive regulation of seed size, especially the components in the SHB1-MINI3-IKU2 pathway, were activated by BR, mostly through direct regulation by BZR1. Genetics interactions provided further evidence that SHB1, MINI3 and IKU2 genes were positive factors for BR regulation of seed size. Overexpression of MINI3 and dominant mutation of SHB1 partly suppressed the small-seed phenotype of det2. Consistent with a role downstream of BR, the shb1, mini3 or iku2 mutants showed normal expressions of the BR-biosynthetic genes DWF4 and CPD (Supplementary information, Figure S7). The suppression of the seed size but not seed shape of det2 by MINI3-overexpression and shb1-D was consistent with previous studies showing that the SHB1-MINI3-IKU2 pathway regulated endosperm and embryo but not integument development to affect seed size (Zhou et al., 2009). Our molecular experiments indicated that BZR1 bound to the promoter region of SHB1, which might in turn activated the expressions of MINI3 and IKU2. BZR1 also binds to the promoter region of IKU1, which can interact with MINI3 at the protein level (Wang et al., 2010). The expression of MINI3 and IKU2 is altered in iku1 and mini3 (Luo et al., 2005), indicating BZR1 might target and regulate IKU1 and thus affect MINI3, which led to IKU2 activation too. The molecular and genetic data together supported that the SHB1-MINI2-IKU2 pathway acted downstream of BR signaling to positively regulated embryo development and seed size (Figure 8).

Our studies showed that BR also suppressed the expression of genes that negatively regulated seed size, including APETALA2 (AP2) and AUXIN RESPONSE FACTOR 2 (ARF2). These two genes were confirmed to be direct targets BZR1 and are expressed at high levels in the det2 and bri1 mutants (Figure 3D). The loss-of-function mutations of AP2 and ARF2 increased seed size and suppressed the small-seed phenotype of det2, indicating that the elevated expression levels of these genes contributed to the small-seed phenotype of det2.

We confirmed that AP2 expression was repressed by BR signaling (Huang et al.,
The expression levels of the BR-biosynthetic genes *DWF4* and *CPD* were decreased in the *ap2* mutants (Supplementary information, Figure S7). Due to feedback regulation, expression levels of *CPD* and *DWF4* were increased in *det2* and decreased in *bzr1-1D*. The *ap2* mutations reduced *CPD* and *DWF4* expression in the *det2* background, but not in the *bzr1-1D* background (Supplementary information, Figure S7). AP2 might negatively regulate BR signaling. The reduced expression of BR biosynthetic genes in the *ap2* mutant could be due to feedback inhibition by enhanced BR signaling. Alternative, AP2 might positively regulate BR biosynthetic genes directly, and thus would form another feedback loop as BR inhibition of AP2 expression would lead to reduced *CPD* and *DWF4* expression again. In contrast to AP2, ARF2 seems not to affect the expression of *CPD* and *DWF4*, suggesting that AP2 and ARF2 act through distinct pathways, which is consistent with the reports that *AP2* influences development of maternal integuments, the zygotic embryo and endosperm to repress seed size (Ohto et al., 2009) and *ARF2* represses seed size by regulating the development of integuments (Schruff et al., 2006).

**BR and Other Signals Co-regulate Seed Size**

Besides the SHB1-MINI3-IKU2 pathway, several other pathways affecting seed size were regulated by BR. *KLU* expresses in the inner integument of developing ovules, and promotes cell proliferation in integuments to determine the growth potential of seed coat and seed. The *klu* mutant has smaller seeds, and by contrast, overexpression of the *KLU* gene results in significantly enlarged seeds (Adamski et al., 2009). The expression level of *KLU* was up regulated by BR, and *KLU* was a direct target of BZR1. These results suggested that BR might positively regulate integument development through BZR1 activating *KLU*. *FIS2* also regulates seed development through epigenetic regulation pathway of endosperm development (Pien and Grossniklaus, 2007). Our results confirmed that BR activated *FIS2* by BZR1 targeting *FIS2*, *RGE1* and *ANT*, which were direct targets of BZR1, and were up-regulated by
BR, regulate positively embryo proliferation. BR might regulate seed development through regulation of embryo proliferation through BZR1 targeting and regulating \textit{RGE1} and \textit{ANT}.

Recent studies reported that BR and GA regulate common cellular activities and developmental processes (Bai et al., 2012); and BZR1 interacts with PIF4 to control a core transcription network, which indicating that plant growth can be co-regulated by the steroid and environmental signals (Oh et al., 2012). Our results showed that the important genes for cytokinin signaling, \textit{ARABIDOPSIS HISTIDINE KINASE (AHK)} and \textit{CYTOKinin INDEPENDENT 1 (CKI1)} (Riefler et al., 2006; Deng et al., 2010), were the target genes of BZR1 and regulated by BR (Supplementary information, Figure S4). BR and cytokinin might crosstalk to co-regulate seed development, but the detailed mechanism needs further investigation.

**BR Regulates Seed Shape through Unknown Maternal Tissue Factors After Fertilization**

Our results showed that BR might have a major effect on seed shape. The ratio of length to width in wild type seed is ranged from 1.5 to 1.7, depends on growth conditions and metrical methods, which resembles the Golden Ratio of 1.61803399 (Cervantes et al., 2010), and this ratio was notably lower in \textit{det2}, \textit{dwf4} or \textit{bri1-116} (Figure 2). Cross pollination of these mutants with wild type pollen, restoring BR signaling in the embryo and endosperm, rescued seed size/mass to that of wild type, but did not rescue the seed shape. Before pollination, the ovule shape, including length/width/area, of \textit{det2} was not significantly different from the wild type (Supplementary information, Figure S8), indicating the BR regulated seed shape after fertilization. Although the \textit{SHB1-MINI3-IKU2} pathway, \textit{AP2}, and \textit{ARF2} mediated the BR control of seed weight and seed size, the BR regulation of seed shape was independent of these genes. Therefore, mechanism for BR regulation of seed shape was different from that for seed size. BR synthesis and signaling in the maternal
tissues such as integument determined the seed shape, whereas the BR produced in
the embryo/endosperm had little effect on seed shape. Such distinct cell-type specific
effects of BR suggested that BR did not transport over long distance from source cells
(for our case, from zygote to integument cells) and thus can function as a local signal
in morphogenesis, which was consistent with previous report (Symons et al., 2008).
Also was in accord with the recent finding of a role of BR in regulating organ
boundary formation (Bell et al., 2012; Gendron et al., 2012). Our study indicated that
BR regulated distinct pathways in specific cell types to control the seed size and shape.
Further study of these pathways at the molecular and developmental levels would be
important for increasing grain yield.
METHODS

Plant Materials and Growth Conditions
The det2, dwf4, bri1-1I6, bzc1-1D, shb1, mini3, aku2, ap2-5, ap2-6, arf2-8 mutants and transgenic Arabidopsis thaliana plants are with Columbia (Col) background. bri1-5 and shb1-D mutants are with Wassilewskija (Ws) ecotype. Plants were grown at 23°C and seeds were harvested when the plant was completely mature. At later developmental stages, the plants were also fitted with a plastic well to catch all seeds released by dehiscing siliques. Isolated siliques of 4 to 5 d after hand-pollination of Col and det2 were dipped with mock solution or 10 µM 24-epibrassinolide (BR) treatment, and mock solution or 1 µM brassinazole (BRZ) for 3 hr as determined by qRT-PCR analysis. All siliques were cut in the same length of centre joint position for easily absorbing the solutions.

Measurement of Seed Weight, Seed Length, Seed Width and Seed Area
For seed weight, plants were grown concurrently under identical conditions and seeds were harvested when the plant was mature. 1000 seeds per genotype were dried at 24°C for 7 d, and weighed. Data are presented as means ± SD from at least three independent experiments. For seed length and width, dried seeds were photographed using a ZEISS Axio Imager A1 microscope, and then measured by ImageJ software. The measured seed length and width were used to calculate the ratio of length to width. ImageJ software was used to calculate the seed area. Young seeds at early heart or middle heart stage were cleared in Hoyer’s solution overnight and photographed using a Leica differential interference contrast (DIC) microscope, followed by using ImageJ software to calculate the seed dimensions.

Developmental Alteration Analysis
To examine the developmental alterations of det2, ap2-6, ap2-6×det2 mutants, flowers of Col and det2, ap2-6, ap2-6×det2 mutants were hand-pollinated, and the developing seeds were harvested at different days after pollination. The siliques were dissected under an OLYMPUS SZX9 microscope to isolate the young seeds, and then these young seeds were cleared in Hoyer’s solution overnight and photographed using a Leica differential interference contrast (DIC) microscope. To investigate the endosperm development, young seeds were fixed in FAA solution overnight at 4°C, and dehydrated through an ethanol series (50, 70, 80, 90, and 100%, each for 30 min). The samples were then embedded in LR White (SIGMA-ALDRICH). Sections were stained with 0.1% (w/v) toluidine blue O in distilled water. Stained sections were photographed using a ZEISS Axio Imager A1 microscope.

**Scanning Electron Microscopy Analysis**

Mature dried seeds were imbibed for 1 hr and dissected under an OLYMPUS SZX9 microscope to isolate the mature embryos. The embryos were incubated for 12 hr in buffer that contains 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 1% Triton X-100, and 1% DMSO at 37°C, fixed for 24 hr in FAA (10% formalin, 5% acetic acid, 45% ethanol, and 0.01% Triton X-100). After fixation, the samples were dehydrated through an ethanol series (70, 85, 95, and 100%, each for 30 min) and then dehydrated through an ethanol and isopentyl acetate series (75% ethanol and 25% isopentyl acetate, 50% ethanol and 50% isopentyl acetate, 25% ethanol and 75% isopentyl acetate, and 100% isopentyl acetate, each for 30 min). The embryos were then dried using a HITACHI Critical Point Dryer. The individual embryos were mounted on scanning electron microscopy stubs, sputter-coated with platinum using the HITACHI Ion Sputter, and examined under a Hitachi S-4800 scanning electron microscope. Around 50 embryos were photographed and the cell length and width were determined using ImageJ software. Data are presented as the average of three
independent experiments.

**Total RNA Isolation and qRT-PCR Analysis**

Total RNA was extracted from siliques 4 to 5 d after hand-pollination using the TRIzol RNA extraction kit (Invitrogen, USA). The first-strand cDNA was synthesized by using M-MLV reverse transcriptase (Promega, USA) and used as templates for RT-PCR analysis. qRT-PCR analyses were carried out on Mx3000P (Stratagene, USA) by using the SYBR Green reagent (Toyobo, Japan) according to the manufacturer’s instructions. The primer sequences for qRT-PCR were listed in Table S2. Data were calculated from three biological replicates, and each biological replicate was examined in triplicate.

**Reciprocal Crosses and Double Mutant Analysis**

The reciprocal crosses between Col and det2, dwf4, bri1-116 were performed with flowers at identical positions (11th to 14th flowers) on secondary inflorescences. A series of double mutants of det2 with mutants showing large seed size ap2-5, ap2-6 and arf2-8 were generated and the mutant of bri1-5 with mutant showing large seed size shb1-D was also generated. A succession of double mutants of bzr1-1D with mutants showing small seed size shb1, mini3 and iku2 and with mutants showing large seed size ap2-5 and ap2-6 were generated. The T-DNA specific primer for shb1, mini3 and iku2 is T-DNA-1 and for arf2-8 is T-DNA-2. For shb1-D genotyping, the T-DNA specific primer is T-DNA-3. For ap2-5 genotyping, the PCR fragment from WT but not from ap2-5 can be cut by SacII. For ap2-6 genotyping, the PCR fragment from WT but not from ap2-6 can be cut by PstI. For det2 genotyping, MnlI cuts the PCR fragment amplified from WT but not det2. For bri1-5 genotyping, PstI cuts the PCR fragment amplified from WT but not bri1-5. For bzr1-1D genotyping, the PCR fragment from WT but not from bzr1-1D can be cut by Cfr10I. The primer sequences
for mutants screening were listed in Table S3.

**ChIP Analysis**

For ChIP experiments, *Arabidopsis* wild type and pBZR1::BZR1-CFP transgenic *Arabidopsis* plants were grown at 23°C under 16-h light and 8-h dark condition. Siliques 4 to 5 d were cross-linked for 15 minutes in 1% formaldehyde by vacuum filtration. ChIP analysis was performed by using an affinity purified anti-GFP polyclonal antibody and qRT-PCR was performed using SYBR Green reagent (Toyobo, Japan). Results were presented as the ratio of the amount of DNA immunoprecipitated from BZR1-CFP samples to that of the wild type samples. The *PP2A* and *CNX5* genes were used as the negative control, and the *DWF4* gene was used as the positive control. The ChIP experiments were performed three times, from which the means and standard deviations were calculated. The primer sequences for ChIP-qPCR were listed in Table S4.

**Accession Numbers**

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AUTHOR CONTRIBUTIONS

WB.J. designed the project, did genes expression, cross, microscope observation, data analysis, and wrote the paper. HH.Y. did the mutant phenotypes observation and ChIP-qPCR. YW.H. observed mutant phenotypes and culture plants. SW.Z. did part of the mutant phenotypes. ZY.W. designed the project and edited the paper. WH.L. designed the project, analyzed data, wrote and edited the paper.
FIGURE LEGENDS

Figure 1. BR Deficient Mutants and BR Insensitive Mutants Have Small Seeds.
Mature seeds of Col (A), det2 (B), bzh1-1D (C), Ws (D), and bri1-5 (E). Mature embryos of Col (F) and det2 (G). Seed weight per 1000 mature dried seeds (H), seed length and seed width (I), the ratio of length to width (J), and seed area (K) of mature dried seeds of Col, det2, bzh1-1D, Ws, and bri1-5. Hypocotyl cell length and width of Col and det2, *P<0.05. (L). Average cell numbers from three columns in the central region of the hypocotyl plus the embryonic (Em) root (left) and the cotyledon (right) of Col and det2, *P<0.05 (M). Bars = 750 µm for (A) to (E), and 50 µm for (F) and (G). Values that differ at the 0.05 significance level are labeled with different letters.

Figure 2. BR Regulates Seed Size and Shape.
Cleared seeds of the plants from reciprocal crosses between Col and det2, dwf4 and bzh1-116 (shown 116) in middle heart stage were imaged with differential contrast optics. Bar = 60 µm. Col det2 indicates Col pistils pollinated with det2 pollen, and so on (A). Seed length and seed width (B), the ratio of length to width (C) and seed area (D) from seeds of Col Col, Col det2, Col dwf4, Col 116, det2 det2, det2 Col, dwf4 dwf4, dwf4 Col, bzh1-116 bzh1-116, bzh1-116 Col in middle heart stage. Col det2 indicates Col pistils pollinated with det2 pollen, and so on. Seed weight per 1000 mature seeds from seeds of these reciprocal crosses (E). Values that differ at the 0.05 significance level are labeled with different letters.

Figure 3. BR increases Seed Size and Affects Seed Shape; and BR Regulates the Expression Levels of Seed Size-related Genes through directly binding to their promoters by BZR1.

(A) Statistics of seed weight, seed area and the ratio of seed length to width indicated that BR treatment resulted in Col seeds with enhanced weight and det2 seeds with rescued seed area and partially rescued seed weight/ratio of seed length to width.
Values that differ at the 0.05 significance level are labeled with different letters. The inflorescence of Col and det2 Mutants was dipped with 10 µM 24-epibrassinolide (BR) or mock solution once a day. The inflorescence was dipped 10 seconds and then grew normally. (B) Expression levels of the above-mentioned genes in siliques 4 to 5 d after hand-pollination of Col with mock solution or 10 µM 24-epibrassinolide (BR) treatment, det2 with mock solution or 10 µM 24-epibrassinolide (BR) treatment for 3 hr as determined by qRT-PCR analysis. (C) Expression levels of the above-mentioned genes in siliques 4 to 5 d after hand-pollination of Col with mock solution or 1 µM brassinazole (BRZ) treatment, det2 with mock solution or 1 µM BRZ treatment for 3 hr as determined by qRT-PCR analysis. (D) Expression levels of SHB1, IKU1, MINI3, IKU2, AP2, ARF2, HSF15 and KLU in siliques 4 to 5 d after hand-pollination of Col, det2, bzrl-1D, Ws, bri1-5 as determined by qRT-PCR analysis. (E) ChIP analysis revealed that BZR1 binds to the promoters of SHB1, IKU1, IKU2, AP2 and ARF2. Means were calculated from three biological samples, and each biological sample was examined in triplicate. Error bars indicate SD.

Figure 4. Increased MINI3 Expression can Partially Restore the Seed Phenotypes of det2 Mutant and shb1-D also can Partially Restore the Seed Phenotypes of bri1-5 Mutant.

(A) Overexpression of MINI3 in det2 mutants produces larger seeds in comparison to det2 mutant. Mature dried seeds from MINI3 OE 1/det2, MINI3 OE 2/det2, MINI3 OE 3/det2, shb1-D and bri1-5 shb1-D were shown. Col and det2, WS and bri1-5 data showed in Figure 1, and all the seeds came from parallel experiments. Bar = 750 µm. Seed weight per 1000 mature dried seeds (B), seed length and seed width (C), the ratio of length to width (D), seed area of mature dried seeds (E) of Col, det2, MINI3 OE 1/det2, MINI3 OE 2/det2, and MINI3 OE 3/det2 were shown. Seed weight per 1000 mature dried seeds (F), seed length and seed width (G), the ratio of length to width (H), seed area of mature dried seeds (I) of Ws, bri1-5, shb1-D and bri1-5 shb1-D were shown. Values that differ at the 0.05 significance level are labeled with different letters.
Figure 5. SHB1, MINI3 and IKU2 Act Downstream of BZR1

(A) Mature dried seeds of shb1, shb1 bzr1-1D, mini3, mini3 bzr1-1D, iku2 and iku2 bzr1-1D were shown. Col and bzr1-1D data showed in Figure 1, and all the seeds came from parallel experiments. Bar = 750 µm. Seed weight per 1000 mature dried seeds (B), seed length and seed width (C), the ratio of length to width (D), seed area of mature dried seeds (E) of Col, bzr1-1D, shb1, shb1 bzr1-1D, mini3, mini3 bzr1-1D, iku2 and iku2 bzr1-1D. Values that differ at the 0.05 significance level are labeled with different letters.

Figure 6. The AP2 Mutation in det2 Mutants Suppressed the Small Seed Phenotype of det2 and AP2 Acts Downstream of BZR1

(A) Mature dried seeds from Col, det2, bzr1-1D, ap2-5, ap2-5 det2, ap2-5 bzr1-1D, ap2-6, ap2-6 det2 and ap2-6 bzr1-1D were shown. Col, det2 and bzr1-1D data showed in Figure 1, and all the seeds came from parallel experiments. Bar = 750 µm. Seed weight per 1000 mature dried seeds (B), seed length and seed width (C), the ratio of length to width (D), seed area of mature dried seeds (E) of Col, det2, bzr1-1D, ap2-5, ap2-5 det2, ap2-5 bzr1-1D, ap2-6, ap2-6 det2 and ap2-6 bzr1-1D. Values that differ at the 0.05 significance level are labeled with different letters.

Figure 7. The ap2-8 det2 Double Mutants Presented arf2-8 Phenotypes

(A) Mature dried seeds from arf2-8 and arf2-8 det2 were shown. Col and det2 data showed in Figure 1, and all the seeds came from parallel experiments. Bar = 750 µm. Seed weight per 1000 mature dried seeds (B), seed length and seed width (C), the ratio of length to width (D), seed area of mature dried seeds (E) of Col, det2, arf2-8 and arf2-8 det2. Values that differ at the 0.05 significance level are labeled with different letters.

Figure 8. Hypothetical Model for BR-Regulated Seed Development.

BR regulates embryo and endosperm development to determine seed size through
binding to the promoter region of *SHB1* by BZR1, which further regulates the expression of *MINI3* and *IKU2*, or through BZR1 binding to the promoter region of *IKU1*, which interacts with MINI3 to regulate the expression of *MINI3* and *IKU2*, or alternatively through BZR1 binding to the promoter of *IKU2* and regulates the expression of *IKU2*. BR regulates integuments, endosperm and embryo development through BZR1 binding to the *AP2* promoter, and then affects seed size. BR also modulates the integument development through BZR1 binding to the promoter of *ARF2*. 
REFERENCES


functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell 18: 40-54


Figure 1. BR Deficient Mutants and BR Insensitive Mutants Have Small Seeds.
Mature seeds of Col (A), det2 (B), bezl-1D (C), Ws (D), and bri1-5 (E). Mature embryos of Col (F) and det2 (G). Seed weight per 1000 mature dried seeds (H), seed length and seed width (I), the ratio of length to width (J), and seed area (K) of mature dried seeds of Col, det2, bezl-1D, Ws, and bri1-5. Hypocotyl cell length and width of Col and det2, *P<0.05. (L). Average cell numbers from three columns in the central region of the hypocotyl plus the embryonic (Em) root (left) and the cotyledon (right) of Col and det2, *P<0.05 (M). Bars = 750 µm for (A) to (E), and 50 µm for (F) and (G). Values that differ at the 0.05 significance level are labeled with different letters.
Jiang et al., Figure 2

Figure 2. BR Regulates Seed Size and Shape.
Cleared seeds of the plants from reciprocal crosses between Col and det2, dwf4 and bri1-116 (shown 116) in middle heart stage were imaged with differential contrast optics. Bar = 60 µm. Col det2 indicates Col pistils pollinated with det2 pollen, and so on (A). Seed length and seed width (B), the ratio of length to width (C) and seed area (D) from seeds of Col Col, Col det2, Col dwf4, Col 116, det2 det2, det2 Col, dwf4 dwf4, dwf4 Col, bri1-116 bri1-116, bri1-116 Col in middle heart stage. Col det2 indicates Col pistils pollinated with det2 pollen, and so on. Seed weight per 1000 mature seeds from seeds of these reciprocal crosses (E). Values that differ at the 0.05 significance level are labeled with different letters.
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Figure 5. SHB1, MINI3 and IKU2 Act Downstream of BZR1

(A) Mature dried seeds of shb1, shb1 bzr1-1D, mini3, mini3 bzr1-1D, iku2 and iku2 bzr1-1D were shown. Col and bzr1-1D data showed in Figure 1, and all the seeds came from parallel experiments. Bar = 750 µm. Seed weight per 1000 mature dried seeds (B), seed length and seed width (C), the ratio of length to width (D), seed area of mature dried seeds (E) of Col, bzr1-1D, shb1, shb1 bzr1-1D, mini3, mini3 bzr1-1D, iku2 and iku2 bzr1-1D. Values that differ at the 0.05 significance level are labeled with different letters.
Figure 6. The AP2 Mutation in det2 Mutants Suppressed the Small Seed Phenotype of det2 and AP2 Acts Downstream of BZR1

(A) Mature dried seeds from Col, det2, bzh1-1D, ap2-5, ap2-5 det2, ap2-5 bzh1-1D, ap2-6, ap2-6 det2 and ap2-6 bzh1-1D were shown. Col, det2 and bzh1-1D data showed in Figure 1, and all the seeds came from parallel experiments. Bar = 750 µm. Seed weight per 1000 mature dried seeds (B), seed length and seed width (C), the ratio of length to width (D), seed area of mature dried seeds (E) of Col, det2, bzh1-1D, ap2-5, ap2-5 det2, ap2-5 bzh1-1D, ap2-6, ap2-6 det2 and ap2-6 bzh1-1D. Values that differ at the 0.05 significance level are labeled with different letters.
Figure 7. The *ap2-8 det2* Double Mutants Presented *arf2-8* Phenotypes

(A) Mature dried seeds from *arf2-8* and *arf2-8 det2* were shown. Col and *det2* data showed in Figure 1, and all the seeds came from parallel experiments. Bar = 750 µm. Seed weight per 1000 mature dried seeds (B), seed length and seed width (C), the ratio of length to width (D), seed area of mature dried seeds (E) of Col, *det2, arf2-8* and *arf2-8 det2*. Values that differ at the 0.05 significance level are labeled with different letters.
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BR regulates embryo and endosperm development to determine seed size through binding to the promoter region of \textit{SHB1} by BZR1, which further regulates the expression of \textit{MINI3} and \textit{IKU2}, or through BZR1 binding to the promoter region of \textit{IKU1}, which interacts with MINI3 to regulate the expression of \textit{MINI3} and \textit{IKU2}, or alternatively through BZR1 binding to the promoter of \textit{IKU2} and regulates the expression of \textit{IKU2}. BR regulates integuments, endosperm and embryo development through BZR1 binding to the \textit{AP2} promoter, and then affects seed size. BR also modulates the integument development through BZR1 binding to the promoter of \textit{ARF2}.