Transcription Factors SOD7/NGAL2 and DPA4/NGAL3 Act Redundantly to Regulate Seed Size by Directly Repressing KLU Expression in Arabidopsis thaliana

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Although seed size is one of the most important agronomic traits in plants, the genetic and molecular mechanisms that set the final size of seeds are largely unknown. We previously identified the ubiquitin receptor DA1 as a negative regulator of seed size, and the Arabidopsis thaliana da1-1 mutant produces larger seeds than the wild type. Here, we describe a B3 domain transcriptional repressor NGATHA-like protein (NGAL2), encoded by the suppressor of da1-1 (SOD7), which acts maternally to regulate seed size by restricting cell proliferation in the integuments of ovules and developing seeds. Overexpression of SOD7 significantly decreases seed size of wild-type plants, while the simultaneous disruption of SOD7 and its closest homolog DEVELOPMENT-RELATED PcG TARGET IN THE APEX4 (DPA4/NGAL3) increases seed size. Genetic analyses indicate that SOD7 and DPA4 act in a common pathway with the seed size regulator KLU to regulate seed growth, but do so independently of DA1. Further results show that SOD7 directly binds to the promoter of KLUH (KLU) in vitro and in vivo and represses the expression of KLU. Therefore, our findings reveal the genetic and molecular mechanisms of SOD7, DPA4, and KLU in seed size regulation and suggest that they are promising targets for seed size improvement in crops.

INTRODUCTION

Seed size is an important agronomic trait and is also a key ecological trait that influences many aspects of a species’ regeneration strategy, such as seedling survival rates and seed dispersal syndrome (Harper et al., 1970; Westoby et al., 2002; Moles et al., 2005; Fan et al., 2006; Orsi and Tanksley, 2009; Gegas et al., 2010). In higher plants, seed development starts with a double fertilization process, in which one of the two haploid pollen nuclei fuses with the haploid egg cell to produce the diploid embryo, while the other sperm nucleus fuses with the diploid central cell to form the triploid endosperm (Lopes and Larkins, 1993). The integuments surrounding the ovule are maternal tissues and form the seed coat after fertilization. Therefore, seed size is determined by the growth of the embryo, the endosperm, and the maternal tissues. However, the genetic and molecular mechanisms setting the limits of seed growth are almost unknown in plants.

Several factors that function maternally to regulate seed size have been identified in Arabidopsis thaliana. For example, TRANSPARENT TESTA GLABRA2 (TTG2) influences seed growth by increasing cell elongation in the maternal integuments (Garcia et al., 2005; Ohto et al., 2009), while APETALA2 (AP2) may regulate seed growth by limiting cell elongation in the maternal integuments (Jofuku et al., 2005; Ohto et al., 2005, 2009). By contrast, AUXIN RESPONSE FACTOR2 (ARF2) acts maternally to control seed growth by restricting cell proliferation (Schruff et al., 2006). Similarly, the ubiquitin receptor DA1 acts synergistically with the E3 ubiquitin ligases DA2 and EOD1/BB to regulate seed size by limiting cell proliferation in the maternal integuments (Li et al., 2008; Xia et al., 2013). Mutations in the suppressor of da1-1 (SOD2), which encodes the ubiquitin-specific protease (UBP15), suppress the large seed phenotype of da1-1 (Du et al., 2014). DA1 physically associates with UBP15/SOD2 and modulates the stability of UBP15. These studies show that the ubiquitin pathway plays an important part in the maternal control of seed size. KLU/CYTOCHROME P450 78A5 (CYP78A5) regulates seed size by increasing cell proliferation in the maternal integuments of ovules (Adamski et al., 2009). KLU has also been suggested to generate mobile plant growth substances that promote cell proliferation (Anastasiou et al., 2007; Adamski et al., 2009). By contrast, overexpression of CYP78A6/EOD3 increases both cell proliferation and cell elongation in the integuments, resulting in large seeds (Fang et al., 2012). Seed size is also determined by zygotic tissues. Several factors have been described to influence seed size via the zygotic tissues in Arabidopsis, including HAIKU1 (IKU1), IKU2, MINISEED3 (MINI3), and SHORT HYPOCOTYL UNDER BLUE1 (SHB1) (Garcia et al., 2003; Luo et al., 2005; Zhou et al., 2009; Wang et al., 2010; Kang et al., 2013). iku and mini3 mutants form small seeds due to precocious cellularization of the endosperm (Garcia et al., 2003; Luo et al., 2005; Wang et al., 2010). SHB1 associates with MINI3 and...
**RESULTS**

**sod7-1D Suppresses the Seed Size Phenotype of da1-1**

We previously identified the ubiquitin receptor DA1 as a negative regulator of seed size in Arabidopsis (Li et al., 2008). The da1-1 mutant formed large seeds due to increased cell proliferation in the maternal integuments (Li et al., 2008; Xia et al., 2013). To identify additional factors that influence seed size, we initiated a T-DNA activation-tagging screen for modifiers of da1-1 (Fang et al., 2012). A dominant sod7-1D was isolated from seeds produced from −16,000 T1 plants (Figure 1A). Seeds of the sod7-1D da1-1 double mutant were significantly smaller and lighter than da1-1 seeds (Figures 1A, 1E, and 1F). The embryo constitutes the major volume of a mature seed in Arabidopsis. sod7-1D da1-1 embryos were smaller than da1-1 embryos (Figure 1B). The size of sod7-1D da1-1 cotyledons was significantly reduced compared with that of da1-1 cotyledons (Figure 1G). In addition, the sod7-1D da1-1 double mutant formed smaller leaves and flowers than da1-1 (Figures 1C, 1D, 1H, and 1I). Thus, the sod7-1D mutation suppressed the seed and organ size phenotypes of da1-1.

**sod7-1D Produces Small Seeds**

We isolated the single sod7-1D mutant among F2 progeny derived from a cross between the wild type (Columbia-0 [Col-0]) and sod7-1 D da1-1. The sod7-1D seeds were significantly smaller and lighter than wild-type seeds (Figures 2A, 2B, 2G, and 2H). We further isolated and visualized embryos from mature wild-type and sod7-1D seeds. The sod7-1D embryos were smaller than wild-type embryos (Figures 2C and 2D). The changes in seed size were also reflected in the size of seedlings (Figures 2E and 2F). The 10-d-old sod7-1D cotyledons were significantly smaller than wild-type cotyledons (Figure 2E, 2F, and 2I). In addition, the sod7-1D mutants exhibited small leaves and flowers compared with the wild type (Supplemental Figures 1A to 1D). The decreased size of sod7-1D leaves and petals was not caused by smaller cells (Supplemental Figures 1E and 1F), indicating that the sod7-1D mutation results in a decrease in cell number. In fact, the average area of epidermal cells in sod7-1D petals was larger than that in wild-type petals (Supplemental Figure 1E), suggesting a possible compensation mechanism between cell number and cell size.

**Figure 1.** Isolation of a Suppressor of da1-1 (sod7-1D).

(A) to (D) Seeds (A), mature embryos (B), flowers (C), and 30-d-old plants (D) of the wild type, da1-1, and sod7-1D da1-1 (from left to right).

(E) Seed area of the wild type, da1-1, and sod7-1D da1-1 (n = 100).

(F) Seed weight of the wild type, da1-1, and sod7-1D da1-1. The weights of five sample batches were measured for each seed lot (n = 5).

(G) Cotyledon area of 10-d-old wild-type, da1-1, and sod7-1D da1-1 seedlings (n = 20).

(H) and (I) Petal area and the fifth leaf area of Col-0, da1-1, and sod7-1D da1-1. Fifty petals and eight leaves were used to measure petal area and the fifth leaf area, respectively.

Values in (E) to (I) are given as mean ± se relative to the respective wild-type values, set at 100%. **P < 0.01 compared with da1-1 (Student’s t test). Bars = 0.5 mm in (A), 0.2 mm in (B), 1 mm in (C), and 5 cm in (D).
SOD7 Encodes a B3 Domain Transcriptional Repressor NGAL2

To determine whether the seed and organ size phenotypes of sod7-1D were caused by the T-DNA insertion, we first analyzed the genetic linkage of the mutant phenotypes with Basta resistance, which is conferred by the selectable marker of the activation-tagging vector (Fan et al., 2009). In a T2 population, 181 plants with sod7-1D da1-1 phenotypes were resistant, whereas 55 plants with da1-1 phenotypes were sensitive, indicating that the insertion cosegregates with the sod7-1D phenotypes. To clone SOD7, we isolated the T-DNA flanking sequences using thermal asymmetric interlaced PCR (TAIL-PCR; Liu et al., 1995). DNA sequencing revealed that the T-DNA had inserted ~5.6 kb upstream of At3g11580 and ~3.7 kb upstream of At3g11590 gene (Figure 3A). To determine which gene is responsible for the sod7-1D phenotypes, we examined the mRNA levels of these two genes. The mRNA of At3g11590 accumulated at a similar level in sod7-1D da1-1 and da1-1, suggesting that At3g11590 is not the SOD7 gene (Figure 3B). By contrast, the expression level of At3g11580 in sod7-1D da1-1 plants was dramatically higher than that in da1-1 plants, suggesting that At3g11580 is the SOD7 gene (Figure 3B). To further confirm whether the sod7-1D phenotypes were caused by ectopic At3g11580 expression, we overexpressed the At3g11580 gene (35S:GFP-SOD7) in wild-type plants (Col-0) and isolated 37 transgenic plants. Most transgenic lines had small seeds and organs (Figures 3D to 3F; Supplemental Figure 2), similar to those observed in the sod7-1D single mutant, indicating that At3g11580 is the SOD7 gene.

SOD7 encodes a NGATHA-like protein (NGAL2) containing a B3 DNA binding domain and a transcriptional repression motif (Figure 3C) (Alvarez et al., 2009; Ikeda and Ohme-Takagi, 2009; Trigueros et al., 2009). SOD7 belongs to the RAV (RELATED TO ABI3 AND VP1) gene family that consists of 13 members in Arabidopsis (Supplemental Figure 3 and Supplemental Data Set 1) (Swaminathan et al., 2008). Several members of the RAV family contain the putative transcriptional repression motifs, including NGA1, NGA2, NGA3, NGA4, NGAL1, NGAL2/SOD7, and NGAL3 (Supplemental Figure 3) (Ikeda and Ohme-Takagi, 2009). The transcriptional repression motifs in NGA1, NGAL1, and NGAL2/SOD7 have been shown to possess repressive activity (Ikeda and Ohme-Takagi, 2009), indicating that they are transcriptional repressors. SOD7 exhibits the highest similarity to Arabidopsis NGAL3/DPA4 (Supplemental Figure 3), which is an established regulator of leaf serrations (Engelhorn et al., 2012), but no previously identified function in seed size control.

Expression Pattern and Subcellular Localization of SOD7

To monitor SOD7 expression pattern during development, the ProSOD7:GUS and ProSOD7:SOD7-GFP vectors were constructed and transformed into wild-type plants, respectively. The tissue-specific expression patterns of SOD7 were examined using a histochemical assay for GUS activity. In seedlings, relatively higher GUS activity was detected in younger leaves than in older leaves (Figures 4A to 4C). In flowers, GUS activity was observed in sepals, petals, stamens, and carpels (Figures 4D to 4K). GUS activity was stronger in younger floral organs than in older ones (Figures 4D to 4K). Expression of SOD7 was also detected in the nucellus, inner integuments, and outer integuments of developing ovolves (Figures 4L to 4Q). Thus, these results are consistent with the function of SOD7 in cell proliferation.

As SOD7 encodes a B3 domain transcriptional repressor, we speculated that SOD7 is localized in the nucleus. To determine subcellular localization of SOD7, we expressed a GFP-SOD7 fusion protein under the control of the 35S promoter in wild-type plants. Transgenic lines overexpressing GFP-SOD7 formed smaller seeds than the wild type (Figure 3D), indicating that the GFP-SOD7 fusion protein is functional. As shown in Figures 4P to 4Q and Supplemental Figures 4D to 4L, GFP fluorescence in Pro35S:GFP-SOD7 transgenic plants was observed exclusively in nuclei. We further transformed sod7-2 dpa4-3 plants with ProSOD7:SOD7-GFP. Transgenic plants produced smaller seeds and petals than sod7-2 dpa4-3, indicating that the SOD7-GFP protein is functional (Supplemental Figures 4A and 4B). Interestingly, transgenic lines had significant increases in SOD7 mRNA compared with wild-type plants (Supplemental Figure 4C). It is possible that the integration regions of transgenes in the genome affect the promoter activity of ProSOD7:SOD7-GFP because these transgenic lines contain one copy of the transgene (Supplemental Figure 4). We further observed GFP fluorescence in ProSOD7:SOD7-GFP transgenic plants. As shown in Figures 4P to 4S and Supplemental Figures 4D to 4L, GFP signal was only detected in nuclei. Thus, these results show that SOD7 is a nuclear-localized protein.

Figure 2. Seed and Organ Size in the sod7-1D Mutant.

(A) and (B) Seeds of Col-0 (A) and sod7-1D (B).
(C) and (D) Mature embryos of Col-0 (C) and sod7-1D (D).
(E) and (F) Ten-day-old seedlings of Col-0 (E) and sod7-1D (F).
(G) Seed area of Col-0 and sod7-1D (n = 120).
(H) Seed weight of Col-0 and sod7-1D. The weights of five sample batches were measured for each seed lot (n = 5).
(I) Cotyledon area of 10-d-old Col-0 and sod7-1D seedlings (n = 20).
Values in (G) to (I) are given as mean ± se relative to the respective wild-type values, set at 100%. **P < 0.01 compared with the wild type (Student’s t test). Bars = 0.5 mm in (A) and (B), 0.2 mm in (C) and (D), and 1 mm in (E) and (F).
SOD7/NGAL2 Acts Redundantly with DPA4/NGAL3 to Regulate Seed Size

To further investigate the function of SOD7 in seed size control, we isolated dSpm transposon insertional loss-of-function mutants for SOD7 and DPA4/NGAL3, the most closely related family member. sod7-2 (SM_3.34191) was identified with a dSpm transposon insertion in the first exon of the SOD7 gene (Figure 5A). dpa4-3 (SM_3.36641) had a dSpm transposon insertion in the first exon of DPA4/NGAL3 (Figure 5B). The dSpm transposon insertion sites were confirmed by PCR and sequencing PCR products (Supplemental Figures 5A and 5B). sod7-2 and dpa4-3 mutants had no detectable full-length transcripts of SOD7 and DPA4 (Supplemental Figures 5C and 5D), respectively.

Seeds from sod7-2 and dpa4-3 mutants were slightly larger and heavier than seeds from wild-type plants (Figures 5C, 5G, and 5H). The cotyledon area of sod7-2 and dpa4-3 mutants was increased compared with that of the wild type (Figure 5I). Considering that SOD7 shares the highest similarity with DPA4, we speculated that SOD7 may act redundantly with DPA4 to influence seed size. To test this, we generated the sod7-2 dpa4-3 double mutant. As shown in Figures 5C, 5D, 5G, and 5H, the seed size and weight phenotypes of the sod7-2 mutant were synergistically enhanced by the disruption of DPA4, indicating that SOD7 functions redundantly with DPA4 to regulate seed size. We further measured the cotyledon area of 10-d-old seedlings. A synergistic enhancement of cotyledon size of sod7-2 by the dpa4-3 mutation was also observed (Figure 5I). In addition, the sod7-2 dpa4-3 double mutant formed larger leaves and flowers than their parental lines (Figures 5E and 5F; Supplemental Figure 6). The flowering time of the sod7-2 dpa4-3 double mutant was similar to that of the wild type (Supplemental Figure 7). Thus, these results indicate that SOD7 and DPA4 act redundantly to control seed and organ growth.

SOD7 and DPA4 Act Maternally to Regulate Seed Size

As the size of a seed is determined by the zygotic and/or maternal tissues (Garcia et al., 2005; Xia et al., 2013; Du et al., 2014), we asked whether SOD7 and DPA4 function maternally or zygotically. We therefore performed reciprocal cross experiments between the wild type and sod7-2 dpa4-3. The effect of sod7-2 dpa4-3 on seed size was observed only when sod7-2 dpa4-3 was used as maternal plants (Figure 6A). The size of seeds from sod7-2 dpa4-3 plants pollinated with wild-type pollen was similar to that from self-pollinated sod7-2 dpa4-3 plants (Figure 6A). By contrast, the size of seeds from wild-type plants pollinated with sod7-2 dpa4-3 mutant pollen was similar to that from the self-pollinated wild-type plants (Figure 6A). These results indicate that sod7-2 dpa4-3 acts maternally to influence seed size. We further investigated the size of Col-0/Col-0 F2, Col-0/sod7-2 dpa4-3 F2, sod7-2 dpa4-3/Col-0 F2, and sod7-2 dpa4-3/sod7-2 dpa4-3 F2 seeds. As shown in Figure 6B, sod7-2 dpa4-3/ sod7-2 dpa4-3 F2 seeds were larger than wild-type seeds, while the size of Col-0/sod7-2 dpa4-3 F2 and sod7-2 dpa4-3/Col-0 F2 seeds was similar to that of wild-type seeds. Thus, these results...
indicate that the embryo and endosperm genotypes for \textit{SOD7} and \textit{DPA4} do not determine seed size, and \textit{SOD7} and \textit{DPA4} are required in the sporophytic tissue of the mother plant to control seed growth.

\textbf{SOD7 and DPA4 Regulate Cell Proliferation in the Maternal Integuments}

The reciprocal crosses showed that \textit{SOD7} and \textit{DPA4} function maternally to influence seed size. The integuments surrounding the ovule are maternal tissues, which could set the growth potential of the seed coat after fertilization. Consistent with this idea, several studies showed that the integument size influences the final size of seeds in Arabidopsis (Garcia et al., 2005; Schruff et al., 2006; Adamski et al., 2009; Xia et al., 2013; Du et al., 2014). We therefore asked whether \textit{SOD7} acts through the maternal integuments to determine seed size. To test this, we characterized mature ovules of the wild type and \textit{sod7-2 dpa4-3}. As shown in Figures 6C and 6D, the \textit{sod7-2 dpa4-3} ovules were obviously larger than wild-type ovules. The outer integument length of \textit{sod7-2 dpa4-3} ovules was significantly increased compared with that of wild-type ovules (Figure 6E).

As the size of the integument is determined by cell proliferation and cell expansion, we examined the number and size of outer integument cells in wild-type and \textit{sod7-2 dpa4-3} ovules. As shown in Figure 6F, the number of outer integument cells in \textit{sod7-2 dpa4-3} ovules was increased compared with that in wild-type ovules. By contrast, the length of outer integument cells in \textit{sod7-2 dpa4-3}
ovules was similar to that in wild-type ovules (Figure 6G). These results showed that SOD7 is required for cell proliferation in the maternal integuments of ovules. After fertilization, cells in the integument mainly undergo expansion but still divide. We further examined the number and size of outer integument cells in wild-type and sod7-2 dpa4-3 seeds at 6 and 8 d after pollination (DAP). In wild-type seeds, the number of outer integument cells at 6 DAP was comparable with that at 8 DAP (Figure 6F), indicating that cells in the outer integuments of wild-type seeds completely stop dividing by 6 DAP. Similarly, in the outer integuments of sod7-2 dpa4-3 seeds also cease division by 6 DAP. The number of outer integument cells in sod7-2 dpa4-3 seeds was significantly increased compared with that in wild-type seeds (Figure 6F). By contrast, the length of outer integument cells in sod7-2 dpa4-3 seeds was not increased in comparison to that in wild-type seeds (Figure 6G). We further counted cells in the outer integuments of sod7-1D ovules and developing seeds. As shown in Supplemental Figure 8, the outer integuments of sod7-1D contained fewer cells than those of the wild type. Thus, SOD7 and DPA4 influence cell proliferation in the maternal integuments of ovules and developing seeds.

**SOD7 and DPA4 Act Maternally to Affect Embryo and Endosperm Growth**

The reciprocal experiments showed that the embryo and endosperm genotypes for SOD7 and DPA4 do not determine seed

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**Figure 5.** SOD7 Acts Redundantly with DPA4 to Regulate Seed Size.

(A) SOD7 gene structure. The start codon (ATG) and the stop codon (TGA) are shown. Closed boxes indicate the coding sequence, and the lines between boxes indicate introns. The dSpm transposon insertion site (sod7-2) in SOD7 is indicated.

(B) DPA4/NGAL3 gene structure. The start codon (ATG) and the stop codon (TGA) are shown. Closed boxes indicate the coding sequence, and the line between boxes indicates intron. The dSpm transposon insertion site (dpa4-3) in NGAL3 is indicated.

(C) to (F) Seeds (C), mature embryos (D), 25-d-old plants (E), and flowers (F) of Col-0, sod7-2, dpa4-3, and sod7-2 dpa4-3 (from left to right).

(G) and (H) Seed area (G) and seed weight (H) of Col-0, sod7-2, dpa4-3, and sod7-2 dpa4-3. One hundred and twenty seeds were used to measure seed area (n = 120). The weights of five sample batches were measured for each seed lot (n = 5).

(I) Cotyledon area of Col-0, sod7-2, dpa4-3, and sod7-2 dpa4-3 seedlings (n = 20).

Values in (G) to (I) are given as mean ± se relative to the respective wild-type values, set at 100%. *P < 0.01 compared with the wild type (Col-0) (Student’s t test). Bars = 0.5 mm in (C), 0.2 mm in (D), 5 cm in (E), and 1 mm in (F).

**Figure 6.** SOD7 and DPA4 Act Maternally to Determine Seed Size.

(A) Area of Col-0 × Col-0 (C/C) F1, Col-0 × sod7-2 dpa4-3 (C/d) F1, sod7-2 dpa4-3 × Col-0 (d/C) F1, and sod7-2 dpa4-3 × sod7-2 dpa4-3 (d/d) F1 seeds. Values are given as mean ± se relative to the respective wild-type values, set at 100% (n = 120).

(B) Area of Col-0 × Col-0 (C/C) F2, Col-0 × sod7-2 dpa4-3 (C/d) F2, sod7-2 dpa4-3 × Col-0 (d/C) F2, and sod7-2 dpa4-3 × sod7-2 dpa4-3 (d/d) F2 seeds. Values are given as mean ± se relative to the respective wild-type values, set at 100% (n = 120).

(C) and (D) Mature ovules of Col-0 (C) and sod7-2 dpa4-3 (D) (n = 60).

(E) Outer integument length of mature Col-0 and sod7-2 dpa4-3 ovules. Values are given as mean ± se (n = 60).

(F) The number of cells in the outer integuments of Col-0 and sod7-2 dpa4-3 at 0, 6, and 8 DAP. Values are given as mean ± se (n = 30).

(G) The length of cells in the outer integuments of Col-0 and sod7-2 dpa4-3 at 0, 6, and 8 DAP. Values are given as mean ± se (n = 100).

*P < 0.01 compared with the wild type (Col-0) (Student’s t test). Bars = 50 μm in (C) and (D).
size, and SOD7 and DPA4 are required in the sporophytic tissue of the mother plant to regulate seed growth (Figure 6). The sod7-2 dpa4-3 had larger seed coats than the wild type. The maternal integument or seed coat has been known to act as a physical constraint on embryo and endosperm growth and maternally affect endosperm and embryo development (Jofuku et al., 2005; Ohto et al., 2005, 2009; Adamski et al., 2009; Fang et al., 2012; Xia et al., 2013; Du et al., 2014). Consistent with this, mature sod7-2 dpa4-3 embryos were significantly larger than wild-type embryos (Figure 5D). The cotyledon area of mature sod7-2 dpa4-3 embryos was ~163% that of wild-type embryos (Supplemental Figure 9A). Embryo size is determined by both cell number and cell size. We measured palisade cells in wild-type and sod7-2 dpa4-3 cotyledons to determine which parameter is affected. As shown in Supplemental Figures 9B and 9C, cells in sod7-2 dpa4-3 cotyledons were slightly larger than those in wild-type cotyledons. The number of cells in sod7-2 dpa4-3 cotyledons was significantly higher than that in wild-type cotyledons. Thus, these results suggest that SOD7 acts maternally to influence embryo cell proliferation and cell expansion.

We then asked whether sod7-2 dpa4-3 integuments could influence embryo development. To test this, we manually pollinated wild-type and sod7-2 dpa4-3 plants with their own pollen grains and examined developing embryos at specific times after pollination. In the siliques of wild-type plants, most embryos reached the globular stage at 2 DAP, the heart stage at 4 DAP, the torpedo stage at 6 DAP, and the stage of the fully filled seed cavity at 11 DAP (Figure 7A). Morphological development of sod7-2 dpa4-3 embryos was delayed, compared with that of wild-type embryos (Figure 7A). We further investigated whether sod7-2 dpa4-3 integuments could affect endosperm development. As shown in Figure 7B, the endosperm cellularization in sod7-2 dpa4-3 seeds was also delayed, compared with that in wild-type seeds. Taken together, these results indicate that SOD7 acts maternally to influence embryo and endosperm development because SOD7 is solely required in the sporophytic tissue of the mother plant to control seed growth (Figure 6). It is possible that sod7-2 dpa4-3 forms a large seed cavity, which provides more space for embryo and endosperm growth and development. This phenomenon of embryo and endosperm development has also been observed in other Arabidopsis large seed mutants that act maternally to regulate seed size (Schuff et al., 2006; Ohto et al., 2009; Fang et al., 2012).

**SOD7 and DPA4 Act in a Common Pathway with KLU to Regulate Seed Size, but Do So Independently of DA1**

The Arabidopsis klu mutants formed small seeds due to the decreased cell proliferation in the integuments, while plants overexpressing KLU/CYP78A5 produced large seeds as a result of the increased cell proliferation in the integuments (Adamski et al., 2009), suggesting that SOD7 and KLU function antagonistically in a common pathway to regulate seed growth. To test for genetic interactions between SOD7 and KLU, we generated the klu-4 sod7-2 dpa4-3 triple mutant and measured the size of seeds from wild-type, klu-4, sod7-2 dpa4-3, and klu-4 sod7-2 dpa4-3 plants. As shown in Figures 8A and 8B, the average size and weight of klu-4 sod7-2 dpa4-3 seeds were similar to those of the klu-4 single mutant, indicating that klu-4 is epistatic to sod7-2 dpa4-3 with respect to seed size and weight. We further investigated the mature ovules from wild-type, klu-4, sod7-2 dpa4-3, and klu-4 sod7-2 dpa4-3 plants. The outer integument length of klu-4 sod7-2 dpa4-3 ovules was comparable with that of klu-4 ovules (Figure 8C). Similarly, the outer integument length of klu-4 sod7-2 dpa4-3 seeds was indistinguishable from that of klu-4 seeds at 8 DAP (Figure 8C). In addition, the size of klu-4 sod7-2 dpa4-3 petals was more similar to that of klu-4 petals than to that of sod7-2 dpa4-3 petals (Supplemental Figure 10A). Expression patterns of SOD7 and KLU in young petals partially overlapped (Supplemental Figure 10B). Thus, klu-4 is epistatic to sod7-2 dpa4-3 with respect to seed and organ size, indicating that SOD7 and DPA4 act in a common pathway with KLU to regulate seed and organ growth.

To further understand the cellular basis of epistatic interactions between SOD7 and KLU, we investigated the outer integument cell number of ovules and developing seeds from wild-type, klu-4, sod7-2 dpa4-3, and klu-4 sod7-2 dpa4-3 plants. The number of outer integument cells in klu-4 sod7-2 dpa4-3 ovules was similar to that in klu-4 ovules (Figure 8D). Similarly, the number of outer integument cells in klu-4 sod7-2 dpa4-3 seeds was comparable with...
that in klu-4 seeds (Figure 8D). These results indicate that klu-4 is epistatic to sod7-2 dpa4-3 with respect to the number of outer integument cells. We also observed that cells in the outer integuments of klu-4 and klu-4 sod7-2 dpa4-3 seeds were slightly longer than those in wild-type seeds (Supplemental Figure 11), suggesting the existence of a possible compensation mechanism between cell proliferation and cell expansion. Together, these findings show that SOD7 and DPA4 function antagonistically in a common pathway with KLU to regulate cell proliferation in the maternal integuments.

Considering that sod7-1D was identified as a suppressor of da1-1 in seed size, we further asked whether SOD7 and DA1 could act in the same genetic pathway. To test this, we measured the size of wild-type, da1-1, sod7-1D, and sod7-1D da1-1 seeds. The genetic interaction between sod7-1D and da1-1 was essentially additive for seed size, compared with that of sod7-1D and da1-1 single mutants (Supplemental Figure 12A), indicating that SOD7 might function independently of DA1 to regulate seed size. We further crossed sod7-2 dpa4-3 with da1-1 and generated the sod7-2 dpa4-3 da1-1 triple mutant and measured its seed size. The genetic interaction between sod7-2 dpa4-3 and da1-1 was also additive for seed size, compared with their parental lines (Supplemental Figure 12B), further supporting the notion that SOD7 and DPA4 regulate seed growth separately from DA1.

**DISCUSSION**

Seed size is crucial for plant fitness and agricultural purposes. Several factors that act maternally to influence seed size have been described in Arabidopsis, such as TTG2, AP2, ARF2, KLU, DA1, DA2, and SOD2/UBP15. DA1 acts synergistically with E3 ubiquitin ligases DA2 and EOD1 to restrict seed and organ growth (Du et al., 2014). DA1 interacts with SOD2 and modulates the stability of SOD2 to regulate seed and organ size (Du et al., 2014). In this study, we identify another SOD7, which acts maternally to regulate seed size by restricting cell proliferation in the integuments of ovules and developing seeds. SOD7 encodes a B3 domain transcriptional repressor NGAL2 and acts redundantly with its

**Figure 8.** *klu-4* Is Epistatic to *sod7-2 dpa4-3* with Respect to Seed Size.

(A) Seed area of Col-0, klu-4, sod7-2 dpa4-3, and klu-4 sod7-2 dpa4-3. Values are given as mean ± so relative to the respective wild-type values, set at 100% (n = 120).

(B) Seed weight of Col-0, klu-4, sod7-2 dpa4-3, and klu-4 sod7-2 dpa4-3. Values are given as mean ± so relative to the respective wild-type values, set at 100%. The weights of five sample batches were measured for each seed lot (n = 5).

(C) The outer integument length of Col-0, klu-4, sod7-2 dpa4-3, and klu-4 sod7-2 dpa4-3 at 0 and 8 DAP. Values are given as mean ± so relative to the respective wild-type values, set at 100%.

(D) The number of cells in the outer integuments of Col-0, klu-4, sod7-2 dpa4-3, and klu-4 sod7-2 dpa4-3 at 0 and 8 DAP. Values are given as mean ± so (n = 30).

**P < 0.01 compared with their respective controls (Student’s t test).**

**SOD7 Directly Binds to the Promoter of KLU and Represses the Expression of KLU**

Considering that SOD7 acts antagonistically in a common pathway with KLU to regulate seed size, we asked whether the transcription repressor SOD7 could repress the expression of KLU. We therefore investigated the expression of KLU in the chemically-inducible SOD7 (pER8-SOD7) transgenic plants. After the pER8-SOD7 transgenic plants were treated with the inducer (β-estradiol), the expression of SOD7 was induced at 4 and 8 h (Figure 9A). As expected, the expression of KLU was significantly repressed at 4 and 8 h (Figure 9A). We further detected the expression of KLU in wild-type and sod7-2 dpa4-3 ovules using in situ hybridization. As shown in Figure 9B, the expression of KLU in the inner integuments of sod7-2 dpa4-3 ovules was markedly increased, with that in the inner integuments of wild-type ovules. Thus, these results indicate that SOD7 represses the expression of KLU and also suggest that KLU is a direct target of SOD7. To determine whether SOD7 can directly bind to the promoter of KLU, we performed a chromatin immunoprecipitation (ChIP) assay with Pro35S:GFP and Pro35S:GFP-SOD7 transgenic plants. It has been reported that the CACCTG sequence is recognized by the B3 domain of RAV1, one member of the RAV family (Kagaya et al., 1999; Yamasaki et al., 2004). We therefore analyzed the 2-kb promoter sequence of KLU, but did not find an intact CACCTG sequence. However, we found a similar sequence (CAGTG) in the promoter region of KLU (Figure 9B), which could be the potential SOD7 binding site. To test this, we examined the enrichment of a KLU promoter fragment (PF1) containing the CAGTG sequence by ChIP analysis and found that PF1 was strongly enriched in the chromatin-immunoprecipitated DNA with anti-GFP antibody (Figures 9C and 9D). By contrast, we did not detect significant enrichment of an ACTIN7 promoter sequence and the KLU promoter fragment PF2, which does not contain the CAGTG sequence (Figures 9C and 9D). Therefore, SOD7 associates with the KLU promoter in vivo. We further expressed SOD7 as a MBP fusion protein (MBP-SOD7) and performed a DNA electrophoretic mobility shift assay (EMSA). As shown in Figures 9C and 9E, MBP-SOD7 bound to a biotin-labeled probe A containing the CAGTG sequence, and the binding was reduced by the addition of an unlabeled probe A. By contrast, MBP-SOD7 failed to bind to a probe A-m with mutations in the CAGTG sequence (Figures 9C and 9E). Taken together, these results show that SOD7 directly binds to the promoter of KLU and represses KLU expression.
SOD7 and DPA4 Act Maternally to Regulate Seed Size

The sod7-1D gain-of-function mutant was identified as a suppressor of the large seed phenotype of da1-1. However, genetic analyses showed that SOD7 functions independently of DA1 to regulate seed growth (Supplemental Figure 12). The sod7-1D single mutant produced small seeds and organs (Figure 2), while the simultaneous disruption of SOD7 and the closely related family member DPA4/NGAL3 resulted in large seeds and organs (Figure 5), indicating that SOD7 is a negative regulator of seed and organ size. Several previous studies suggest that there is a possible link between seed size and organ growth. For instance, arl2, da1-1, da2-1, and eod3-1D mutants produced large seeds and organs (Schruff et al., 2006; Li et al., 2008; Fang et al., 2012; Xia et al., 2013), whereas klu and sod2/ubp15 mutants formed small seeds and organs (Anastasiou et al., 2007; Adamski et al., 2009; Du et al., 2014). However, seed size is not invariably associated with organ size. For example, eod8/med25 mutants with large organs formed normal-sized seeds (Xu and Li, 2011), while ap2 mutants with normal-sized organs produced large seeds (Jofuku et al., 2005; Ohto et al., 2005). Thus, these findings suggest that seeds and organs not only possess distinct pathways but also share common mechanisms to regulate their size. In this study, our results show that SOD7 and DPA4 redundantly restrict both seed and organ growth in Arabidopsis.

Reciprocal cross experiments showed that SOD7 and DPA4 act maternally to restrict seed growth, and the endosperm and embryo genotypes for SOD7 and DPA4 do not determine seed size (Figure 6). The integuments surrounding the ovule are closest homolog NGAL3/DPA4 to regulate seed size. Genetic analyses indicate that SOD7 and DPA4 function in a common pathway with the maternal factor KLU to regulate seed growth, but do so independently of DA1. Further results reveal that SOD7 directly binds to the promoter region of KLU and represses KLU expression. Thus, our findings identify SOD7 and DPA4 as negative factors for seed size and define the genetic and molecular mechanisms of SOD7, DPA4, and KLU in seed size control.
maternal tissues and form the seed coat after fertilization. Arabidopsis arf2, ap2, da1-1, da2-1, and ecd3-1D mutants with large integuments formed large seeds (Jofuku et al., 2005; Ohno et al., 2005; Schruff et al., 2006; Li et al., 2008; Fang et al., 2012; Xia et al., 2013), while klu-4 and ubp15/sod2 mutants with small integuments produced small seeds (Adamski et al., 2009; Du et al., 2014), indicating that the maternal integuments are crucial for determining seed size in Arabidopsis. Consistent with this notion, mature sod7-2 dpa4-3 ovules were larger than wild-type ovules (Figures 6C and 6D). The outer integument length of sod7-2 dpa4-3 ovules and developing seeds was significantly increased compared with that of wild-type ovules and seeds (Figures 6E and 7C). Thus, the regulation of maternal integument size is an important mechanism for seed size control. The maternal integument acts as a physical constraint on embryo and endosperm growth and influences endosperm and embryo development (Jofuku et al., 2005; Ohno et al., 2005, 2009; Adamski et al., 2009; Fang et al., 2012; Xia et al., 2013; Du et al., 2014). Consistent with this, embryo and endosperm development in sod7-2 dpa4-3 was delayed, compared with that in the wild type (Figures 7A and 7B). Thus, it is plausible that sod7-2 dpa4-3 has a large seed cavity, which provides more space for embryo and endosperm growth and affects embryo and endosperm development. In support of this notion, large seed mutants that act maternally to influence seed size usually exhibited a prolonged period of seed growth and delayed embryo and endosperm development in Arabidopsis (Schruff et al., 2006; Ohno et al., 2009; Fang et al., 2012).

The size of the integument is determined by cell proliferation and cell expansion; these two processes are assumed to be coordinated. The number of outer integument cells in sod7-2 dpa4-3 ovules and seeds was significantly increased compared with that in wild-type ovules and seeds (Figure 6F), indicating that SOD7 and DPA4 regulate seed growth by limiting cell proliferation in the maternal integuments. Similarly, several mutants with the increased number of cells in the maternal integuments produced large seeds in Arabidopsis (Schruff et al., 2006; Li et al., 2008; Xia et al., 2013). By contrast, several other mutants with a decreased number of cells in the maternal integuments formed small seeds in Arabidopsis (Adamski et al., 2009; Du et al., 2014). Considering that cells in the integuments mainly undergo expansion after fertilization (Garcia et al., 2005), it is possible that the number of cells in the integuments determines the growth potential of the seed coat after fertilization.

**The Genetic and Molecular Mechanisms of SOD7 and KLU in Seed Size Regulation**

The sod7-1D mutant had small seeds and organs (Figure 2), as had been seen in klu mutants (Anastasiou et al., 2007; Adamski et al., 2009). KLU encodes a cytochrome P450 CYP78A5 that has been proposed to generate mobile plant growth substances (Anastasiou et al., 2007; Adamski et al., 2009). KLU regulates seed size by promoting cell proliferation in the maternal integments of ovules (Anastasiou et al., 2007; Adamski et al., 2009). By contrast, SOD7 acts maternally to control seed size by limiting cell proliferation in the integuments of ovules and developing seeds (Figure 6). These results suggest that SOD7 could function antagonistically in a common pathway with KLU to regulate seed size. In our growth conditions, klu-4 formed slightly smaller seeds than the wild type due to the decreased cell number and the slightly increased cell length in the integuments of developing seeds (Figures 8A and 8D; Supplemental Figure 11), suggesting a possible compensation mechanism between cell proliferation and cell expansion in klu-4 integuments. Importantly, our genetic analyses showed that klu-4 is epistatic to sod7-2 dpa4-3 with respect to seed and organ size (Figures 8A and 8B; Supplemental Figure 10A). klu-4 is also epistatic to sod7-2 dpa4-3 for the outer integument length (Figure 8C). Further results revealed that the number of cells in the outer integuments of klu-4 sod7-2 dpa4-3 ovules and developing seeds was similar to that of klu-4 ovules and developing seeds (Figure 8D). Thus, these genetic results demonstrate that SOD7 and DPA4 act in a common pathway with KLU to modulate seed size by regulating cell proliferation in the maternal integuments.

SOD7 encodes a B3 domain transcriptional repressor NGAL2 that is localized in nuclei of Arabidopsis cells (Figures 4P to 4S; Supplemental Figures 4D to 4I). Thus, it is possible that SOD7 directly binds to the promoter of KLU and represses KLU expression. SOD7 and KLU have overlapping expression patterns during ovule and seed development. For example, SOD7 and KLU expression was stronger in younger ovules than in older ones (Figures 4L to 4O) (Adamski et al., 2009). After fertilization, the expression of both SOD7 and KLU was hardly detected (Supplemental Figure 13). Importantly, the inducible expression of SOD7 resulted in a significantly reduction of KLU expression (Figure 9A). Our ChIP-quantitative PCR data showed that SOD7 associates with the promoter region of KLU in vivo (Figures 9B and 9C). EMSA experiments revealed that SOD7 directly binds to the CACTTG sequence in the promoter of KLU (Figures 9B and 9D). Thus, these results illustrate that SOD7 directly targets the promoter region of KLU and represses the expression of KLU, thereby determining seed size. Interestingly, the gain-of-function mutant sod7-1D had smaller seeds than klu-4, suggesting that overexpression of SOD7 might also repress the expression of other target genes. Taken together, these findings reveal the genetic and molecular mechanisms of SOD7, DPA4, and KLU in regulating Arabidopsis seed size.

For many plants, the seeds are the main product to be harvested, and an increase in seed size would be beneficial for growers. In this study, we identify SOD7 and DPA4 as negative regulators of seed size and demonstrate that SOD7 and DPA4 act in a common genetic pathway with KLU to regulate seed size. Our current knowledge of SOD7 functions suggests that SOD7 (and its homologs in other plant species) could be used to engineer large seed size in crops. Considering that crop plants have undergone selection for large seed size during domestication (Fan et al., 2006; Song et al., 2007; Gegas et al., 2010), it will be worthwhile establishing whether beneficial alleles of SOD7 homologs have already been utilized by plant breeders.

**METHODS**

**Plant Materials and Growth Conditions**

*Arabidopsis thaliana* Col-0 was used as the wild-type line. The da1-1, sod7-1D, sod7-2, and dpa4-3 were in the Col-0 background. sod7-1D was identified as a suppressor of da1-1 using a T-DNA activation-tagging method. sod7-2 (SM_3.34191) and dpa4-3 (SM_3.36641) were identified in SIGnAL (http://signal.salk.edu/cgi-bin/tdnaexpress) and obtained from the Arabidopsis stock center NASC collection. dSpm transposon
insertions were confirmed by PCR and sequencing using the primers described in Supplemental Table 1. Arabidopsis plants were grown under long-day conditions (16 h light/8 h dark) at 22°C.

**Activation-Tagging Screening**

The activation-tagging plasmid pJFAT260 was introduced into the *da1-1* mutant plants using *Agrobacterium tumefaciens* strain GV3101 (Fan et al., 2009; Fang et al., 2012), and T1 plants were selected using the herbicide Basta. Seeds produced from T1 plants were used to isolate modifiers of *da1-1*.

**Morphological and Cellular Analysis**

To measure seed size, we photographed dry seeds of the wild type and mutants under a Leica microscope (Leica S8APO) using Leica CCD (DFC420). The area of wild-type and mutant seeds was measured using ImageJ software. Average seed weight was determined by weighing mature dry seeds in batches of 100 using an electronic analytical balance (Mettler Toledo AL104). The weights of five sample batches were measured for each seed lot.

Fully expanded cotyledons, petals (stage 14), and leaves were scanned to produce digital images for area measurement. To measure cell number and cell size, petals, leaves, ovules, and seeds were placed in a drop of clearing solution (50 mL water, 80 g chloral hydrate [Sigma-Aldrich C8383], and 10 mL 100% glycerol [Sigma-Aldrich G6279]). Cleared samples were imaged under a Leica microscope (Leica DM2500) with differential interference contrast optics and photographed with a Spot Flex cooled CCD digital imaging system. Area measurement was made using ImageJ software.

**Cloning of SOD7**

The flanking sequences of the T-DNA insertion of the *sod7-1D* mutant were identified by TAIL-PCR according to a previously reported method (Liu et al., 1995). Briefly, TAIL-PCR uses three nested specific primers (OJF22, OJF23, and OJF24) within the T-DNA region of the pJFAT260 vector and a shorter arbitrary degenerate primer (AD1). Thus, the relative amplification efficiencies of specific and nonspecific products can be thermally controlled. TAIL-PCR products were sequenced using the primer OJF24. The specific primers OJF22, OJF23, and OJF24 and an arbitrary degenerate (AD1) primer are described in Supplemental Table 1.

** Constructs and Plant Transformation**

The 35S::GFP-SOD7, ProSOD7::SOD7::GFP, and ProSOD7::GUS constructs were made using a PCR-based Gateway system. The coding sequence (CDS) of SOD7 was amplified using the primers SOD7CDS-F and SOD7CDS-R (Supplemental Table 1). PCR products were cloned into the pCR8/TOPO TA cloning vector. The SOD7 CDS was then subcloned into the binary vector pMDC43 with the GFP gene to generate the transformation plasmid Pro35S::GFP-SOD7. The SOD7 genomic sequence containing a 2040-bp promoter sequence and 2104-bp genomic sequence (CDS) of SOD7 was amplified using the primers SOD7G-F and SOD7G-R (Supplemental Table 1). PCR products were cloned into the pCR8/TOPO TA cloning vector. The SOD7 genomic sequence was then subcloned into the binary vector pMDC107 with the GFP gene to generate the transformation plasmid pSOD7-SOD7-GFP. The 2262-bp SOD7 promoter sequence was amplified using the primers SOD7P-F and SOD7P-R (Supplemental Table 1). PCR products were cloned into the pCR8/TOPO TA cloning vector. The SOD7 promoter was then subcloned into the binary vector pGW30 with the GUS gene to generate the transformation plasmid pSOD7::GUS. The plasmids 35S::GFP-SOD7, pSOD7-SOD7-GFP, and pSOD7::GUS were introduced into Col-0 and *sod7*-2 dap4-3 plants using *Agrobacterium* GV3101, respectively, and transformants were selected on medium containing 30 µg/mL hygromycin.

The SOD7 cDNA was cloned into the Apal and SpeI sites of the binary vector pER8 to generate a chemically inducible construct pER8-SOD7. The specific primers for the pER8-SOD7 construct were SOD7ER-F and SOD7ER-R. The plasmid pER8-SOD7 was introduced into Col-0 plants using *Agrobacterium* GV3101, and transformants were selected on medium containing 30 µg/mL hygromycin.

**GUS Staining**

Samples (ProSOD7::GUS) were stained in a GUS staining solution [1 mM X-Gluc, 50 mM NaPO4 buffer, 0.4 mM each KFe(III)CN6KFe(III)CN6, and 0.1% (v/v) Triton X-100] and incubated at 37°C for 3 h. After GUS staining, chlorophyll was removed by washing with 70% ethanol.

**RT-PCR and Quantitative Real-Time RT-PCR**

Total RNA was extracted from Arabidopsis seedlings using an RNAprep pure Plant Kit (Tiangen). mRNA was reverse transcribed into cDNA using SuperScriptII reverse transcriptase (Invitrogen). cDNA samples were standardized on ACTIN2 transcript amount using the primers ACTIN2-F and ACTIN2-R (Supplemental Table 1). Quantitative real-time RT-PCR analysis was performed with a Lightcycler 480 machine (Roche) using the Lightcycler 480 SYBR Green I Master (Roche). ACTIN2 mRNA was used as an internal control, and relative amounts of mRNA were calculated using the comparative threshold cycle method. The primers used for RT-PCR and quantitative real-time RT-PCR are described in Supplemental Table 1.

**DNA EMSA**

The coding sequence of SOD7 was cloned into the Ndel and BamHl sites of the pMAL-C2 vector to generate the MBP-SOD7 construct. MBP-SOD7 fusion proteins were expressed in *Escherichia coli* BL21 (DE3) (Biomed) and purified on Amylose resin (New England Biolabs). The blutin-labeled and unlabeled probes were synthesized as forward and reverse strands. The forward and reverse strands were then incubated in a solution (50 nM Tris-HCl, 5 mM EDTA, and 250 nM NaCl) at 95°C for 10 min and renatured to double stranded probes at room temperature.
The gel shift assay was performed according to a method described previously (Smaczniak et al., 2012).

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**AUTHOR CONTRIBUTIONS**

Y.Z. and Y.L. designed the research. Y.Z. performed most of the experiments. L.D. and R.X. identified the SOD7 gene. R.C. performed the in situ hybridization experiment. J.H. and C.S. conducted the TAIL-PCR assay. Y.Z. and Y.L. analyzed the data. Y.L. wrote the article.

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Transcription Factors SOD7/NGAL2 and DPA4/NGAL3 Act Redundantly to Regulate Seed Size by Directly Repressing KLU Expression in Arabidopsis thaliana

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