Regulation of *Arabidopsis* Embryo and Endosperm Development by the Polypeptide Signaling Molecule CLE8 [C][W]

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The plant seed is a major nutritional source for humans as well as an essential embryo development and dispersal unit. To ensure proper seed formation, fine spatial and temporal coordination between the embryo, endosperm, and maternal seed components must be achieved. However, the intercellular signaling pathways that direct the synchronous development of these tissues are poorly understood. Here we show that the *Arabidopsis thaliana* peptide ligand CLAVATA3/EMBRYO SURROUNDING REGION-RELATED8 (CLE8) is exclusively expressed in young embryos and endosperm, and that it acts cell and noncell autonomously to regulate basal embryo cell division patterns, endosperm proliferation, and the timing of endosperm differentiation. *CLE8* positively regulates expression of the transcription factor gene *WUSCHEL-LIKE HOMEOBOX8* (*WOX8*), and together *CLE8* and *WOX8* form a signaling module that promotes seed growth and overall seed size. These results demonstrate that seed development is coordinated by a secreted peptide ligand that plays a key early role in orchestrating cell patterning and proliferation in the embryo and endosperm.

INTRODUCTION

Angiosperm seed formation is characterized by the process of double fertilization, during which the female gametophyte receives a pollen tube that delivers two sperm cells to the ovule. One sperm cell fertilizes the egg cell to produce a zygote, which develops into an embryo, whereas the second sperm cell targets the central cell polar nuclei, leading to endosperm formation. The endosperm surrounds and nourishes the embryo as it develops (Dumas and Rogowsky, 2008), and although both tissues seem to develop autonomously during early seed development, the endosperm plays an important role in regulating embryo growth at later stages (Weijers et al., 2003; Kondou et al., 2008). Both the diploid embryo and the triploid endosperm are enclosed by the maternally derived integument layers that form the seed coat, and successful seed formation requires the simultaneous development of all three of these genetically distinct tissues (Garcia et al., 2005; Berger et al., 2006; Ingouf et al., 2006).

Although little is known about the molecular mechanisms that coordinate the genetic programs of the three seed components, evidence indicates that intercellular signal transduction pathways play major roles (Saulsberry et al., 2002; Nodine et al., 2011). In *Arabidopsis thaliana*, nearly 400 kinases are expressed in embryos (Nodine et al., 2011), and among these, several receptor-like kinases (RLKs) have been shown to be required for proper endosperm and/or embryo development. *HAIKU2* (*IKU2*)

encodes a Leu-rich repeat RLK that is expressed in the endosperm of developing seeds (Luo et al., 2005). *IKU* mutants display precocious endosperm cellularization as well as decreased endosperm size, which ultimately results in a smaller embryo and a decrease in seed size. Several other RLKs, including RPK1/TOAD2 and ACR4/ALE2, are required during embryogenesis for pattern formation and cell fate maintenance (Nodine et al., 2011). In addition, two cytoplasmic kinases regulate early embryonic patterning (Lukowitz et al., 2004; Bayer et al., 2009). An important unresolved question is the identity of the extracellular signals that trigger these kinase pathways, because despite the presence of >1000 putative peptide ligand genes in the *Arabidopsis* genome (Lease and Walker, 2006), no small signaling molecule has yet been shown to influence seed development.

Candidates for polypeptide signaling molecules that could regulate seed formation include members of the CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (CLE) family. The *CLE* genes encode small proteins that consist of a signal peptide or membrane anchor sequence, a 40- to 90-amino-acid variable domain, and a highly conserved 14-amino-acid motif near their carboxyl termini, called the CLE domain (Cock and McCormick, 2001). Full-length *Arabidopsis* CLV3 is proteolytically processed (Ni and Clark, 2006) to a mature 12- or 13-amino-acid arabinosylated glycopeptide consisting of the CLE domain, which has full biological function (Kondo et al., 2006; Ohyama et al., 2009). Synthetic peptides corresponding to the CLE domains of other CLE proteins also exhibit activity in various bioassays (Fiers et al., 2005; Ito et al., 2006), suggesting that this region represents the bioactive form of the protein.

Thirty-two members of the *CLE* gene family have been identified in *Arabidopsis*, yet the biological functions of only three are known. *CLV3* is specifically expressed in the stem cell reservoirs of shoot and floral meristems (Fletcher et al., 1999) but has a noncell autonomous effect on the interior meristem cells, where

the CLV3 mature polypeptide is perceived by three transmembrane receptor complexes (Ogawa et al., 2008; Guo et al., 2010; Kinoshita et al., 2010). Intercellular signaling through the CLV3 pathway limits to the organizing center the expression domain of the *WOX* family transcription factor gene *WUSCHEL* (Laux et al., 1996), which in turn promotes stem cell activity (Schoof et al., 2000) and directly activates *CLV3* transcription (Yadav et al., 2011). Thus, this signal transduction pathway functions as part of a negative feedback loop that modulates stem cell accumulation during plant development (Brand et al., 2000). A comparable signaling module involving CLE40, the receptor kinase ACR4, and the WUS-related protein WOX5 regulates stem cell homeostasis in the root apical meristem (Stahl et al., 2009), whereas a module consisting of CLE41, PXY/TDR, and WOX4 regulates vascular meristem activity (Hirakawa et al., 2008; Etchells and Turner, 2010; Hirakawa et al., 2010).

Although the activities of CLV3, CLE40, and CLE41 are restricted to meristems, the expression patterns of the other CLE family members suggest that the polypeptides may play diverse signaling roles during the plant life cycle. At least one *CLE* gene is expressed in each *Arabidopsis* tissue and at each stage of development (Jun et al., 2010), including more than six during embryogenesis (Fiers et al., 2004; Fiume et al., 2011). Most tissues and cell types express multiple *CLE* genes (Sharma et al., 2003; Jun et al., 2010; Fiume et al., 2011). These data, along with the observation of *CLE* overexpression phenotypes ranging from dwarfism to anthocyanin overproduction (Ito et al., 2006; Ni and Clark, 2006; Strabala et al., 2006; Whitford et al., 2008; Jun et al., 2010), indicate that CLE-mediated signaling pathways are likely to function in many biological processes. Here we show that the *CLE8* gene, which is expressed in early embryos and in endosperm, regulates embryo and suspensor proliferation. It also promotes seed size by regulating both endosperm proliferation and the timing of endosperm differentiation. We additionally demonstrate that CLE8 positively regulates expression of the *WUS*-related gene *WOX8*, uncovering a novel *CLE-WOX* signaling module that directs *Arabidopsis* suspensor and endosperm growth.

RESULTS

CLE8 Is Expressed in Embryos and Endosperm

CLE8 is the only *Arabidopsis CLE* gene that is expressed exclusively in seed-bearing siliques (Sharma et al., 2003), and thus we hypothesized that *CLE8* might play a specific role in seed development. RNA in situ hybridization experiments detected *CLE8* mRNA expression in young embryos (Figures 1A to 1D) and endosperm (Figure 1F). *CLE8* mRNA was expressed throughout the embryo proper, beginning at the one-cell stage (Figure 1A) and continuing through the globular and the triangular stages (Figures 1B to 1D). By the heart stage, *CLE8* expression was no longer detectable (Figure 1E). In contrast with the strong signal in the embryo proper, the weak staining observed in developing suspensor cells was indistinguishable from the surrounding background signal, indicating that *CLE8* is not highly transcribed in the suspensor.

We verified the *CLE8* expression pattern by generating transgenic lines carrying the *CLE8* 2.9-kb putative promoter region

fused to the *uid-A* reporter gene. This *ProCLE8*:*GUS* construct drove expression solely in seeds of stably transformed *Arabidopsis* plants. We detected β-glucuronidase (GUS) activity in developing embryos (Figures 1H to 1L) prior to the heart stage (Figure 1M). To investigate whether *ProCLE8*:*GUS* activity was present in the suspensor cells that connect the embryo to the maternal seed coat, we modified our staining procedure to prevent the solution from completely penetrating the tissues (see Methods). This experiment resulted in preferential GUS staining in the outer layers of the isolated embryos and confirmed the absence of *ProCLE8*:*GUS* activity in the suspensor (Figures 1I, 1J, and 1L). We also detected *ProCLE8*:*GUS* activity in all endosperm compartments at early stages of seed development (Figures 1H, 1N, and 1O), whereas from the torpedo stage onward, it became restricted to the chalazal endosperm in the nucellar region of the seed (Figure 1P). This expression analysis suggested a role for *CLE8* in the initial stages of seed formation, potentially acting as a signal coordinating the early development of the various seed tissues.

CLE8 Acts Noncell Autonomously to Regulate Early Embryo Development

To functionally characterize *CLE8*, we obtained an ethyl methanesulfonate–induced allele via TILLING (McCallum et al., 2000). The *cle8-1* mutation is a C to T nucleotide substitution at position 200 in the *CLE8* coding region, which results in a nonconservative amino acid substitution of the second Pro residue of the CLE domain with a Leu residue (Figure 2A). This Pro is believed to be important for CLE peptide function, because it undergoes posttranslational modifications in CLV3 (Kondo et al., 2006; Ohyama et al., 2009) and is conserved in all *Arabidopsis* A-type CLE genes except CLE40 (Jun et al., 2008). This Pro is adjacent to another functionally crucial amino acid, a highly conserved Gly that, when mutated in two *CLV3* alleles, causes a moderate phenotype (Fletcher et al., 1999).

Careful morphological analysis of *cle8-1* homozygous plants throughout vegetative and reproductive growth did not reveal any aberrant phenotype. However, analysis of open mature *cle8-1* siliques revealed the presence of a variety of defective seeds, ranging from wrinkled or misshapen seeds to discolored seeds to seeds aborted at early stages of development (Figures 2B and 2C). Although *cle8-1* seeds that aborted early were generally not recovered during seed harvesting, we also observed a significant germination defect (see Supplemental Table 1 online), indicating that morphologically normal-looking seeds were among those that failed to germinate.

Further examination of wild-type and mutant seeds revealed a low frequency of defective wild-type accession Columbia (Col) seeds (1.21%), whereas ~15% of *cle8-1* seeds displayed morphological defects by the early globular to triangular stage (see Supplemental Table 2 online). In addition, we determined using quantitative analysis that mature *cle8-1* seeds with a morphologically normal appearance were significantly smaller than wild-type seeds (Figures 2D and 2E). *CLE8* full-length genomic sequence rescued the *cle8-1* phenotype (Figure 2F), confirming that the observed aberrant seeds arose solely because of the mutation in *CLE8*. Furthermore, reciprocal crosses between *cle8-1* and wild-type plants indicated that the *cle8-1* mutation did not produce a maternal sporophytic effect (Figure 2G).
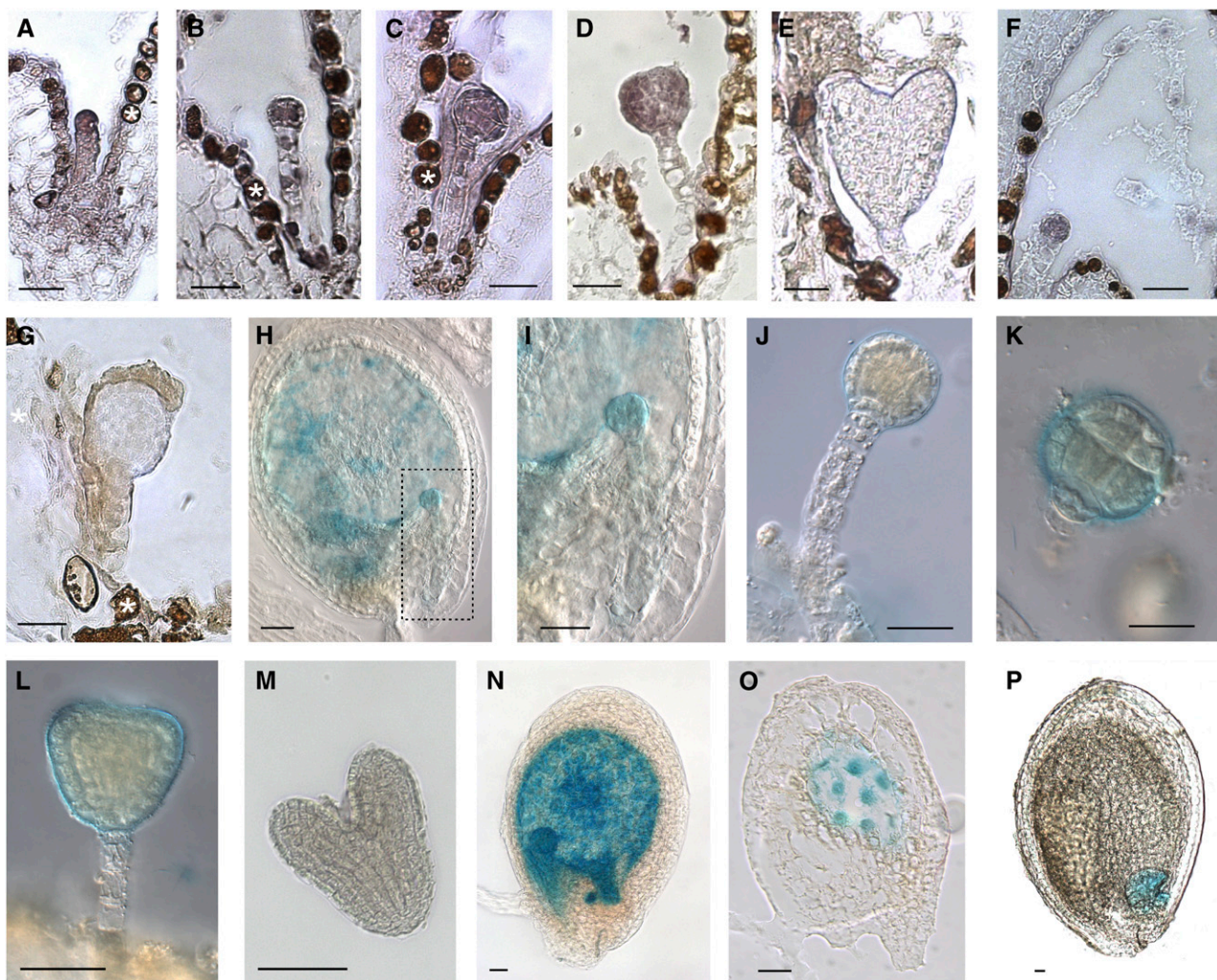
Figure 1. *CLE8* Expression Analysis in Developing Seed Tissues.

(A) to **(F)** *CLE8* mRNA expression pattern in a one-cell stage embryo **(A)**, a four-cell stage embryo **(B)**, a globular stage embryo **(C)**, a triangular stage embryo **(D)**, a heart stage embryo **(E)**, and in the endosperm nuclei **(F)**.
(G) Control hybridization with a *CLE8* sense probe.
(H) to **(P)** *CLE8* promoter-driven GUS activity in embryos (**[H]** to **[M]**) and endosperm (**[N]** to **[P]**). **(I)** shows a higher magnification image of the embryo boxed with the dotted lines in **(H)**.
White stars indicate background browning of the seed coat. Bars = 20 µm.

We tested whether *cle8-1* is a hypomorphic allele by generating a constitutively expressed artificial microRNA (amiRNA) that specifically targeted the *CLE8* sequence (Ossowski et al., 2008). The phenotypes of three independent *CLE8 amiRNA* transgenic lines showing substantially reduced *CLE8* transcript levels (Figure 2H) were analyzed. The frequency of aberrant seeds in *CLE8 amiRNA* plants was significantly higher than that observed in wild-type plants but was the same as in *cle8-1* plants (Figure 2I), consistent with *cle8-1* acting as a hypomorphic allele. However, reciprocal crosses using *cle8-1* resulted in a significant number of defective F1 seeds (Figure 2G). These data suggest that although the seed phenotype results from reduced *CLE8* expression levels, the *cle8-1* allele causes haploinsufficiency; thus, plants are sensitive to the dosage of *CLE8*.

We isolated *cle8-1* seeds containing developing embryos from the two-cell to the early globular stage and analyzed their morphology using Nomarski microscopy (Figures 3A to 3H; see Supplemental Table 2 online). Among the aberrant seeds, 33.9% showed embryo defects, 38.8% showed suspensor defects, and 21.8% showed endosperm defects. Approximately 5.5% of defective seeds showed more than one defect. All wild-type embryos progressed through early development with normal cell division patterns, and their boundary with the suspensor was always clearly recognizable (Figures 3A to 3D). By contrast, *cle8-1* embryos prematurely ceased developing (Figure 3E) or underwent uncontrolled cell divisions in their basal portion (Figures 3F and 3G). We did not observe any aberrant embryo
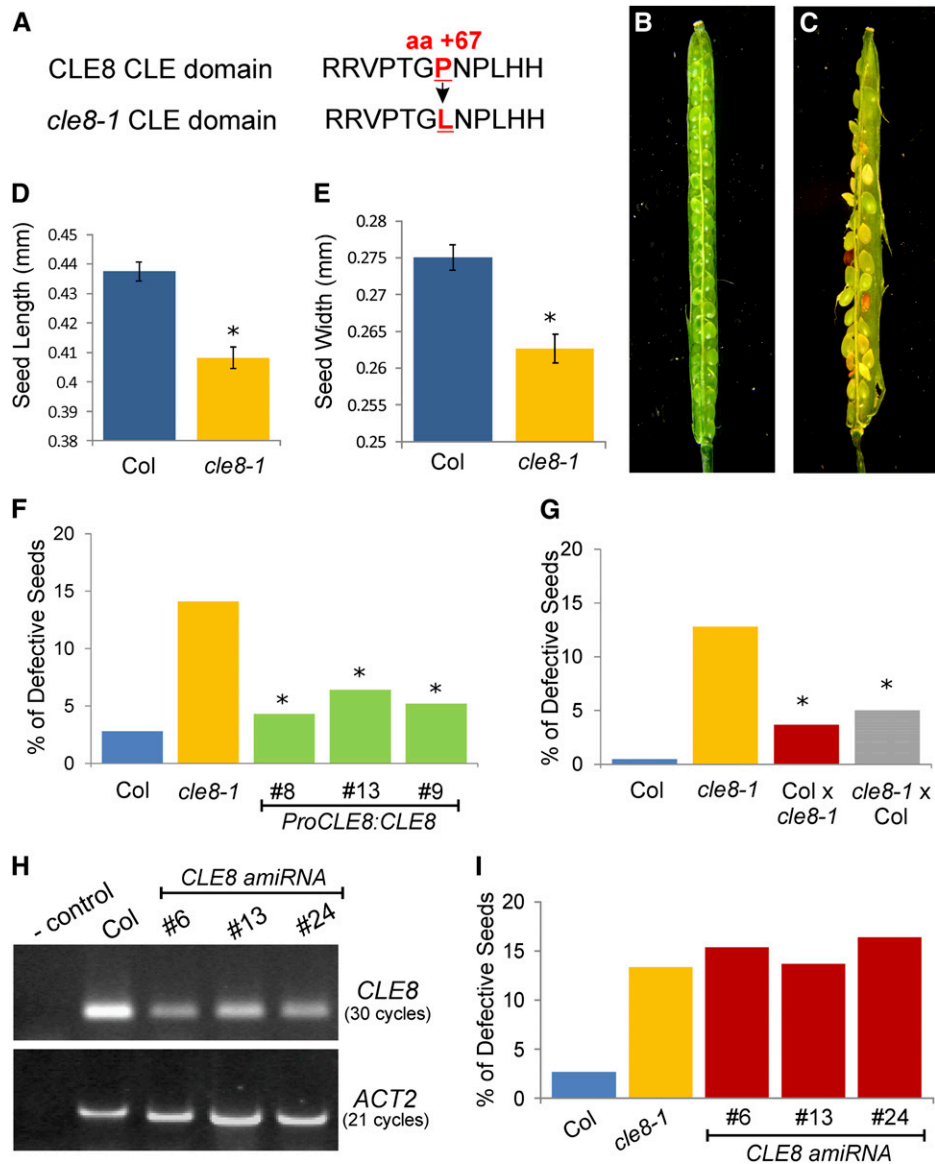
Figure 2. *cle8-1* and *CLE8 amiRNA* Seed Phenotypes.

(A) Wild-type CLE8 CLE domain sequence **(Top)** and cle8-1 CLE domain sequence **(Bottom)**. The mutated amino acid and its position in the protein sequence are indicated in red.

(B) and **(C)** A wild-type **(B)** and a *cle8-1* **(C)** open silique containing mature seeds. This *cle8-1* silique contains more than the average number of abnormal seeds but illustrates the range of seed defects observed.

(D) and **(E)** Length **(D)** and width **(E)** of wild-type Col and *cle8-1* seeds (*n* = 100).

(F) *cle8-1* complementation experiment (*n* = 118 to 186). All transgenic lines are in the *cle8-1* homozygous background. Graph values are means ± SE, * indicates the difference is statistically significant (Z test, P < 0.05) when compared with the *cle8-1* value but not when compared with the wild-type value.

(G) Reciprocal crosses between wild-type Col and *cle8-1* plants (*n* = 179 to 226). * indicates the values are statistically different from wild-type and *cle8-1* values (Z test, P < 0.05). The difference in the percentage of defective seeds between the two reciprocal crosses is not significant.

(H) RT-PCR analysis performed on RNA extracts from siliques of Col and three independent transgenic *CLE8 amiRNA* lines, using two technical replicates.

(I) Quantification of defective seeds in Col, *cle8-1*, and the *CLE8 amiRNA* transgenic lines (*n* = 122 to 275). The difference between wild-type Col and the other genotypes is statistically significant (Z test, P < 0.01), whereas the *CLE8 amiRNA* transgenic line values are not significantly different from the *cle8-1* values.
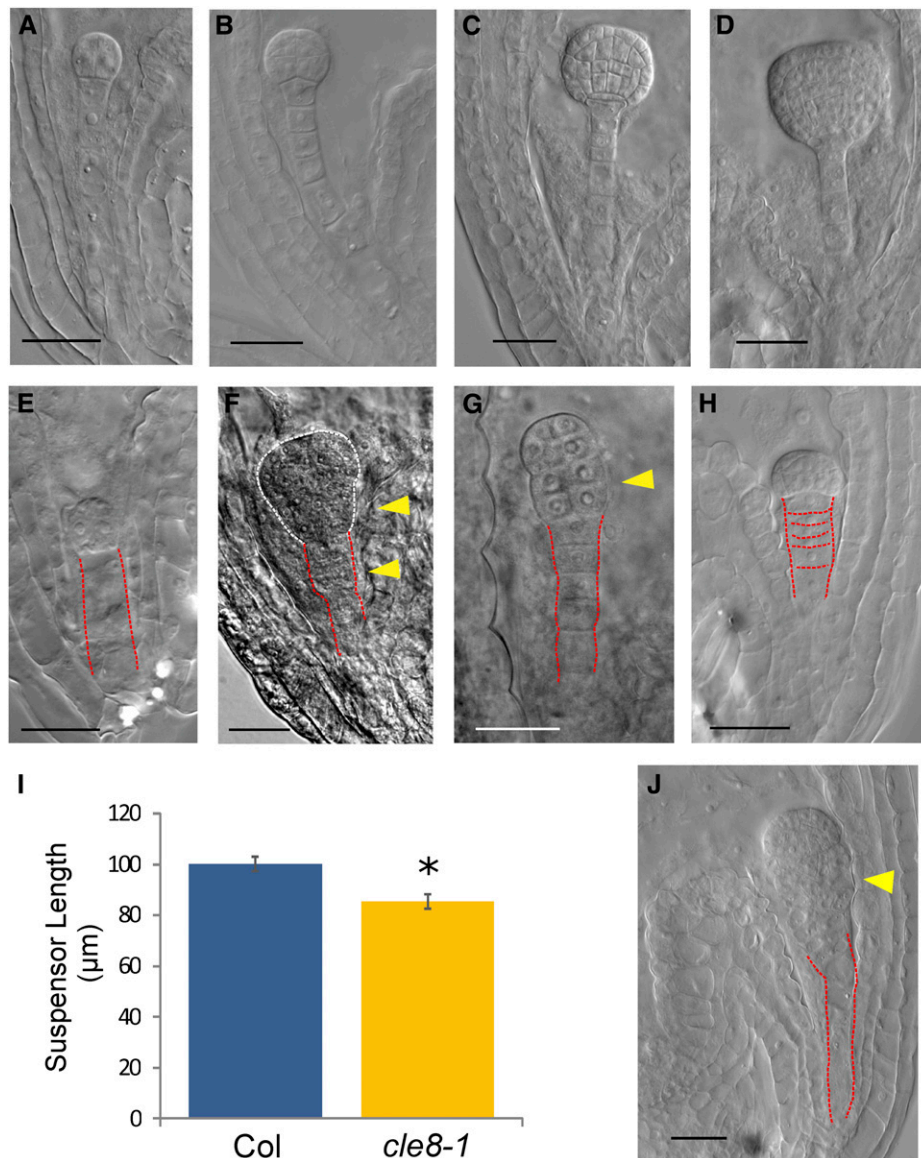
Figure 3. *cle8-1* and *CLE8 amiRNA* Embryo Phenotypes.

(A) to **(D)** Wild-type embryos at the two-cell **(A)**, 16-cell **(B)**, globular **(C)**, and triangular **(D)** stage.

(E) to **(H)** *cle8-1* aberrant embryos in the same developmental window.

(I) Wild-type and *cle8-1* suspensor length measured at the dermatogen embryo stage (*n* = 30). Graph values are means ± SE, * indicates statistically significant values (Student's *t* test, P < 0.05).

(J) *CLE8 amiRNA* aberrant embryo.

Red dashed lines outline the suspensors; white dashed lines outline the embryo proper; yellow arrowheads indicate unusual planes of cell division. Bars = 20 μm.

morphology at later stages of development, suggesting that these early defects lead to seed abortion. Wild-type embryos were subtended by suspensors consisting of a single file of elongated cells (Figures 3A to 3D), whereas in *cle8-1* embryos, the uppermost suspensor cell often acquired the wrong plane of cell division. This produced suspensors with their uppermost portion consisting of more than one tier of cells (Figure 3F) and occasional embryos with an indistinct boundary between the

embryo proper and the suspensor (Figure 3G). On average, wild-type and *cle8-1* embryos at the dermatogen stage had the same number of suspensor cells (Col = 6.37 and *cle8-1* = 6.33; *n* = 20). However, in most of the *cle8-1* defective suspensors, the cells failed to elongate properly (Figure 3H), and suspensor length was significantly shorter in *cle8-1* than in wild-type embryos (Figure 3I). We likewise observed uncontrolled division of the uppermost suspensor cells and the basal cells of the embryo proper in *CLE8*

amiRNA transgenic seeds (Figure 3J). We conclude that *CLE8* plays a key role in embryo development by regulating cell proliferation in the basal domain of the embryo proper, as well as organizing suspensor cell division planes and regulating suspensor cell proliferation and elongation. Because *CLE8* is not transcribed in suspensor cells, CLE8 plays a noncell autonomous role in this tissue.

CLE8 Regulates Endosperm Proliferation and Differentiation

More than 24% of defective *cle8-1* seeds showed aberrations in the developing endosperm. In wild-type seeds containing globular stage embryos, the syncytial cytoplasm of the embryo-surrounding region (ESR) surrounded the developing embryo, and the multinucleate peripheral endosperm syncytium was a thin layer with evenly spaced nuclei (Figure 4A). In corresponding *cle8-1* seeds, we observed endosperm tissue that lacked this organization and showed uneven distribution of the peripheral endosperm nuclei (Figure 4B) or seeds that altogether lacked the peripheral endosperm nuclei and contained only a few endosperm nuclei at the micropylar and chalazal pole of the embryo sac (Figure 4C). We determined that wild-type seeds with embryos at either the one-cell or two-cell stage contained an average of 22.3 endosperm nuclei, whereas *cle8-1* seeds that had fewer endosperm nuclei contained an average of 7.4. Seeds from transgenic *CLE8 amiRNA* plants likewise either lacked peripheral endosperm (Figure 4D) or showed aberrant distribution of the endosperm nuclei (Figure 4E).

To further characterize these defects, we used *ProMEA:GUS* (Luo et al., 2000) as a marker for early endosperm development. In wild-type seeds, this marker is expressed throughout the developing endosperm (Figure 4F) until 2 d after fertilization, when its expression fades in the peripheral endosperm but persists at the posterior pole (Figure 4G). At early stages of development, even *cle8-1* seeds that did not exhibit morphological endosperm defects displayed weak *ProMEA:GUS* activity throughout the peripheral endosperm, whereas the chalazal endosperm exhibited stronger marker activity characteristic of endosperm at later stages of development (Figure 4H). This result suggests that *cle8-1* endosperm undergoes an accelerated acquisition of characteristics of more mature endosperm. We monitored endosperm proliferation using the *ProFIS2:GUS* marker, which is specifically expressed in the central cell and endosperm upon fertilization (Luo et al., 2000). In wild-type seeds containing two-cell stage embryos, the endosperm had
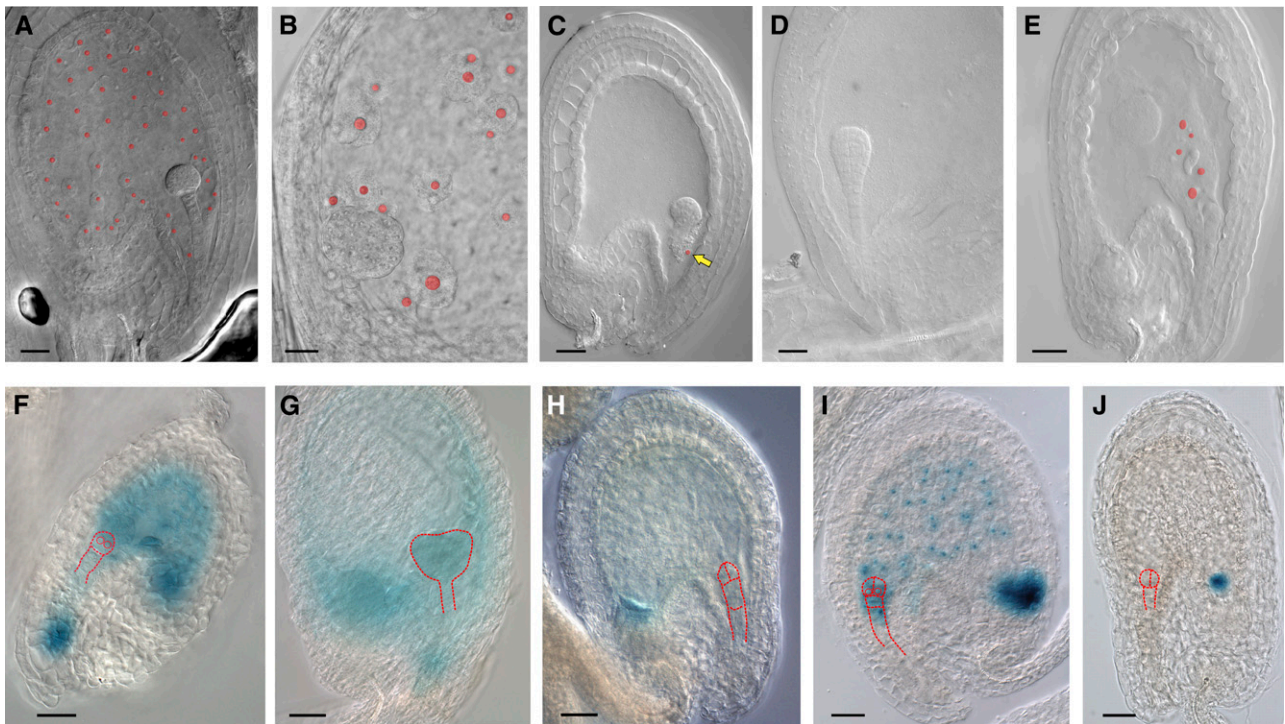

Figure 4. *cle8-1* and *CLE8 amiRNA* Endosperm Phenotypes.

(A) to **(C)** Wild-type seed **(A)** and *cle8-1* seeds (**[B]** and **[C]**). Free endosperm nuclei are highlighted in red, and the yellow arrow indicates a solitary endosperm nucleus near the suspensor.

(D) and **(E)** *CLE8 amiRNA* seeds.

(F) to **(H)** Wild-type **(F)** and **(G)** and *cle8-1* **(H)** seeds expressing the *ProMEA:GUS* marker.

(I) and **(J)** Wild-type **(I)** and *cle8-1* **(J)** seeds expressing the *ProFIS2:GUS* marker. **(F)** to **(J)** Red dashed lines outline the embryos.

Bars = 20 μm.

undergone many rounds of nuclear division and completely filled the seed cavity (Figure 4I), whereas comparable *cle8-1* seeds with normal or abnormal morphology had fewer endosperm nuclei, indicative of a delay in endosperm proliferation (Figure 4J). Thus, *CLE8* promotes the proliferation and organized distribution of the free endosperm nuclei and prevents premature endosperm differentiation.

CLE8 Is Sufficient to Regulate *Arabidopsis* Seed Size

Our results demonstrate that *CLE8* function is necessary to produce normal-sized seeds. To determine whether increasing *CLE8* activity is sufficient to enhance seed size, we generated *CLE8*-overexpressing plants under the control of a double cauliflower mosaic virus 35S promoter. We obtained multiple independent lines that displayed significantly increased levels of *CLE8* mRNA expression (Figure 5A). We examined the endosperm of *Pro35S:CLE8* seeds at early stages of development and detected no difference from wild-type endosperm in terms of proliferation or differentiation. Because of technical limitations, quantifying endosperm nuclei at later stages was not feasible, so it remains an open question whether *CLE8* overexpression can induce excess endosperm proliferation. Nonetheless, we observed that *Pro35S:CLE8* plants contained 6 to 11.5% longer mature embryos than wild-type plants (Figures 5B and 5C) and produced 6 to 7% larger seeds (Figures 5D and 5E). These data indicate that *Arabidopsis* seed size is positively influenced by the level of the *CLE8* signal.

A CLE8-WOX8 Signaling Pathway Functions in Suspensor and Endosperm Development

Although little is known about the molecular targets of CLE signal transduction pathways, several *Arabidopsis* CLE peptides act upstream of *WOX* family transcription factor genes (Brand et al., 2000; Stahl et al., 2009; Hirakawa et al., 2010). *WOX8* and *WOX9* function redundantly during seed development to regulate patterning and cell proliferation in the embryo. *wox8 wox9* embryos form irregular cell division planes or enlarged, misshapen cells in the suspensor (Wu et al., 2007; Breuninger et al., 2008), phenotypes strikingly similar to those of *cle8-1* embryos. We focused on *WOX8* as a likely *CLE8* target gene candidate, because it is the only *WOX* family member expressed in both the endosperm and the embryo basal lineage after the zygotic division (Haecker et al., 2004; Breuninger et al., 2008).

We analyzed *WOX8* expression in *cle8-1* seeds using a *WOX8gΔ:NLS-venusYFP3* marker line that recapitulates the endogenous *WOX8* expression pattern (Breuninger et al., 2008). In early-stage wild-type embryos, *WOX8* is expressed in all suspensor cells except the hypophysis (Figure 6A) and, at later stages, is also detected in the hypophysis (Figure 6A, inset). In *cle8-1* seeds, we observed strong *WOX8* expression only in the most basal suspensor cell, whereas its expression in the other suspensor cells was dramatically reduced or absent (Figure 6B). To exclude the possibility that reduced *CLE8* function generally perturbs gene expression levels in suspensor cells, we analyzed *ProWOX2:NLS-venusYFP3* expression and observed no significant difference between wild-type and *cle8-1* seeds (see
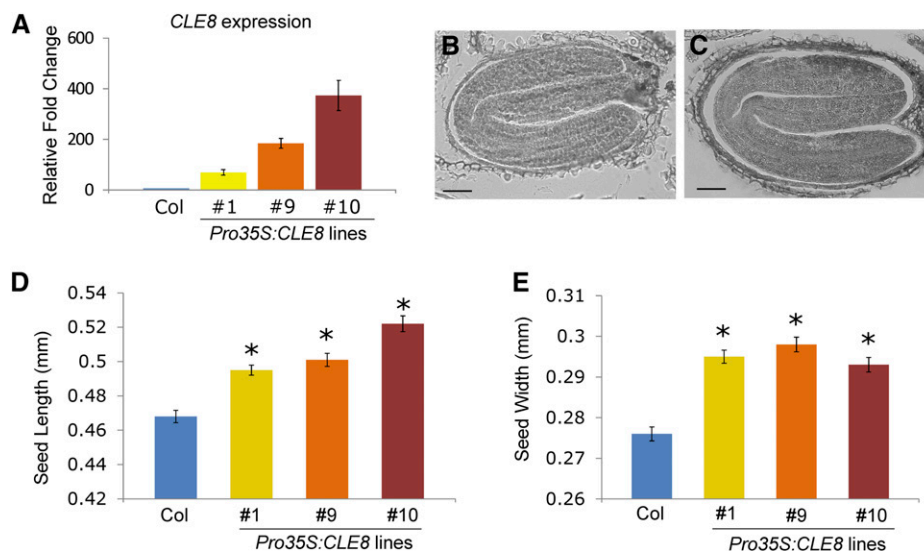

Figure 5. *Pro35S*:*CLE8* Seed Phenotypes.

(A) *CLE8* mRNA transcription levels in wild-type and three independent *Pro35S*:*CLE8* transgenic lines determined by real-time quantitative RT-PCR. Graph values are means of three biological replicates $\pm$ SE. All experimental values are statistically significant (Student's *t* test, P < 0.05).
(B) and **(C)** Mature wild-type **(B)** and *Pro35S*:*CLE8* transgenic **(C)** seeds.
(D) and **(E)** Length **(D)** and width **(E)** of wild-type and *Pro35S*:*CLE8* seeds from three independent transgenic lines. Graph values are means $\pm$ SE, * indicates statistically significant values (*n* = 100, Student's *t* test, P < 0.01).
Bars = 50 μm.
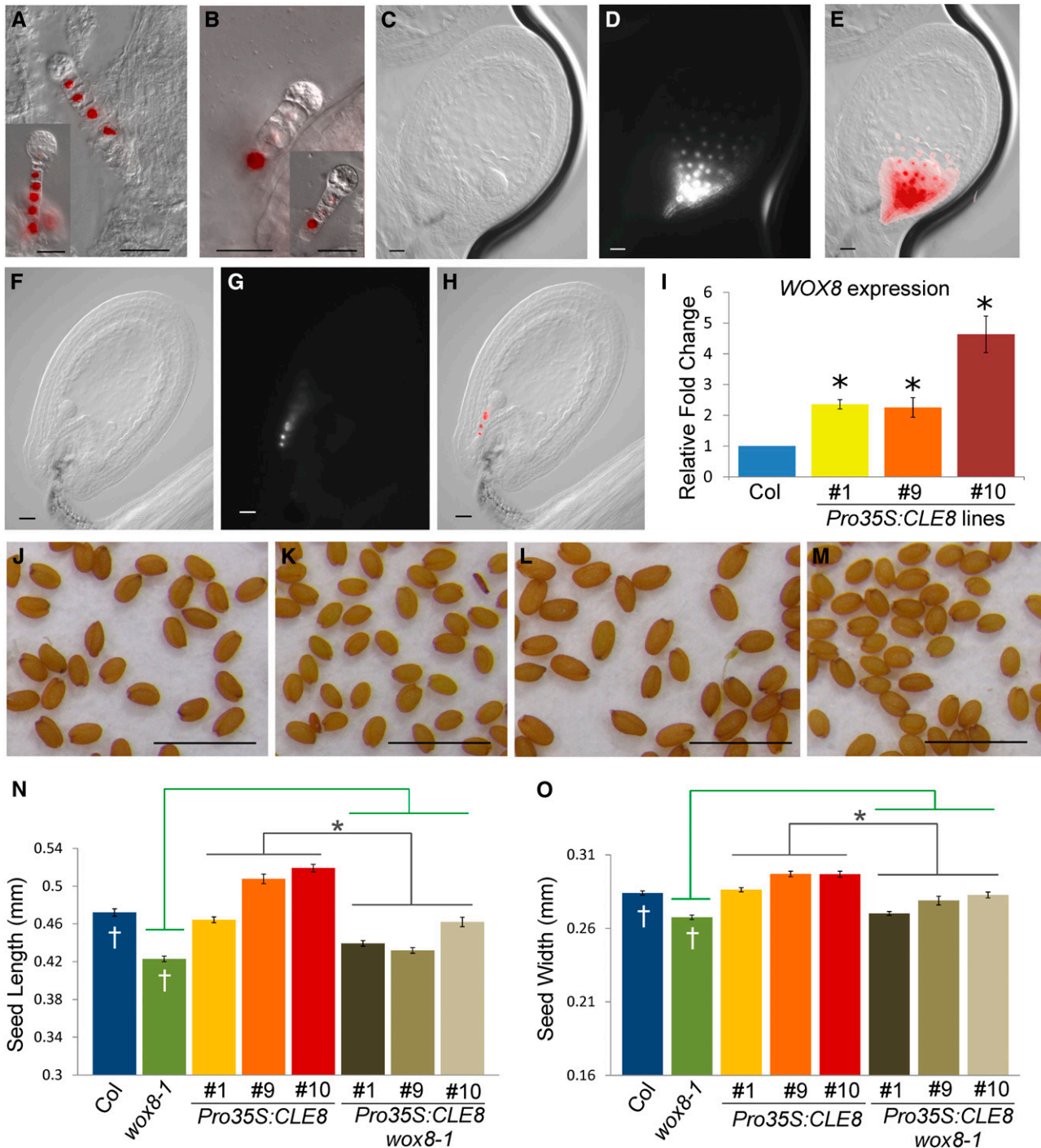[See online article for color version of this figure.]

Figure 6. *WOX8* Activity in *cle8-1* and *Pro35S:CLE8* Plants.

(A) to **(H)** Merged Nomarski and fluorescence images of wild-type (**[A]** and **[C]** to **[E]**) and *cle8-1* (**[B]** and **[F]** to **[H]**) seeds expressing *WOX8gΔ:NLS-venusYFP₃*. Insets show slightly older embryos displaying the same expression patterns.

(I) *WOX8* mRNA transcription levels in three independent *Pro35S:CLE8* transgenic lines. Graph values are means of three biological replicates ± SE, * indicates statistically significant values (Student's *t* test, P < 0.05).

(J) to **(M)** Wild-type **(J)**, *wox8-1* **(K)**, *Pro35S:CLE8* **(L)**, and *Pro35S:CLE8 wox8-1* **(M)** seeds.

(N) and **(O)** Length **(N)** and width **(O)** of wild-type, *wox8-1*, *Pro35S:CLE8*, and *Pro35S:CLE8 wox8-1* seeds from three independent transgenic lines. * indicates that the values of the *Pro35S:CLE8 wox8-1* seeds are significantly different (*n* = 100, Student's *t* test, P < 0.05) from those of the *Pro35S:CLE8* seeds (gray bars) but not from those of the *wox8-1* seeds (green bars). In this generation, *Pro35S:CLE8* line 1 transgenic plants were silenced (*n* = 100). Bars in **(A)** to **(H)** = 20 μm; bars in **(J)** to **(M)** = 1 mm.

Supplemental Figure 1 online). Therefore, *CLE8* acts upstream of *WOX8* and is required for high levels of *WOX8* transcription in the upper suspensor cells.

In early-stage wild-type seeds, *WOX8* was also strongly expressed in the ESR endosperm cells (Figures 6C to 6E). However, in *cle8-1* seeds with wild-type endosperm morphology, *WOX8* expression was restricted to a few cells of the micropylar endosperm (Figures 6F to 6H). This reveals that *CLE8* establishes the proper gene expression programs in both the embryo basal lineage and the ESR endosperm. Conversely, *CLE8*-overexpressing lines showed a significant elevation of *WOX8* mRNA levels (Figure 6I), demonstrating that *CLE8* is sufficient to induce *WOX8* expression. The biological relevance of *WOX8* regulation by CLE8 was demonstrated by the observations that *wox8-1* seeds were smaller than wild-type seeds (Figures 6J, 6K, 6N, and 6O) and that the *wox8-1* mutation suppressed the enlarged seed phenotype of *CLE8*-overexpressing lines (Figures 6L to 6O). Together, these data indicate that *CLE8* and *WOX8* form a signaling module that regulates suspensor and endosperm development, with CLE8 acting upstream in the pathway to positively regulate *WOX8* and promote seed growth.

DISCUSSION

Angiosperm seed development is a complex process that requires the coordinated growth and development of the embryo, endosperm, and seed coat tissues. Here we have shown that in wild-type *Arabidopsis* plants, CLE8 peptide is produced by the embryo proper and the endosperm, and that it is involved in essential signaling–mediated regulation of transcriptional programs that regulate fundamental cell proliferation and cell division processes. *CLE8* is the only *Arabidopsis CLE* gene whose expression is restricted to the developing seed. We detect *CLE8* mRNA throughout the embryo proper from the one-cell stage to the early heart stage, making it, to our knowledge, the earliest expressed polypeptide signaling molecule identified to date (De Smet et al., 2010; Katsir et al., 2011). Previous work indicated that *CLE* genes are generally not expressed at high levels (Sharma et al., 2003), and we likewise observed low GUS activity in *ProCLE8:GUS* transgenic seeds. Nonetheless, the pattern of GUS activity driven by *CLE8* promoter sequences that complement the *cle8-1* phenotype confirms the in situ analysis and demonstrates that *CLE8* is expressed in both young embryos and in the endosperm. By contrast, *CLE8* expression is low in or absent from the suspensor cells.

Most *Arabidopsis CLE* genes lack introns and consist of small open reading frames of 240 to 360 nucleotides (Cock and McCormick, 2001; Jun et al., 2008), making them small targets for mutagenesis. To date, no *CLE8* insertion mutants have been identified. We were able to obtain an ethyl methanesulfonate–induced *cle8-1* allele via TILLING that altered a conserved amino acid in the CLE domain. The seed phenotype caused by the *cle8-1* missense mutation shows incomplete penetrance and is indistinguishable from that conditioned by our *CLE8* amiRNA construct, which substantially reduces but does not completely abolish *CLE8* expression. The most parsimonious interpretation

of these results is that *cle8-1* represents a hypomorphic, partial loss-of-function allele. Residual activity of cle8-1 mutant peptide may allow most *cle8-1* seeds to develop normally, explaining why only 15% display aberrant phenotypes. However, we cannot rule out that *cle8-1* is a null allele that produces a nonfunctional protein, in which case other embryo-expressed *CLE* genes (Fiers et al., 2004; Fiume et al., 2011) may act partially redundantly with *CLE8*.

Although the CLE proteins constitute the largest known family of plant-specific signaling peptides, only a few members have been assigned a clear function. Our study reveals a seed-specific role for *CLE8* in regulating the development of the embryo proper, the suspensor, and the endosperm. We observe that nearly one-quarter of *cle8* aberrant seeds contain defective endosperm. In most of these seeds, endosperm development initiates but fails to proliferate beyond the globular stage of embryogenesis. In other instances, the distribution of the free endosperm nuclei in the embryo sac lacks organization, and the nuclei are enlarged. Analysis of the early endosperm-specific marker *ProMEA:GUS* indicates that early-stage *cle8* seeds displayed a GUS staining pattern similar to that of later-stage wild-type seeds (Luo et al., 2000), suggesting that *cle8* endosperm undergoes a faster rate of maturation. This premature endosperm differentiation may limit nutrient availability to the developing embryo.

We additionally find that *CLE8* regulates cell division and proliferation in the embryo proper as well as cell elongation and patterning in the suspensor. Most *cle8* aberrant seeds show defects only in basal embryo cells, although *CLE8* transcripts accumulate throughout the embryo proper, from the one-cell stage until the heart stage. This suggests either that completely eliminating *CLE8* function is required to affect the apical embryo domain or that other *CLE* genes act redundantly with *CLE8* in apical embryo cells. The identification of null alleles in *CLE8* and other embryo-expressed *CLE* genes will be necessary to resolve this issue. It has been observed that some mutations that cause embryo defects also lead to abnormalities in the suspensor (Hamann et al., 1999; Lukowitz et al., 2004; Bureau et al., 2010). It is therefore possible that the *cle8* suspensor phenotype may be a secondary effect of a primary embryo defect, although both are a consequence of reduced *CLE8* function.

Our results from loss-of-function and gain-of-function analyses demonstrate that CLE8 has a qualitative, positive effect on seed size. *cle8* seeds are smaller on average than wild-type seeds, even those in which the embryos inside are morphologically normal. We observed that in *cle8* seeds, the endosperm fails to sufficiently proliferate and seems to undergo accelerated maturation. Endosperm proliferation and growth are key determinants of embryo size and ultimately seed size (Garcia et al., 2005; Kang et al., 2008), and therefore reduced proliferation and premature differentiation of the endosperm is likely to be one cause of the *cle8* small seed phenotype. We note that *CLE8* expression during later stages of wild-type seed development is restricted to the chalazal endosperm, the compartment responsible for nutrient uptake from the maternal tissue (Costa et al., 2004). *CLE8* activity in this compartment may be important to positively regulate nutrient uptake at the maternal–filial interface and may indirectly promote embryo growth. Conversely, transgenic

Pro35S:CLE8 seeds are significantly larger than wild-type seeds. In this case, the embryos within them are clearly larger than wild-type embryos, whereas early endosperm development seems to proceed normally. Given that *CLE8* acts to promote proliferation and prevent premature differentiation of the endosperm, we predict that broader and more prolonged *CLE8* expression may delay endosperm maturation and cause it to overproliferate at later developmental stages, thereby supporting excess embryo growth. These specific effects on endosperm and embryo development make *CLE8* an important determinant of seed size.

Many studies have shown that ectopic overexpression of almost every *CLE* gene results in the termination of the shoot and/or root meristems or other pleiotropic phenotypes (reviewed in Jun et al., 2008, 2010). These data imply that multiple CLE peptides have the capacity to activate the same receptors, and thus that the tissue distribution of *CLE* gene expression may be an important factor in determining the specificity of CLE function. Interestingly, *CLE8* behaves differently than other *CLE* genes in this respect. Ectopic *CLE8* expression does not generate any aberrant phenotypes in tissues other than seeds, where it is normally transcribed. Because *CLE8* is the only member of the family whose expression is restricted to the seed (Sharma et al., 2003; Jun et al., 2010), it can be speculated that CLE8 evolved to specifically fulfill a role in seed development and that it diverged from other CLE proteins to exclusively interact with its receptor(s). Therefore, top candidates for a CLE8 cognate receptor(s) would be those that are specifically and uniquely expressed in the developing seed.

Finally, it has been well established that signaling pathways comprising CLE polypeptides and WOX transcription factors regulate aspects of shoot, root, and vascular meristem function. Our findings that *CLE8* signaling positively regulates *WOX8* expression in endosperm and suspensor cells and that *WOX8* is required to condition the *CLE8* overexpression phenotype indicates that CLE-WOX regulatory module activity extends beyond the meristems and likely has been repeatedly recruited during evolution to regulate a variety of developmental processes. It is important to emphasize that downregulation of the *WOX8gΔ:NLS-venusYFP3* marker in *cle8* seeds was also observed in morphologically wild-type–looking suspensors, indicating that it is not merely a secondary consequence of a physical defect, but rather an informative molecular characteristic of *cle8* seeds. Interestingly, *WOX8* is a target of a cell-autonomous CLE8 function in the endosperm as well as potentially also a noncell autonomous function in the suspensor. A role for *WOX8* in endosperm formation has not been defined, but because expression and functional studies suggest that *WOX* genes are generally involved in promoting cell division and/or preventing differentiation (van der Graaff et al., 2009), it is reasonable to speculate that *WOX8* might play a role in promoting endosperm proliferation. *WOX8* regulates embryo and suspensor development by activating *WOX2*, which in turn induces expression of the auxin transport gene *PINFORMED1* (*PIN1*) and formation of auxin gradients during early embryogenesis (Breuninger et al., 2008). Thus, in embryos, CLE8 functions as a key upstream component of a regulatory cascade that drives hormone-mediated developmental patterning.

METHODS

Plant Material

All *Arabidopsis thaliana* genotypes were in the Col accession. Plants were grown either on Murashige and Skoog medium or on soil (1:1:1 mixture of perlite:vermiculite:topsoil), under continuous light (120 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22°C. Seeds were stratified at 4°C for 4 d prior to light exposure. Prior to in vitro culture, seeds were surface-sterilized for 40 min with chlorine gas. Control seeds were collected and grown side by side with experimental seeds for each experiment.

Plant Transformation

Agrobacterium tumefaciens strain GV3101 was used to stably transform *Arabidopsis* plants by the floral dip method (Clough and Bent, 1998). Transient transformation was performed by particle bombardment of onion epidermis cells using a Biolistic PDS-1000/He unit (Bio-Rad), as previously described (Sanford et al., 1993).

Cloning

PCR-amplified products were cloned into the pENTR/D-TOPO vector (Invitrogen) and then moved into the appropriate destination vector from the pMDC series (Curtis and Grossniklaus, 2003) or pBGWFS7 (Karimi et al., 2002) through an LR reaction (Invitrogen), according to the manufacturer's instructions. The *CLE8* promoter sequence, consisting of 2959 bp from the stop codon of the nearest upstream gene to the *CLE8* start codon, was PCR-amplified and cloned into the binary vector pBGWFS7 for expression analysis. The *CLE8* coding sequence, which does not contain introns, was PCR-amplified and cloned into the binary vector pMDC32 under the control of a double cauliflower mosaic virus 35S promoter for overexpression analysis. An *Arabidopsis* genomic sequence consisting of the *CLE8* promoter described above and the *CLE8* coding region was PCR-amplified and cloned into the binary vector pMDC107 for *cle8-1* complementation analysis. The *CLE8* amiRNA was designed using the publicly available online tool WMD3 (http://wmd3.weigelworld.org) and cloned into the binary vector pMDC32. *CLE8* mRNA transcripts were PCR-amplified and cloned into the pGEM-T Easy vector (Promega) for in vitro transcription of an in situ hybridization probe. Primer sequences are listed in Supplemental Table 3 online.

Plant DNA and RNA Extraction

Preparation of genomic DNA for PCR analysis was conducted as previously described (Edwards et al., 1991). Genomic DNA was extracted for cloning using the DNeasy Plant Kit (Qiagen). Total RNA isolated using the RNeasy Plant Kit (Qiagen) was treated with RNase-free DNase (Roche) for 30 min at 37°C and then purified with phenol/chloroform.

RT-PCR and Quantitative PCR

The first-strand cDNA synthesis was performed on 1 μg of total RNA using Superscript III RNase H⁻ reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The annealing temperature was 58 to 60°C for all primer pairs. For the RT-PCR amplification from the amiRNA lines, 30 cycles of PCR were used to amplify *CLE8* from all samples, and 21 cycles of PCR were used to amplify *ACT2* from all samples. Quantification of *CLE8* and *WOX8* cDNAs by real-time PCR was performed using the SYBR Green PCR master mix (Applied Biosystems) and a MyIQ Thermal Cycler (Bio-Rad). Three technical replicates were run for each sample. The specificity of the amplification was determined by performing a dissociation curve analysis. Relative quantification values were calculated using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001).

The Ct was calculated using *TUB4* as the endogenous control. Three biological replicates were performed for each genotype. Primer sequences are listed in Supplemental Table 3 online.

RNA in Situ Hybridization

RNA in situ hybridization analyses were conducted as described in Carles et al. (2010), using the *CLE8* full-length coding sequence as the antisense probe.

GUS Histochemical Assays

GUS activity was assayed as described in Jefferson (1989), with the modification that 1 mM potassium ferrocyanide and 1 mM potassium ferricyanide were used. Whole mount incubation times ranged from 8 to 12 h after vacuum infiltration. Subsequent tissue embedding and sectioning were performed as previously described (Sieburth and Meyerowitz, 1997). To stain isolated young embryos, developing seeds were placed in 100 µL of staining solution on a microscope slide. A cover slip was put over the slide, and pressure was applied to squeeze the embryos out of the seed coats. The slides were incubated overnight at 37°C in a tight container to prevent drying.

Microscopy

Nomarski microscopy analysis was performed by placing seeds in a chloral hydrate solution (containing 1:8:3 glycerol:chloral hydrate:water). After a 16-h incubation at room temperature, the seeds were placed onto slides and visualized on a Zeiss Imager A1 microscope equipped with differential interference contrast optics. For fluorescent analysis, ovules were placed on slides containing 10% glycerin and analyzed using the Zeiss filter set 46HE for yellow fluorescent protein. Mature dry seeds were digitally photographed using a Canon D-40 camera attached to a Zeiss Stemi SV11 dissecting microscope. Seed length and width were measured using National Center for Biotechnology Information Image J software for image processing and analysis (http://rsbweb.nih.gov/ij/).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *CLE8*, At1g67775; *WOX2*, At5g59340; *WOX8*, At5g45980.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. *WOX2* Expression Analysis.

Supplemental Table 1. Seed Germination Defect.

Supplemental Table 2. Percentage of Seeds Showing Morphological Defects.

Supplemental Table 3. Primers Used in the Experimental Procedures.

AUTHOR CONTRIBUTIONS

E.F. and J.C.F. designed the research; E.F. performed the research; E.F. and J.C.F. analyzed the data and wrote the article.

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