Research Article

MYB56 Encoding a R2R3 MYB Transcription Factor Regulates Seed Size in *Arabidopsis thaliana*

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Abstract

Plant seed size is tightly regulated by the development of seed coat, embryo, and endosperm; however, currently, its underlying mechanism remains unclear. In this study, we revealed a regulatory role of an R2R3 MYB transcription factor MYB56 in controlling seed size specifically in *Arabidopsis thaliana* L. Loss-of-function or knock-down of *MYB56* yielded smaller seeds as compared with the wild type. Conversely, overexpression of *MYB56* produced larger seeds. Further observation using semi-thin sections showed that *myb56* developed smaller contracted endothelial cells and reduced cell number in the outer integument layer of the seed coat during the seed development; by contrast, *MYB56* overexpressing lines had expanded endothelial cells and increased cell number in the outer integument layer of the seed coat, suggesting the essential role of *MYB56* in regulating seed development. In addition, reciprocal cross-analysis showed that *MYB56* affected the seed development maternally. *MYB56* was shown to be dominantly expressed in developing seeds, consistently with its function in seed development. Moreover, quantitative reverse transcription polymerase chain reaction analysis revealed that *MYB56* regulates the expression of genes involved in cell wall metabolism such as cell division and expansion. Altogether, our results demonstrated that *MYB56* represents an unknown pathway for positively controlling the seed size.

Keywords: Cell wall; endothelium; MYB; seed coat; seed size.

Introduction

Plant seed size is a trait of considerable importance; deciphering the genetic determinants of seed size is of remarkable significance. The *Arabidopsis* seed is composed of three parts: the embryo, the endosperm, and the seed coat. The embryo is surrounded by the endosperm, which is enclosed within the maternal seed coat that is derived from the integument (Kunieda et al. 2008). The seed coat consists of five layers from the inner to the outer: inner integument 1 (ii1), inner integument 1 (ii1'), inner integument 2 (ii2), outer integument 1 (oi1), and outer integument 2 (oi2) (Beeckman et al. 2000). The former three layers constitute the inner integument, and the latter two layers form the outer integument; the developmental programs of the inner and outer integument are relatively independent (Dean et al. 2011). The innermost ii1 layer, so called the endothelium (Beeckman et al. 2000), contacts the endosperm cells of the embryo sac directly and its cytoplasm was stained densely with several histochemical stains (Beeckman et al. 2000; Ondzighi et al. 2008).

The seed development proceeds through two distinct phases during which the growth of the embryo, the endosperm, and the seed coat is tightly coordinated. Therefore, the coordinated growth of maternal sporophytic and zygotic tissues determines the size of a seed (Fang et al. 2012), which accompanies the proliferation and expansion of the endosperm and integument (Boisnard-Lorig et al. 2001). After the previous phase, the embryo grows by consuming most of the endosperm (Lopes and Larkins 1993). The seed size, however, is mainly defined during the first phase (Garcia et al. 2005).

Currently, it is known that there are three main factors affecting the seed size. The first is the parent-of-origin effect evidenced by reciprocal crosses between a diploid and a tetraploid yielding seeds of opposite size, and DNA methylation-associated gene silencing is thought to be involved in this process (Adams et al. 2000; Xiao et al. 2006). Second, zygotic tissues are essential for seed size control, for instance, three zygote expressed genes, *MINISEED3* (*MINI3*), *HAIKU1* (*IKU1*), and *HAIKU2* (*IKU2*), function in the same pathway to promote the development of endosperm via affecting embryo proliferation, and cell elongation of the maternally derived integument (Luo et al. 2005). *mini3* and *haiku* mutants show precocious cellularization of the syncytial endosperm and consequently reduced seed size (Garcia et al. 2003; Luo et al. 2005). Moreover, maternal sporophytic tissues contribute significantly to seed size. The *TRANSPARENT TESTA GLABRA2* (*TTG2*) gene positively regulates the cell expansion in the integument (Johnson et al. 2002; Garcia et al. 2005). *AUXIN RESPONSE FACTOR 2* (*ARF2*) and *DA1* repress the cell proliferation in the integument (Schruff et al. 2006; Li et al. 2008), while *CYP78A5/CYTOCHROME P450 KLUH* (*KLU*) and *CYP78A6/ENHANCER OF DA1-1* (*EOD3*) promotes the cell proliferation in the integument (Adamski et al. 2009; Fang et al. 2012). Nevertheless, despite the significance of seed size, the genetic and molecular mechanisms that control the seed size are poorly understood.

Seed development is tightly controlled by a network of regulatory molecules and pathways (Haughn and Chaudhury 2005; Dubreucq et al. 2010; Bui et al. 2011). In *Arabidopsis*, several transcription factors have been proved to be key regulators of zygotic embryo development, such as three B3 domain factors (*LEAFY COTYLEDON2-LEC2*, *FUSCA3*, and *ABA-INSENSITIVE3* (*ABI3*)), *LEAFY COTYLEDON1* (*LEC1*) (Harada 2001). Loss-of-function of *LEC* genes partially loses embryo identity, while loss-of-function of *FUSCA3* and *ABI3* yield defective and ABA-insensitive embryos, respectively (Meinke et al. 1994; Luerßen et al. 1998). In addition, *SHORT HYPOCOTYL UNDER BLUE1* (*SHB1*) is a positive regulator of seed development, which is required for the proper expression of two other transcription factors that affect endosperm development, *MINI3* and *IKU2*, a WRKY transcription factor gene and a leucine-rich repeat receptor kinase gene (Zhou et al. 2009). Furthermore, *APETALA2* (*AP2*) controls seed size through its effects on embryo, endosperm, and seed coat development. *AP2* restricts endosperm growth by limiting endosperm vacuole growth, controlling the timing of endosperm cellularization, and/or by restricting seed coat integument cell elongation (Jofuku et al. 2005; Ohto et al. 2005, 2009). The targets of these regulators and the entire regulatory network remain unclear in spite of the current findings (Monke et al. 2012; Wang and Perry 2013).

In *Arabidopsis*, the MYB transcription factors are classified into three subfamilies according to the number of adjacent repeats in the MYB domain (Stracke et al. 2001; Chen et al. 2006), participating in various biological processes including primary and secondary metabolism, plant development, cell fate and identity, and responses to biotic and abiotic stresses (Dubos et al. 2010). Some MYBs have been found to be involved in seed development (Dubos et al. 2010). *AtMYB5* regulates outer seed coat differentiation (Gonzalez et al. 2009; Li et al. 2009), *AtMYB123/TRANSPARENT TESTA2* (*TT2*) (subgroup 2) controls the biosynthesis of proanthocyanidins (PAs) in the seed coat (Lepiniec et al. 2006; Buer et al. 2010), and *AtMYB61* is required for the biosynthesis of mucilage in the seed coat (Penfield et al. 2001). There are total seven members in the 21th subgroup of R2R3 MYB, and functions of five of them have been characterized. *AtMYB52*, *AtMYB54*, and *AtMYB69* are regulators of the biosynthesis of lignin, xylan, and cellulose, participating in secondary cell wall thickening (Stracke et al. 2001; Zhong et al. 2008). *AtMYB105/LATERAL ORGAN FUSION2* (*LOF2*) and *AtMYB117/LATERAL ORGAN FUSION1* (*LOF1*) both function redundantly in boundary formation, regulating lateral organ separation, axillary meristem formation, and ovule and fruit development (Lee et al. 2009; Gomez et al. 2011). The sixth member of this group, *AtMYB110*, is an embryo sac expressed gene with unidentified function (Johnston et al. 2007). In this study, we describe functional characterization of *MYB56*, which is mostly close to *AtMYB52* and *AtMYB54* (Dubos et al. 2010), and also designated as one of the QC-enriched transcription factors previously (Nawy et al. 2005; Lee et al. 2006), in positively regulating the seed size by coordinately controlling the expansion of endothelium layer and proliferation of the outer integument in the seed coat.

Results

MYB56 expresses dominantly in developing seeds

In silico analysis using GeneChip Operating Software (GCOS) observed that *MYB56* was highly expressed in developing siliques from seed stage 4 (heart stage) to seed stage 7 (early cotyledons stage; Figure S1A), and the expression level declined sharply thereafter during the later seed developmental stages with very low expression level in mature seeds. The high expression of *MYB56* in developing siliques was also verified by the analysis with Genevestigator (Figure S1B). Consistently, further in-depth analysis with electronic Fluorescent Pictograph (eFP) Browser revealed the expression of *MYB56* in developing seeds (Figure S1C). This result suggests that *MYB56* is likely involved in seed development during early stages.

To confirm the role of *MYB56* during plant development, quantitative reverse transcription polymerase chain reaction (qRT–PCR) was carried out using total RNA from various tissues with *MYB56* specific primers (**Figure 1**). The expression of
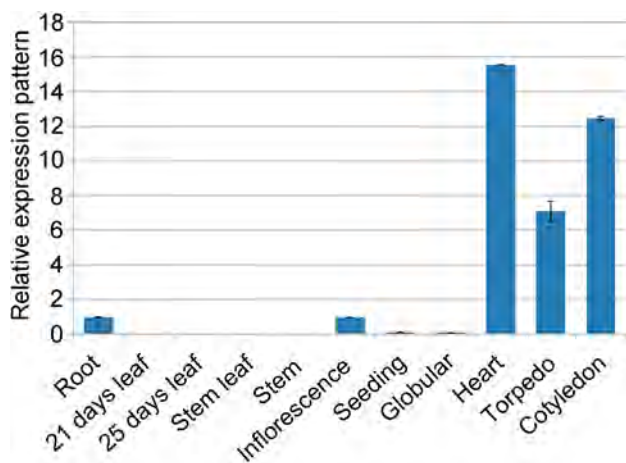
Figure 1. The spatial and temporal expression pattern of MYB56.

Quantitative reverse transcription polymerase chain reaction of *MYB56* in the root, 21 d leaf, 25 d leaf, stem leaf, stem, inflorescence, 10 d seedling, and seeds at globular, heart, torpedo, and cotyledonary stages.

MYB56 was hardly detected in the leaves (20 and 25 d, respectively) and stems, but moderately detected in the roots and inflorescences. In developing seed, the *MYB56* mRNA was weakly detectable at the globular stage, and highly detected at the heart stage and afterwards.

Knockout or knock-down of *MYB56* reduces seed size

To study the function of *MYB56*, we obtained two knockout lines of *MYB56* (At5g17800), SALK_062413C, and SALK_060289C, from the SALK Homozygote T-DNA Collection (http://signal.salk.edu/cgi-bin/homozygotes.cgi), Arabidopsis Biological Resource Center. Their genotypes were confirmed by PCR using corresponding primers, and both T-DNA insertions were found to be in exon 2 (Figure S2A). qRT–PCR analysis showed that the expression of *MYB56* was dramatically reduced in both lines (Figure S2B). Therefore, SALK_062413C and SALK_060289C were named *myb56-1* and *myb56-2*, respectively. Additionally, one SRDX motif-mediated silencing line, *MYB56-SRDX*, was generated by introducing the *MYB56-SRDX* construct containing the coding sequence of a 12-amino acid (LDLDLELRLGFA) motif fused to the end of *MYB56* into the wild-type (WT) *Col-0* (Hiratsu et al. 2003; Ito et al. 2007). The repression construct worked well as evidenced by qRT–PCR confirmation (Figure S2B).

During different vegetative and reproductive developmental stages, as compared with the WT, all mutants and transformation lines used in the current study did not display any detectable phenotypes (Figure S2C) except the seed size (**Figure 2**). The mature seeds of *myb56-1*, *myb56-2*, and *MYB56-SRDX* were significantly smaller than those of WT (**Figure 2A–D**), so were the mature embryos (**Figure 2F–I**). The difference in the seed size of those mutants was further confirmed by the measurement of the seed weight, which was decreased by 10.33%, 7.44%, and 19.01%, respectively, in *myb56-1*, *myb56-2*, and *MYB56-SRDX* lines compared with the WT (**Figure 2K**). Meanwhile, compared with the WT, the seed length of *myb56-1*, *myb56-2*, and *MYB56-SRDX* lines was decreased by 8.87%, 6.91%, and 8.14%, respectively (**Figure 2L**). On the other hand, the seed width of *myb56-1*, *myb56-2*, and *MYB56-SRDX* lines were close to that of the WT (**Figure 2M**). In addition, there were no significant differences in the seed number per silique and in the size of silique among these four lines compared with the control (data not shown).

Overexpression of *MYB56* increases seed size

To understand the role of *MYB56* in regulating seed size, we obtained transgenic plants ectopically expressing *MYB56*. The overexpression lines of *MYB56* displayed no detectable developmental changes during the vegetative and reproductive phases except in seed size. Compared with the WT, mature seeds of the overexpression line were larger (**Figure 2E, J**), and the seed weight, seed length, and seed width were increased by 9.92%, 11.33%, and 21.72%, respectively (**Figure 2K–M**). On the other hand, the seed number per silique in the *MYB56*-overexpressing line was reduced by 15.25% as compared with that of the WT. In addition, the size of silique in most of the overexpression lines did not change significantly (data not shown). Together with the results from knock and repression lines, we propose that *MYB56* is an important seed size regulator.

MYB56 affects the seed development before the heart stage

To elucidate the role of *MYB56* in seed development, we compared the seed development of the above lines with knockdown or overexpression of *MYB56* using the cleared seeds by Hoyer's solution. At the octan stage (**Figure 3A–E**), there were no observable changes between the four lines with altered expression of *MYB56* (*myb56-1*, *myb56-2*, *MYB56-SRDX*, and $2 \times 35S$:*MYB56*) and the WT. At the globular stage (**Figure 3F–J**), the shape and the area of the inner cavity of the mutant (*myb56-1*, *myb56-2*, and *MYB56-SRDX*) and $2 \times 35S$:*MYB56* lines emerged to be different from those of the WT. In WT, the shape of seed was a slender oval shape. In *myb56-1*, *myb56-2*, or the *MYB56-SRDX* lines, the length of the major axis of the seed was shorter, showing a round shape. In the $2 \times 35S$:*MYB56* line, the shape resembled the WT but having longer major and minor axes. At the heart stage (**Figure 3K–O**), the
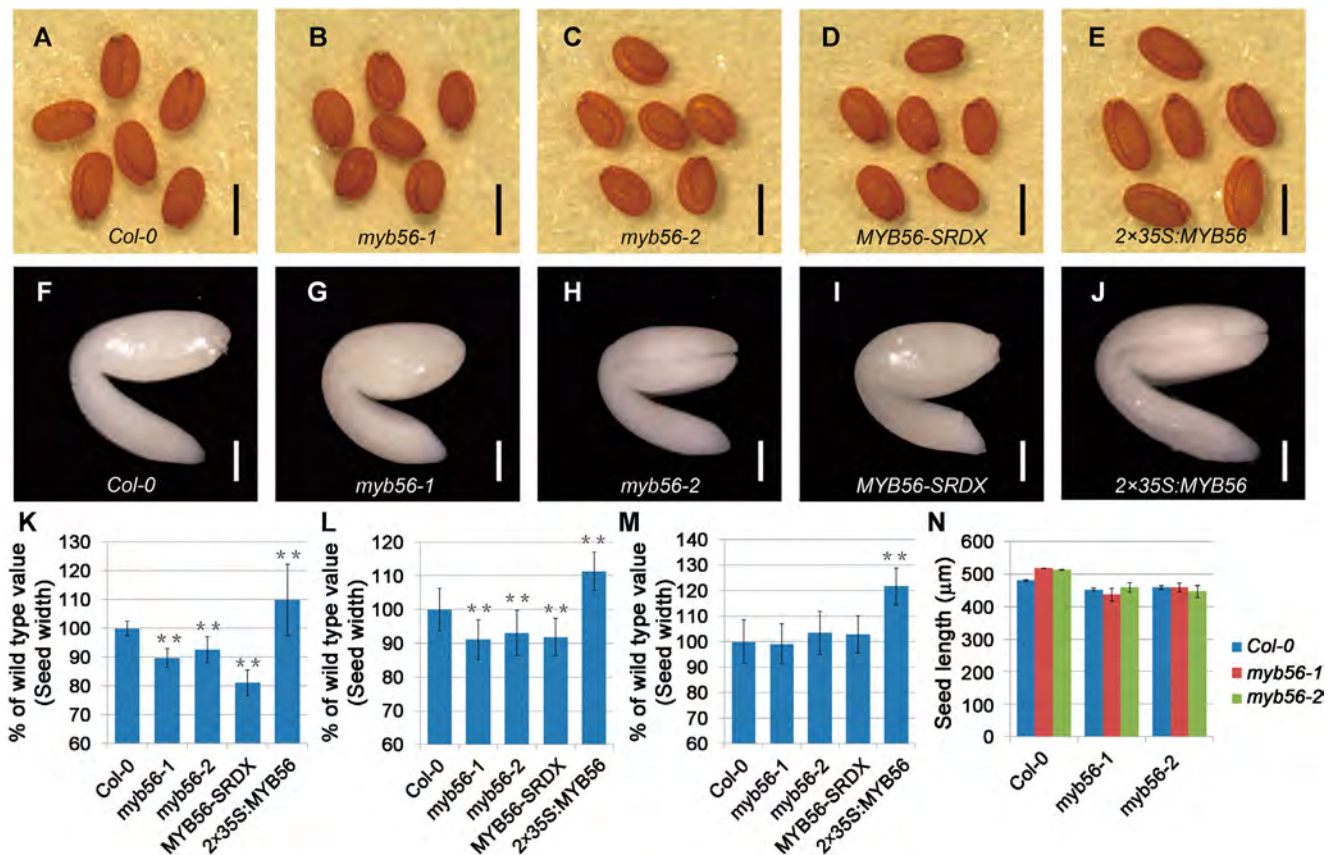
Figure 2. Seed size of the wild-type (WT), *myb56-1*, *myb56-2*, *MYB56-SRDX*, and *2 × 35S*:*MYB56*.

(A–E) The mature seeds of WT (A), *myb56-1* (B), *myb56-2* (C), *MYB56-SRDX* (D), and *2 × 35S*:*MYB56* (E). Scale bars = 500 μm.
(F–J) The mature embryos of WT (F), *myb56-1* (G), *myb56-2* (H), *MYB56-SRDX* (I), and *2 × 35S*:*MYB56* (J). Scale bars = 100 μm.
(K–M) % of WT value of seed weight (K), seed length (L), and seed width (M).
(N) F1 seed length from reciprocal crosses between WT, *myb56-1*, and *myb56-2*. The horizontal coordinate is maternal parent. The data indicates that the seed length is in accordance with the maternal plant.

differences became more obvious. At the torpedo and cotyledonary stages, the volume of all the seeds increased but the shape remained the same as that of the heart stage (**Figure 3P–Y**). We also measured the ratio of the major axis and minor axis or the length/width ratio at the heart and torpedo stages, and observed that the value of *myb56-1* was less than that of the WT, while that of *2 × 35S*:*MYB56* was comparable at both stages (Table S1). Those results indicated that *MYB56* regulates the seed size starting at the early seed developmental stages, and the difference in phenotype became apparent from the heart stage.

MYB56 affects the seed size maternally

To exam whether *MYB56* affects the seed size either maternally or paternally, reciprocal crosses were performed among WT, *myb56-1*, and *myb56-2*. Manually pollinating WT with either *myb56-1* or *myb56-2* produced F1 seeds with comparable seed length, so did manually pollinating either *myb56-1* or *myb56-2* with WT (**Figure 2N**). Therefore, *MYB56* controls seed development maternally through sporophytic genome, in other words, the seed integument from maternal plant.

MYB56 controls the endothelial cell expansion and outer integument layer cell proliferation coordinately

To understand the cellular effect of *MYB56* on seed development, we performed further morphological observation with longitudinal sections of seeds from WT, *myb56-1*, and *2 × 35S*: *MYB56* lines. At the heart stage, as compared with WT (**Figure 4A, D**), *myb56-1* seeds displayed the round-shaped endothelial cell instead of the oval-shaped WT endothelial cell. Furthermore, *myb56-1* endothelial cells had smaller cell diameter and circumference (**Figure 4B, E**). In contrast, *2 × 35S*:

Figure 3. Continued.

MYB56 seeds showed larger cell diameter and circumference compared with the WT (**Figure 4C, F**). From the torpedo to the cotyledeonary stage, as seeds developed, the circumference of endothelial cells reached their maximum, and *myb56-1* and *2 × 35S:MYB56* seeds still had differences in the longitudinal cell diameter, circumference, and volume compared with the WT (**Figure 3U–Y**). Consistently, statistical data confirmed that *myb56-1* developed smaller endothelial cells and *2 × 35S: MYB56* seeds had bigger endothelial cells by measuring the circumference along the inner side of endothelial cells in the maximum longitudinal parafin section of seeds at the cotyledeonary stage (**Figure 4H**). Similar data were also observed by testing the area of seed cavity (**Figure 4I**). Moreover, we counted the total endothelial cell number and observed that there was no significant difference in the total endothelial cell number in *myb56-1* or *2 × 35S:MYB56* compared with the WT (**Figure 4G**), suggesting that *MYB56* affects the endothelial cell size but not cell number.

To investigate whether *MYB56* affects the development of other cell layers within the seed integument, we counted the cell numbers of five layers of the seed integument from a series of longitudinal sections of seeds at early cotyledonary stage when the seed volume reaches the maximum. There were no significant differences in the cell numbers of the three inner integument layers of *myb56-1* and *2 × 35S:MYB56* lines compared with the WT (**Figure 5A**, Table S2). In contrast, the cell numbers of the two outer layers differed obviously in *myb56-1* or *2 × 35S:MYB56* lines compared with the WT, that is, *myb56-1* had smaller cell number of two outer layers and *2 × 35S: MYB56*, with an increased cell number of two outer layers (**Figure 5A**; Table S2). Additionally, we observed that compared with the WT, *myb56-1* had shorter cell length of the inner integument 1 layer at both the curve and side opposite to the embryo (Hughes 2009), and *2 × 35S:MYB56* displayed longer cell length of the inner integument 1 layer at both the curve and side (**Figure 5B**, Table S3). In the outer integument 1 and 2 layers, *myb56-1* had longer cell length, while *2 × 35S:MYB56* had similar cell length as compared with the WT at both sites (**Figure 5B**, Table S3). Furthermore, we cleared the seeds and counted the cell numbers of half the outer integument 2 layer with a differential interference contrast (DIC) microscope (Figure S3),

which showed that *myb56-1* had less cells while *2 × 35S:MYB56* had more cells of the half outer integument 2 layer. Thus, *MYB56* influences not only the endothelial cell expansion but also the outer integument layer cell proliferation, which may ultimately affect final seed size.

MYB56 alters the expression of cell wall-related genes

To decipher the regulatory role of *MYB56* in seed development, we compared the expression of genes relative to seed development in WT and *myb56-1* seeds at different developing stages by qRT–PCR. We observed that *MYB56* altered the expression of 12 genes (**Figure 6**) which included a putative germin-like protein (At5g39130), *PROLINE-RICH PROTEIN 2* (*PRP2*, At2g21140), *IRREGULAR XYLEM 15 LIKE* (*IRX15L*, At5g67210) (Brown et al. 2011), *BETA-GALACTOSIDASE 7* (*BGAL7*, At5g20710), *XYLOGLUCAN ENDOTRANSGLYCO-SYLASE 7* (*XTR7*, At4g14130), *MUCILAGE-MODIFIED2* (*MUM2*, At5g63800), two xyloglucan:xyloglucosyl transferase encoding genes *XYLOGLUCAN*:XYLOGLUCOSYL TRANS-FERASE 33 (*XTH33*, At1g10550) and *XYLOGLUCAN*:XYLO-GLUCOSYL TRANSFERASE 16 (*XTH16*, At3g23730), *SUBTILISIN-LIKE SERINE ENDOPEPTIDASE-LIKE PROTEIN* (*ATSBT5.2*, At1g20160), *PHYTOSULFOKINE 5 PRECURSOR* (*ATPSK5*, At5g65870), and several genes encoding pectines-terase and pectin methylesterase inhibitor family proteins. In addition, one gene encoding cytochrome P450 monooxyge-nase, *EUI-LIKE P450 A1* (*ELA1/CYP714A1*, At5g24910) and *ARL* (*ARGOS-LIKE*, At2g44080), both function in cell expansion (Hu et al. 2006; Zhang et al. 2011).

In addition, we also checked the expression of some known genes that modulate the development of the seed coat in *myb56-1*. Our results showed that the expression of *TRANSPARENT TESTA GLABRA2* (*TTG2*) (Johnson et al. 2002; Debeaujon et al. 2003; Qing and Aoyama 2012), *CYTOCHROME P450 KLUH* (*KLU*) (Adamski et al. 2009), *NACREGULATED SEED MORPHOLOGY1* and *2* (*NARS1* and *NARS2*) (Kunieda et al. 2008), δ-*VACUOLAR PROCESSING ENZYME* (δ*VPE*) (Nakaune et al. 2005), *GORDITA* (*GOA*) (Prasad et al. 2010), *TRANSPARENT TESTA16* (*TT16*) (Nesi et al. 2002), and *DA1* (Li et al. 2008), was not significantly altered in *myb56-1*. These
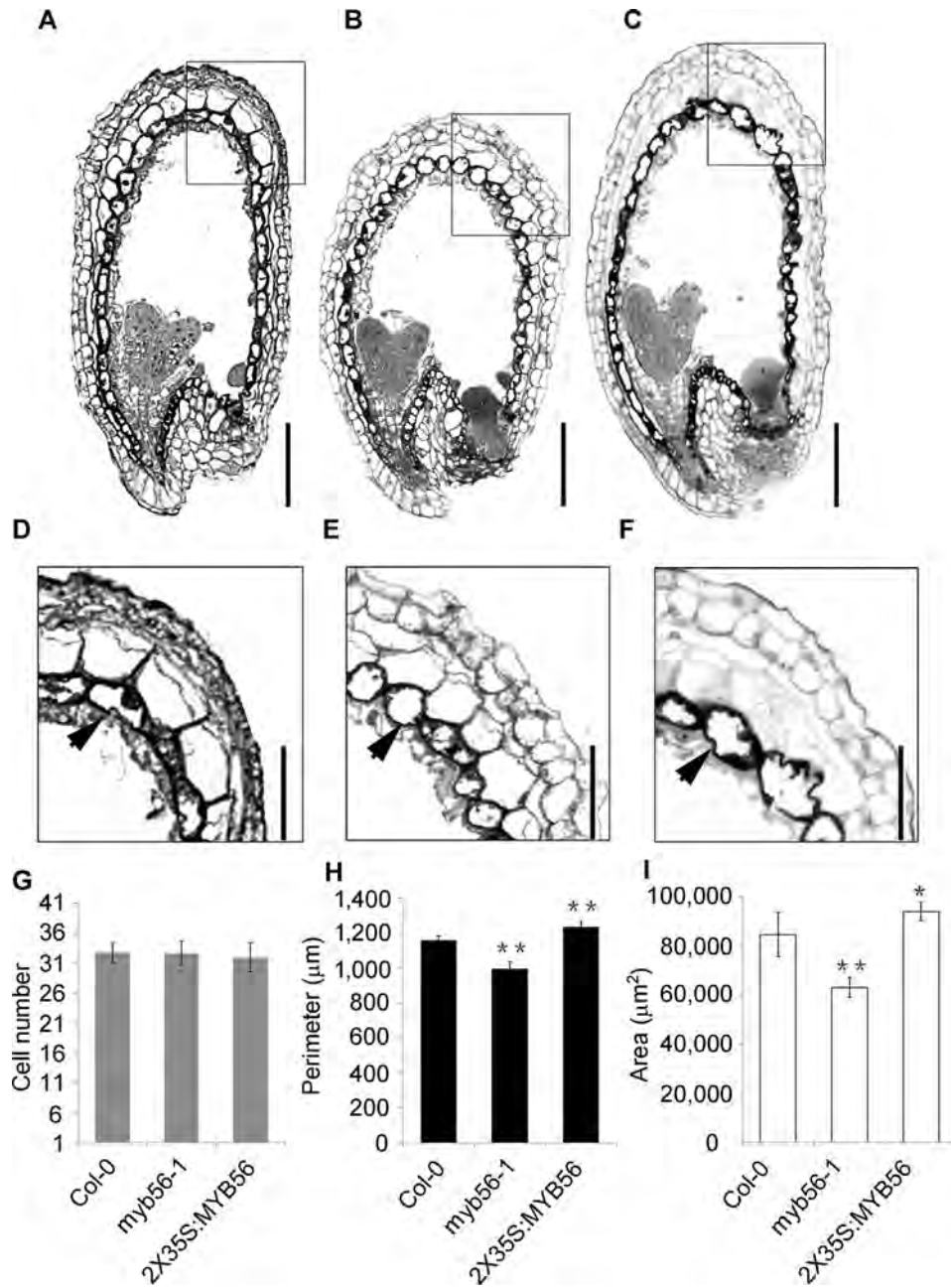
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Figure 3. The seed development of the wild-type (WT), *myb56-1*, *myb56-2*, *MYB56-SRDX*, and *2 × 35S:MYB56*, as evidenced in Hoyer's cleared seeds with differential interference contrast (DIC) optics.

(A–E) Seeds at octan stage. Hoyer's cleared seeds were imaged with DIC optics.
(F–J) Seeds at globular stage.
(K–O) Seeds at heart stage. The seed lengths of *myb56-1* **(L)**, *myb56-2* **(M)**, and *MYB56-SRDX* **(N)** are obviously less than those of WT **(K)**. On the contrary, the seed length of *2 × 35S:MYB56* **(O)** is longer.
(P–T) Seeds at torpedo stage.
(U–Y) Seeds at cotyledonary stage.
Scale bars = 100 μm.

Figure 4. Changes in the characteristics of endothelial (En) cells at different seed developmental stages.

(A–F) Endothelial cell (En) characteristics of wild-type (WT) **(A)**, *myb56-1* **(B)**, and *2 × 35S:MYB56* **(C)** at heart stage. **(D–F)** are the magnification of **(A–C)** within the rectangle, respectively. The arrowheads indicate the En. The magnifications are the same in all three lines. Scale bars = 100 μm in **(A–C)**; 50 μm in **(D–F)**.

(G–I) En cell characteristics of WT, *myb56-1*, and *2 × 35S:MYB56* at cotyledonary stage. Data is shown as mean ± *SD*. **P < 0.01 from *P < 0.05. **(G)** The number of the En cells except those between micropyle and the insertion point of funiculus. Within the continuous longitudinal sections, only the maximal ones were counted. **(H)** The maximal perimeter of endothelial cells facing seed cavity was measured. **(I)** The maximal area of longitudinal seed cavity was measured.
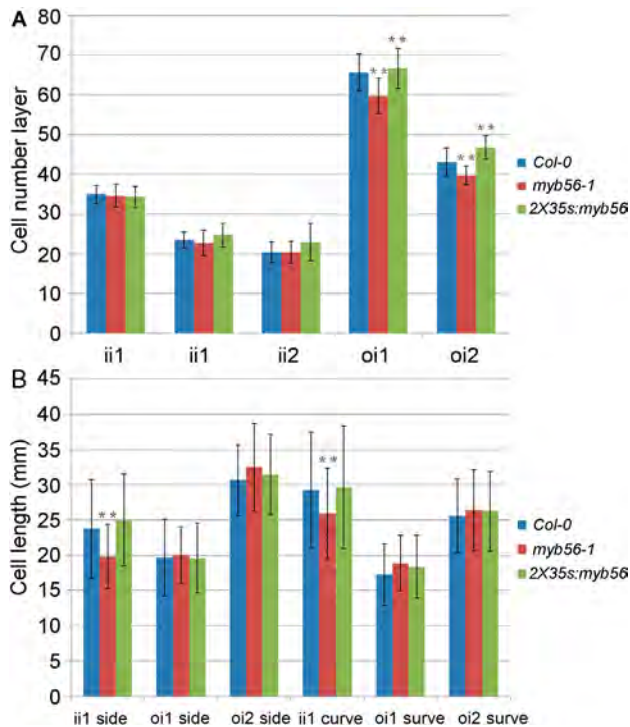
Figure 5. Changes in seed coat characteristics at early cotyledonary stage.

(A) Cell number per layer in the wild-type (WT), *myb56-1*, and *2 × 35S:MYB56* in ii1, ii1', ii2, oi1, and oi2 layers within the longitudinal sections. There is no obvious difference in the three inner integuments (ii1, ii1', and ii2) but there is in the two outer integuments (oi1 and oi2). **P < 0.01.
(B) Cell length of WT, *myb56-1*, and *2 × 35S:MYB56* at the curve and side opposite to the embryo.

results indicated that *MYB56* affects seed size in a distinctive way, which is likely independent of the reported pathways.

Discussion

During the life cycle of *Arabidopsis*, the seed links the start and the end of life. Therefore, the seed is of particular importance regarding to not only economic and nutritional aspects but also physiological, biochemical, and molecular aspects. Although previous studies contributed substantially to the understanding of the individual molecular components involved in the regulation of seed development and seed size in particular, much less information about the regulatory factors and their networks is available. In this study, we identified and characterized a R2R3 MYB gene *MYB56* that controls the seed size in *Arabidopsis*. Because the loss-of-function mutant lines (*myb56-1*, *myb56-2*,

and *MYB56-SRDX*) all generate smaller seeds, while over-expression line *2 × 35S:MYB56* yields larger seeds, as compared with WT, especially from the globular stage to the heart stage, we designated that *MYB56* is a positive regulator of seed size in *Arabidopsis*.

In *Arabidopsis*, several transcription factors have been reported to regulate seed size; most of them also affect the development of other organs rather than seeds. For example, *mnt*, a mutant allele of *AUXIN RESPONSE FACTOR 2* (*ARF2*), displays extra cell division not only in the seed integument but also in vegetative and floral development, resulting in a thick and twisted stem, and more and larger rosette leaves (Okushima et al. 2005; Schruff et al. 2006). Mutant of another B-sister MADS-box transcription factor, *GORDITA*, produce larger seeds and fruits as well (Prasad et al. 2010). Unlike the above-mentioned transcription factors, mutation of *MYB56* only affects the seed development (**Figures 1**, S2C), therefore, *MYB56* represents a specific modulator of seed development. The possible reason for it may be that there are likely different *MYB56*-mediated regulatory networks among different tissues. Thus, although the expression of *MYB56* could be detected in other tissues, there were no detectable phenotypes in these tissues due to the absence of specific regulatory partners or corresponding network; therefore, overexpression of *MYB56* resulted in only seed developmental phenotypes. The effect of the overexpression of *MYB56* on the seed number per silique and the size of silique might have resulted from the pleiotropic effect of the overexpression, because *MYB56*, in this case, was driven by 2 × 35S promoter. In addition, *MYB56* regulates not only the seed size but also the seed shape, as evidenced by the fact that altered expression of *MYB56* (knockout line) yielded similar round seeds as compared with WT. This phenomenon is in agreement with a previous hypothesis that the maternal integument sets an upper limit to final seed size via its physical constraint on embryo and endosperm growth (Fang et al. 2012).

Both maternal and paternal genomic factors affect seed development. The fact that F1 seed size of the reciprocal cross depends on the genotype of the maternal parent irrespective of the paternal parent indicated that *MYB56* regulate seed size maternally. It is known that the three components of the seed are of different origins, while both the embryo and endosperm are derived from zygotic tissues, the integument is from maternal tissue (Haughn and Chaudhury 2005). Consequently, it is reasonable to assume that *MYB56* controls seed size via affecting the development of the integument. This assumption was corroborated by the observation of the longitudinal sections (**Figure 4**). Thus, *MYB56* regulates the seed size via direct action on the development of the integument, which, in turn, influences the growth and development of the embryo through its physical constrain directly.

Another piece of evidence that *MYB56* regulates the development of the integument comes from its dominant
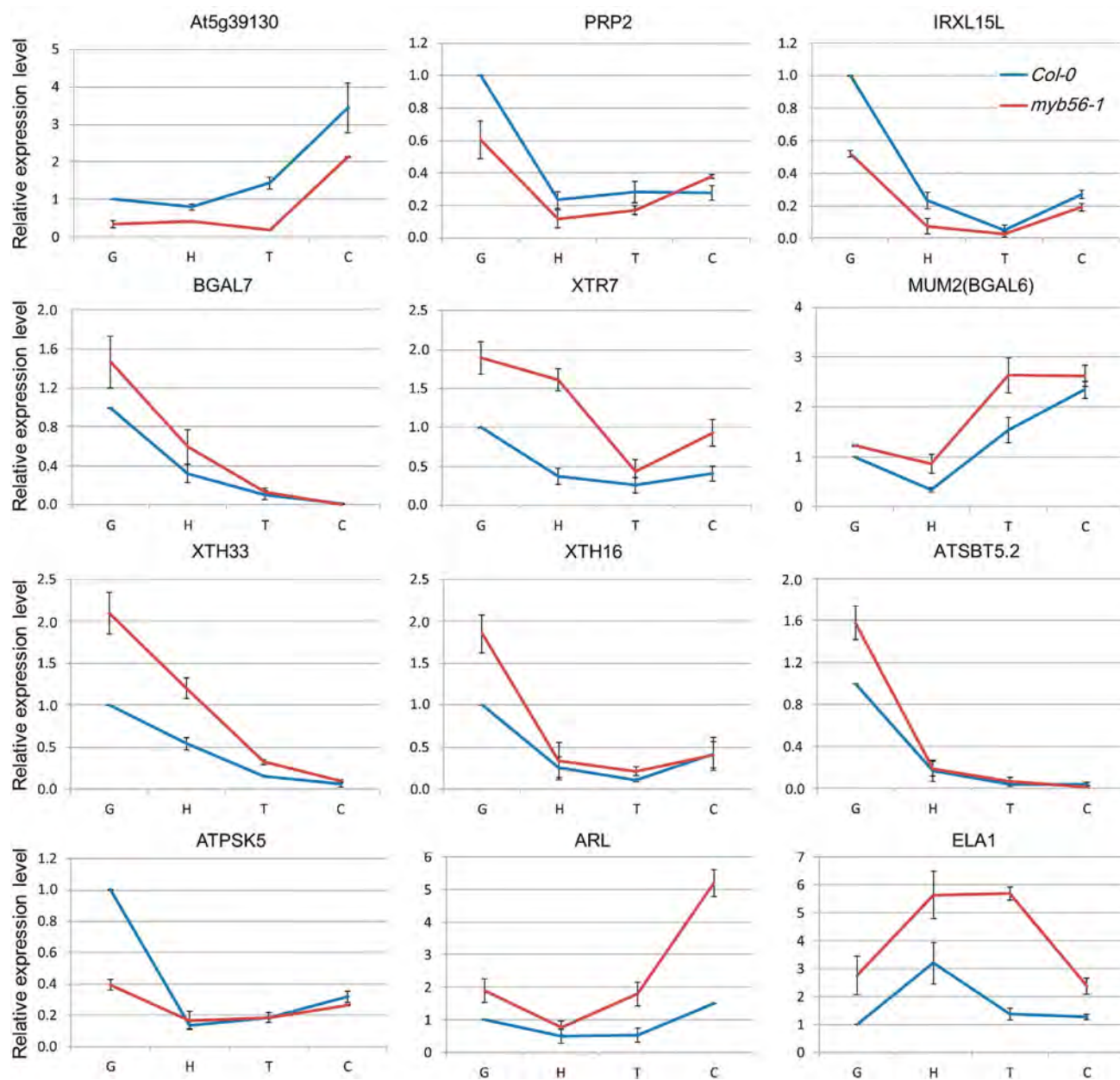
Figure 6. Quantitative reverse transcription polymerase chain reaction analysis of the expression changes of cell wall-related genes.

The expression pattern was detected in the seeds of wild-type (*Col-0*) and *myb56-1* at globular (G), heart (H), torpedo (T), and cotyledonary (C) stages.

expression at the very early stages of seed development as revealed by qRT–PCR (**Figure 1**). Our results indicated the consistency of *MYB56* between its expression and its function in the regulation of the development of the integuments. Because the cell proliferation in the outer integuments nearly stops at the heart stage (Western et al. 2000; Garcia et al. 2003;

Fang et al. 2012), the detectable but low expression level of *MYB56* at globular stage seems to be enough for the control of cell proliferation in the outer integuments, while the high expression level of *MYB56* at the heart and onwards stages obviously accounts for the cell expansion in the inner integument.

The organ size is not only controlled by cell size but also cell number (Mizukami 2001; Horiguchi et al. 2006; Dubreucq et al. 2010), which also has been validated in this study. The fact that altered expression of *MYB56* affects only the endothelial cell size but not the cell number, which was detected during the four developmental stages, indicates that *MYB56* modulates the cell expansion not cell proliferation in the endothelial layer (**Figure 4**). This result is in accord with the fact that cells in integuments mainly undergo expansion after fertilization (Western et al. 2000; Garcia et al. 2003; Fang et al. 2012). On the other hand, altered expression of *MYB56* affects only the cell number but not the cell size in the two layers of the outer integument (Figure S3), which indicates that *MYB56* controls cell proliferation in the outer integuments at the early stage of seed development. It seems that *MYB56* coordinately regulates the cell expansion and cell proliferation locally in the endothelial layer and the two layers of the outer integument, respectively, which fits with the known fact that the developmental mechanism of the inner and outer integument is relatively independent (Dean et al. 2011) and the fact that the expression signal of *MYB56* is present at these specific stages (**Figure 1**). However, the exact regulatory mechanism of *MYB56* on the coordinated development of both the inner and outer integuments remains unrevealed in this study.

In the attempt to find the regulatory network of *MYB56* mediating the seed size, qRT-PCR analysis of seeds at four different developmental stages were performed and we found that knockout of *MYB56* significantly affects the expression of a set of cell wall-related genes. Notably, many of them had higher expression levels at globular stage than those at later stages (**Figure 6**). According to the Cell Wall Navigator (CWN, http://bioweb.ucr.edu/Cellwall/), these differentially expressed cell wall genes were classified into several groups: xyloglucan xylosyltransferases and galactomannan galactosyltransferases (XXT), xyloglucan endotransglycosylases/hydrolases (XTH), beta-galactosidases (BGAL), pectin methyl esterases (PME), hydroxyproline-rich glycoproteins (HRGP), proline-rich proteins (PRP), glycine-rich proteins (GRP), and arabinogalactan proteins (AGP). XXT and XTH participate in hemicellulose synthesis and reassembly (Fry 2004; Nishitani and Vissenberg 2006). BGAL and PME are glycoside hydrolases and esterases in the progress of reassembly (Micheli 2001). HRGP, PRP, GRP, and AGP are all structural proteins (Cassab 1998; Gaspar et al. 2001). It is known that during cell expansion and proliferation, the cell wall-related genes take part in the whole progress (Cassab 1998; Boudart et al. 2007). Therefore, cell wall-related genes were likely the target of *MYB56*, functioning not only for cell expansion in the outer integuments at the late developmental stages but also for cell proliferation in the inner integuments at the early developmental stages. It has been reported that *AtMYB52*, *AtMYB54*, and *AtMYB69*, the closest members of *MYB56* in the 21st subgroup of R2R3 MYBs, are regulators of the biosynthesis of lignin, xylan, and cellulose, participating in secondary cell wall thickening (Stracke et al. 2001; Zhong et al. 2008). *MYB56* may have functions similar to them, with particular association with seed cell wall development. Before this conclusion can be drawn, further biochemical, metabolic, and genetic analyses are required. Furthermore, the network of *MYB56*, particularly the association of *MYB56* with other cell proliferation and cell elongation genes, merits elucidation.

Materials and Methods

Plant materials and growth conditions

The ecotype of *Arabidopsis* in this work was *Columbia-0* (*Col-0*). The plant was grown in the greenhouse at 22 °C with continual light.

Microscopic observation

The mature seeds were harvested and dried for 2 weeks at room temperature. For mature embryo, the dry seeds were imbibed for 16 h and then the seed coats were peeled. Both were digitally photographed with a Leica DFC290. Image J software (http://rsbweb.nih.gov/ij/) was used for measuring seed length and width. The seed weight was measured with analytical microbalance (Mettler Toledo MT5, Columbus, OH, USA).

Characterization of the mutant phenotype

The 25 d seedlings were photographed with a Canon 450D digital camera. For endothelial cell characteristics, the siliques were fixed with FAA solution (ethanol : acetic acid : 37% formaldehyde : water = 63:5:5:27). After dehydration through ethanol series, they were infiltrated and embedded in Paraplast Plus (Sigma-Aldrich, St Louis, MO, USA), cut into 7 μm sections with a rotary microtome (Leica RM2235, Nussloch, Germany), and stained with 0.05% toluidine blue. The photographs were taken with a Nikon eclipse 80i and Nikon Ds-Ri1 digital camera. The classification of seed developmental stage was based on a previous report (Lindsey and Topping 1993).

For longitudinal sections, the siliques were fixed with FAA, dehydrated, and embedded in Spurr's resin (Sigma-Aldrich). The sections were 2 μm using a rotary microtome (Leica RM2265, Nussloch, Germany). The photographs were taken with a Nikon eclipse 80i and Nikon Ds-Ri1 digital camera.

For DIC observations, the immature seeds were dissected from siliques, cleared with Hoyer's solution (chloral hydrate : glycerol: water = 8:1:3, w/v/v) overnight at 4 °C. The Leica DM2500 and Nicon Ds-Ri1 digital cameras were used to take photographs.

Construct building and the transformation of repression and overexpression lines for functional analysis

For the repression line, a 3 854 bp gDNA fragment of *MYB56* with a 3′-SRDX domain was amplified from *Col-0* DNA with primers MYB56SRDX-1/2 (Table S4) and cloned into pMD18-T vector (Takara, Dalian, China). After digesting with *Eco*RI/*Bst*EII, the fragment was subcloned into pCAMBIA1301 (CAMBIA, Canberra, ACT, Australia) and a *MYB56–SRDX* construct produced. For the overexpression line, the full-length cDNA of *MYB56* was amplified with primers MYB56OE-F/R from *Col-0* inflorescence total cDNA (Table S4), cloned into pMD18-T (Takara), digested with *Hin*dIII/*Pst*I, subcloned into pHB vector, and resulted in $2 \times 35S$:*MYB56* construct.

All the constructs were introduced into *Agrobacterium tumefaciens* GV3101, and then transformed into *Col-0*.

qRT-PCR assay

The inflorescence, 21 d leaf, 25 d leaf, root, 10 d seedling, stem leaf, stem, and seeds at globular, heart, torpedo, and cotyledonary stages were collected and the RNA was extracted with TRIZOL (Sigma-Aldrich). The seed stages were decided as described (Lindsey and Topping 1993). One microgram of total RNA was reverse transcribed using an RT reagent Kit with gDNA Eraser (DRR047A; Takara). The RT-PCR primers are listed in Table S4. qRT-PCR was carried out with SsoFast EvaGreen supermix on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The procedure was 40 cycles of 94 °C for 10 s, 54 °C for 10 s, 72°C for 15 s.

Acknowledgements

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's website.

Figure S1. *In silico* analysis of the expression of *MYB56*.
GeneChip Operating Software expression signal **(A)**, developmental expression pattern **(B)**, and electronic Fluorescent Pictograph expression pattern **(C)**.

Figure S2. The alteration of the expression of *MYB56* and its effect on plant vegetative growth.
(A) Simplified diagrammatic display of the gene structure of *MYB56*. The black boxes and lines indicate the exons and introns, respectively. The triangles indicate the T-DNA insertion sites of two mutant lines, SALK_062413C and SALK_060289C, on the second exon of *MYB56*.
(B) Quantitative reverse transcription polymerase chain reaction confirmation of the altered expression level of *MYB56* in knockout (left), repression (middle), and overexpression (right) lines. 1301 and pHB are the control lines with empty vector for the repression and overexpression lines, respectively.
(C) The representative pictures of 25 d seedlings of wild-type *Col-0*, *myb56-1*, *myb56-2*, *MYB56-SRDX*, and *2 × 35S:MYB56* lines, showing similar vegetative growth. Scale bars = 2 cm.

Figure S3. The cell number of half the outer integument two layers at cotyledonary stage.
The seed of *Col-0* **(A)**, *myb56-1* **(B)**, *mby56-2* **(C)**, *MYB56-SRDX* **(D)**, and *2 × 35S:MYB56* **(E)** were cleared with Hoyer's solution, photographed and half of the oi2 layer cell was counted **(F)**. Scale bars = 100 μm.

Table S1 Morphometric measurements of seed shape at different developmental stages.

Table S2. Cell number per layer in the seed coat at early cotyledonary stage.

Table S3. Cell length per layer in the seed coat at early cotyledonary stage.

Table S4. List of primers used in this study.