RESEARCH PAPER

**CLE19 expressed in the embryo regulates both cotyledon establishment and endosperm development in Arabidopsis**

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**Abstract**

Embryo and endosperm development are two well co-ordinated developmental processes in seed formation; however, signals involved in embryo and endosperm interactions remain poorly understood. It has been shown before that CLAVATA3/ESR-RELATED 19 (CLE19) peptide is able to trigger root meristem consumption in a CLV2-dependent manner. In this study, the role of CLE19 in Arabidopsis seed development was explored using antagonistic peptide technology. CLE19 is expressed in the epidermal layers of the cotyledon primordia, hypocotyl, and root cap in the embryo. Transgenic plants carrying an antagonistic CLE19⁶¹⁷ construct expressed under the control of CLE19 regulatory elements exhibited a dominant seed abortion phenotype, with defective cotyledon establishment in embryos and delayed nuclear proliferation and cellularization in endosperms. Ectopic expression of CLE19⁶¹⁷ in Arabidopsis under the control of an endosperm-specific ALE1 promoter led to a similar defect in cotyledon establishment in embryos but without an evident effect on endosperm development. We therefore propose that CLE19 may act as a mobile peptide co-ordinating embryo and endosperm development.

**Key words:** Antagonistic peptide, CLE19, cotyledon establishment, endosperm cellularization, embryo–endosperm interaction.

**Introduction**

Double fertilization in angiosperms, where two male gametes join with the female gametophyte, initiates two parallel developmental events to produce a diploid embryo and a triploid endosperm. Embryo and endosperm development are highly co-ordinated processes (LaFont-Placette and Kohler, 2014). It has been reported previously that several endosperm-expressed genes regulate embryo development: ABNORMAL LEAF SHAPE1 (ALE1) and ZHOUPI (ZOU), which are expressed in the endosperm, regulate the formation of the cuticle layer of the embryo (Tanaka et al., 2001; Yang et al., 2008); and EMBRYO SURROUNDING FACTOR 1 (ESF1) expressed in the endosperm promotes suspensor elongation (Costa et al., 2014). However, how embryo-derived factors regulate endosperm development remains elusive.

As a founding member of the CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (ESR)-RELATED (CLE) family genes, CLV3 acts as 12 or 13 amino acid peptide to interact with several leucine-rich repeat (LRR) receptor kinases to regulate stem cell homeostasis in shoot apical meristems (SAMs) (Clark et al., 1997; Fletcher et al., 1999; Kondo et al., 2006; Ohyama et al., 2009; Shinohara and Matsumiya, 2015). Thirty-two CLE genes with diverse
expression patterns have been identified in the Arabidopsis genome (Cock and McCormick, 2001; Oelkers et al., 2008; Jun et al., 2010), and among them only three have been genetically characterized. Mutation or T-DNA insertion in CLV3 led to plants with an enlarged SAM and increased numbers of floral organs in Arabidopsis (Leyser and Furner, 1992). T-DNA insertion in CLE40 led to plants with a slightly reduced root length and delayed differentiation of columella stem cells in root meristems (Hobe et al., 2003; Stahl et al., 2009). CLE8 is a seed-specific CLE family member expressed in both embryo and endosperm in Arabidopsis (Sharma et al., 2003), and a homozygous cle-1 mutant showed defective embryo and endosperm development in ~15% of the seeds produced (Fiems and Fletcher, 2012). Although CLE41 and CLE44 have been implicated in xylem differentiation (Itou et al., 2006), BnCLE19 in cotyledon development (Fiers et al., 2004), and ESR1 in the embryo–endosperm interaction (Opsahl-Ferstad et al., 1997), since no genetic data are available for these genes, their endogenous functions remain to be elucidated. Studies performed thus far suggest a high level of redundancy among CLE members since mutants carrying T-DNA insertions in CLE1, CLE7, CLE10, CLE16, CLE18, or CLE19 in Arabidopsis showed no visible phenotype (Fiers et al., 2004; Jun et al., 2010), and overexpression of a large group of CLE genes using the cauliflower mosaic virus (CaMV) 35S promoter exhibited a similar dwarf and short-root phenotype (Fiers et al., 2004; Strabala et al., 2006).

BnCLE19 is an embryo-expressed CLE gene that was first identified in microspore embryogenesis of Brassica napus (Fiers et al., 2004). Transgenic Arabidopsis carrying the BnCLE19 regulatory elements fused with β-glucuronidase (GUS) or green fluorescent protein (GFP) reporter genes revealed expression in cotyledon primordia in triangular-stage embryos, and the expression persisted in the epidermal layer of cotyledons during embryogenesis (Fiers et al., 2004). Overexpression of BnCLE19 or Arabidopsis CLE19 under the control of the CaMV 35S promoter, or treatment of Arabidopsis seedlings with synthetic 12–14 amino acid CLE19 peptides, led to a CLV2-dependent premature stem cell differentiation in root meristems (Fiers et al., 2004, 2005). Suppressor screening using RCH1-CLE19 transgenic plants led to the identification of two genetic loci, SOL1 and SOL2 (Casamitjana-Martinez et al., 2003). SOL1 encodes a Zn²⁺-dependent carboxypeptidase that functions to remove the C-terminal arginine residue in CLE19 processing (Casamitjana-Martinez et al., 2003; Tamaki et al., 2013), while SOL2 is allelic to CRN that encodes an extracellular domain-free receptor-like kinase (Miwa et al., 2008; Muller et al., 2008). However, since the T-DNA insertional CLE19 mutant showed no visible phenotype (Fiers et al., 2004), the function of endogenous CLE19 remains unclear.

In this study, detailed expression and functional analyses were performed in CLE19 to elucidate its role in Arabidopsis. Using the recently developed antagonistic peptide technology (Song et al., 2013), it was demonstrated that CLE19, as an embryo-specifically expressed gene, regulates both embryo and endosperm development. Transgenic plants carrying the antagonistic CLE19GST construct expressed under the control of the endogenous CLE19 regulatory elements exhibited a dominant seed abortion phenotype, with defective cotyledon establishment and delayed endosperm development. The phenotype of altered cotyledon establishment was mimicked when CLE19GST was expressed under the control of an endosperm-specific ALE1 promoter.

### Materials and methods

#### Plant materials and growth conditions

Wild-type and transgenic Arabidopsis thaliana plants (Col-0) were grown either on agar plates or in soil as described previously (Song et al., 2012). For transformation, 4- to 5-week-old Arabidopsis plants were transformed with Agrobacterium tumefaciens using the floral dip method (Clough and Bent, 1998).

**Molecular cloning**

A CLE19 genomic fragment containing a 1782bp 5’ upstream region (pCLE19), 225bp coding region, and a 1205bp 3’ downstream region (tCLE19) was amplified and cloned into pDONR201 (Invitrogen) to construct the pDONR201-pCLE19:CLE19:tCLE19 entry clone. To generate a glycine to threonine substitution (G6T) at the sixth amino acid (G6) of the CLE motif, a Fast Mutagenesis Kit (TransGen, Beijing) was used to introduce point mutations into pDONR201-pCLE19:CLE19:tCLE19 to produce pDONR201-pCLE19:CLE19G6T:tCLE19, which was then transferred to the pBGWFS7 binary vector to generate pCLE19:CLE19G6T:tCLE19. pCLE19 and tCLE19 were cloned into the ligation-independent cloning vectors pPLY04 and pPLV15 (De Rybel et al., 2011) to generate pCLE19:SV40-3XGFP:tCLE19 and pCLE19:GUS:tCLE19, respectively. pWOX1:SV40-3XGFP, pWOX3:SV40-3XGFP, and pALE1:SV40-3XGFP were made in a similar manner using pPLV04 (De Rybel et al., 2011). The pALE1:CLE19G6T construct was made by replacing the SV40-3XGFP cassette in pALE1:SV40-3XGFP with the CLE19G6T coding sequence.

**In vitro embryo culture**

Siliques containing ovules with embryos at the heart-shape stage were surface-sterilized, and embryos were isolated and transferred to the top of Gamborg’s B5 basal medium (PhytoTechnology) containing 10% sucrose, 0.6% agar (type A, Sigma-Aldrich), 400 μg ml⁻¹ glutamine, and 500 μg ml⁻¹ inositol, and cultured as described (Liu et al., 1995; Hadfi et al., 1998).

**Microscopy**

Siliques were fixed in pre-cooled acetic acid at −20°C for 1 h. GUS assays were performed on fixed tissue as previously described (Fiers et al., 2004). Dissected ovules were cleared in Hoyers’ solution (Berleth and Jürgens, 1993) for 2 h to overnight depending on developmental stage, and were then observed under a microscope equipped with differential interference contrast (DIC). For fluorescence microscopy, ovules from transgenic plants carrying GFP fusion constructs were mounted with 3% glycerol and observed under a fluorescence microscope. Embryos for confocal laser scanning microscopy (CLSM) were dissected from ovules and transferred to a 9% glucose solution containing 20 μg ml⁻¹ propidium iodide (PI) solution (Sigma-Aldrich) prior to imaging. ImageJ software (version 1.4.3) was used for image processing and analyses. For cytohistological analyses, periodic acid–Schiff’s reagent (PAS) staining (Baum, 2008) was performed on semi-thin sectioned ovules embedded in LR White resin (The London Resin Company).
RNA was extracted from ∼300 embryos or ∼100 ovules using a Plant Total RNA Isolation Kit (GeneMark, Beijing) and reverse transcribed using a First Strand cDNA Synthesis Kit (Tiangen, Beijing). Reverse transcription–PCR (RT–PCR) and RT–PCR analyses were performed with the resultant cDNA for analyzing E1F4A (used as an internal control) and CLE19 expression. Quantitative real-time PCR (qRT-PCR) was performed using a Rotor-Gene 3000 thermocycler (Corbett) with the SYBR Premix ExTaq II kit (TaKaRa, Dalian) to assess relative expression levels of WOX1, WOX3, MEA, FIS2, and AG162. Expression data were normalized to E1F4A using the 2–ΔΔCT method (Livak and Schmittgen, 2001). All primers used are listed in Supplementary Table S1 available at JXB online.

Results

Transgenic plants carrying the pCLE19:CLE19GUS:tCLE19 construct exhibited defective embryo and endosperm development

It has been shown previously that BnCLE19 is an embryo-specific gene expressed in cotyledon primordia in triangular-stage embryos, and in the epidermal layer of cotyledons in heart-shape and torpedo-stage embryos (Fiers et al., 2004). In this study, the upregulatory pCLE19:CLE19GUS:tCLE19 construct (Song et al., 2013) was devised to elucidate the role of CLE19 in Arabidopsis. The construct consisted of a 1782bp 5’ upstream region (pCLE19), a 225bp coding region, and a 1205bp 3’ downstream sequence (tCLE19) of CLE19, with the conserved sixth glycine in the CLE motif substituted by threonine. The construct was transformed into Arabidopsis (Col-0) using the floral dip method (Clough and Bent, 1998). Among 54 independent T1 transgenic lines examined, 12 showed different percentages of seed abortions, and among these 12 lines, three (#1, #2, and #3) exhibited consistently high frequencies of a seed abortion phenotype (Fig. 1A). Examination of seeds at 12 days after pollination (DAP) under a dissection microscope revealed that 36.3, 33.8, and 33.4% of ovules in lines #1, #2, and #3, respectively, were aborted before maturation (Table 1). Progeny plants produced from these three lines showed a consistent seed abortion phenotype for four generations examined so far. The transgenic plants were pollinated with pollen from wild-type plants, and the resultant ovules showed similar frequencies of seed abortion, indicating that the abortion phenotype was a dominant trait. The phenotypes of these three transgenic lines were indistinguishable from one another, and transgenic line #1 was used in all subsequent studies.

Ovules were examined under a DIC microscope after clearing using Hoyers’ solution. Embryos at the early heart-shaped stage (5 DAP) in ovules from pCLE19:CLE19GUS:tCLE19 transgenic plants were morphologically indistinguishable from those in the wild type (Fig. 1B, D). However, the size of embryo sacs in some ovules from the transgenic plants was smaller than those in the wild type at the same stage (Fig. 1B, D). In addition, reduced numbers of endosperm nuclei were often observed in these small embryo sacs (Fig. 1C, E), suggesting that endosperm development was delayed in these transgenic plants. Additionally, evident abnormal embryo development was observed in some ovules from these transgenic plants from 7 DAP onwards, with defective cotyledon formation (Fig. 1F–I, indicated by arrowheads).

Cytohistological examinations of PAS-stained semi-thin sectioned ovules confirmed that at 5 DAP embryos in pCLE19:CLE19GUS:tCLE19 transgenic plants were morphologically indistinguishable from those in the wild type (Fig. 1J, K). Strikingly, no cellularization was seen in endosperms from transgenic plants at this stage (Fig. 1K); in contrast, wild-type endosperms at the same stage showed complete cellularization in the embryo surrounding region, and partial cellularization at the periphery of the endosperm (Fig. 1J, indicated by asterisks). Even at 12 DAP, only partially cellularized endosperms were observed in aborted ovules from pCLE19:CLE19GUS:tCLE19 transgenic plants (Fig. 1L, M; indicated by asterisks), while endosperms in the wild type were cellularized completely by 8 DAP. Moreover, although cotyledon primordia were formed in aborted embryos in the transgenic plants at 12 DAP, further growth of these primordia was arrested (Fig. 1L, M; indicated by arrowheads), suggesting a defect in cotyledon establishment. In wild-type embryos, starch grains (stained dark red by PAS) were accumulated in the whole embryo by 12 DAP (Fig. 1L), while those aborted embryos from the transgenic plants exhibited a substantially lower starch accumulation in cotyledon primordia (Fig. 1M, indicated by arrowheads), suggesting that cotyledon differentiation was delayed. It should be noted that no obvious defect was observed in the hypocotyl and the root tip regions (Fig. 1I, M). Thus, expression of CLE19GUS under the control of CLE19 regulatory elements in Arabidopsis led to defective cotyledon establishment and delayed endosperm nuclear proliferation and cellularization.

Expression of CLE19 in seeds is confined to the embryo

To investigate the expression of CLE19 in Arabidopsis, a pCLE19:GUS:tCLE19 reporter construct was made, with the coding region of pCLE19:CLE19GUS:tCLE19 replaced by the GUS gene, and transformed into Arabidopsis (Col-0). In ovules from the transgenic plants obtained, GUS expression was first observed in embryos at the late globular stage (Fig. 2A, B), and persisted in the triangular (Fig. 2C), heart-shaped (Fig. 2D), and torpedo-stage (Fig. 2E) embryos. No GUS expression was observed in testa or suspensors.

A weak GUS signal was detected in the ESR (Fig. 2C–E) in pCLE19:GUS:tCLE19 transgenic plants. To examine if the signal is from expression or diffusion, RT–PCR was performed using RNA extracted from (i) whole ovules; (ii) isolated embryos; and (iii) the mixed endosperm and testa tissues when embryo development in these ovules was at the heart-shaped stage. Expression of the CLE19 transcript was detected in whole ovules and in embryos, but not in mixed endosperm and testa tissues (Fig. 2F), suggesting that the weak GUS staining signal detected in the ESR was likely to be diffused from the GUS-positive embryo.

A pCLE19:SV40-3XGFP:tCLE19 reporter construct with a nuclear-localized triple GFP (SV40-3XGFP) was made and transformed into Arabidopsis (Col-0) to define further the
Table 1. Frequencies of seed abortions in pCLE19:CLE19<sub>G6T</sub>:tCLE19 and pALE1:CLE19<sub>G6T</sub> transgenic plants

<table>
<thead>
<tr>
<th>Line</th>
<th>Normal</th>
<th>Aborted</th>
<th>Abortion frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCLE19:CLE19&lt;sub&gt;G6T&lt;/sub&gt;:tCLE19</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>#1</td>
<td>272</td>
<td>155</td>
<td>36.3</td>
</tr>
<tr>
<td>#2</td>
<td>290</td>
<td>148</td>
<td>33.8</td>
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<tr>
<td>#3</td>
<td>289</td>
<td>145</td>
<td>33.4</td>
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<tr>
<td>pALE1:CLE19&lt;sub&gt;G6T&lt;/sub&gt;</td>
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<tr>
<td>#1</td>
<td>106</td>
<td>54</td>
<td>33.3</td>
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<tr>
<td>#2</td>
<td>101</td>
<td>40</td>
<td>28.4</td>
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<td>#3</td>
<td>100</td>
<td>49</td>
<td>32.9</td>
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expression pattern of CLE19 in developing embryos. CLSM was performed in embryos dissected from pCLE19:SY40-3XGFP:tCLE19 transgenic plants. GFP expression was first observed in epidermal cells located in the lower portion of the embryo at the late globular stage, and in their progeny cells in triangular-stage embryos (indicated by blue arrowheads; Fig. 3A, B). In heart-shaped and torpedo-stage embryos, additional GFP expression was observed in cotyledon primordia (indicated by white arrowheads; Fig. 3C, D). In walking-stick and cotyledonary-stage embryos, GFP expression was observed in cells located at the edges of the two cotyledons and cells in the root cap (Fig. 3E, F). This expression pattern differs slightly from that observed previously for BnCLE19 from Brassica napus, as no expression was observed in the hypocotyl and root cap regions when the BnCLE19 regulatory elements were examined (Fiers et al., 2004).

Cotyledon establishment, not initiation, is defective in pCLE19:CLE19<sub>G6T</sub>:tCLE19 transgenic plants

The major defect of pCLE19:CLE19<sub>G6T</sub>:tCLE19 transgenic plants was observed in embryos after the heart-shaped stage. Therefore, in vitro embryo culture was used to examine whether the defective cotyledon development phenotype could be rescued. Early heart-shaped embryos were isolated from the wild-type and transgenic plants and cultured in vitro. After 5 d of cultivation, wild-type embryos had progressed...
to the cotyledonary stage and had formed two well-established cotyledons (Fig. 4A). However, embryos isolated from pCLE19:CLE19G6T:tCLE19 transgenic plants exhibited a severe defect in cotyledon establishment (Fig. 4B–D), suggesting that the cotyledon arrest was intrinsic to the embryo.

To elucidate further whether the defect in cotyledon development in pCLE19:CLE19G6T:tCLE19 plants is attributed to failures in cotyledon initiation or cotyledon establishment, two cotyledon marker lines were developed based on published in situ hybridization results for WUSCHEL-RELATED HOMEOBOX 1 (WOX1) and WOX3 (Haeccker et al., 2004). Upstream sequences from WOX1 and WOX3 (4454 bp and 5028 bp, respectively) were fused to the SV40-3XGFP reporter gene and transformed into the wild-type Arabidopsis (Col-0). Transgenic plants were examined and crossed to pCLE19:CLE19G6T:tCLE19 plants, and progeny plants carrying pCLE19:CLE19G6T:tCLE19 and homozygous pWOX1:SV40-3XGFP or pWOX3:SV40-3XGFP constructs were examined under CLSM. As shown in Fig. 5, GFP expression in torpedo-stage embryos from wild-type plants carrying pWOX1:SV40-3XGFP was restricted to cells located at the edges of two cotyledons (Fig. 5A). Embryos from plants carrying both the pCLE19:CLE19G6T:tCLE19 and the pWOX1:SV40-3XGFP constructs exhibited a similar GFP expression pattern despite a severe delay in cotyledon development in these embryos (Fig. 5B). GFP expression in wild-type torpedo-stage embryos carrying the pWOX3:SV40-3XGFP construct was observed at the adaxial side of two cotyledons (Fig. 5C). Similar GFP expression was also observed in transgenic plants carrying both the pWOX3:SV40-3XGFP and pCLE19:CLE19G6T:tCLE19 constructs (Fig. 5D). Real-time PCR analyses confirmed that both WOX1 and WOX3 were expressed in pCLE19:CLE19G6T:tCLE19 plants, albeit with a 30–40% reduction observed in ovules carrying defective embryos compared with those from the wild type (Fig. 5E, F). These data suggest that cotyledon initiation was unaffected in pCLE19:CLE19G6T:tCLE19 transgenic plants.

Endosperm development is delayed in pCLE19:CLE19G6T:tCLE19 transgenic plants

To examine further the endosperm defect in pCLE19:CLE19G6T:tCLE19 transgenic plants, the size of the embryo sac (the total area occupied by the endosperm and the embryo) was measured in ovules when embryos were at the late globular, triangular, heart-shaped, or torpedo stages. Compared with those in the wild type, embryo sacs in ovules from pCLE19:CLE19G6T:tCLE19 transgenic plants exhibited much greater variation in size. Many embryo sacs from the transgenic plant were smaller than those from the wild type at the same stage (Fig. 6A). The smallest embryo sac in pCLE19:CLE19G6T:tCLE19 transgenic plants was only half the size of those from the wild type, while the largest embryo sac was not significantly bigger than those from the wild type (Fig. 6A). This result suggests that endosperm development is delayed in pCLE19:CLE19G6T:tCLE19 transgenic plants.

Next, the number of endosperm nuclei was counted in ovules from wild-type and pCLE19:CLE19G6T:tCLE19 transgenic plants. A highly variable number of nuclei was observed in ovules from siliques of pCLE19:CLE19G6T:tCLE19 transgenic plants, and progeny plants when embryos inside these ovules were at the triangular stage. In general, the number of endosperm nuclei in these transgenic plants was significantly lower than that in the wild type (Fig. 6B), which was consistent with the smaller embryo sac observed in transgenic plants.

Expression of early endosperm-specific genes is prolonged in ovules from pCLE19:CLE19G6T:tCLE19 transgenic plants

To determine whether expression of the early endosperm-specific genes was altered in pCLE19:CLE19G6T:tCLE19 transgenic plants, expression of MEA, FIS2, and AGL62, which are expressed in the endosperm prior to celluarization...
Fig. 3. Dynamic expression of CLE19 during embryogenesis. As examined under a confocal microscope in embryos excised from pCLE19:SV40-3XGFP::tCLE19 transgenic plants, GFP expression was first observed in protodermal cells (indicated by blue arrowheads) at the lower portion of the 32-cell stage embryo (A), and persisted in their progeny cells in the triangular-stage embryo (B). Additional GFP expression was observed in epidermal cells at the tips and abaxial sides of the cotyledon (indicated by white arrowheads) in heart-shaped (C) and torpedo-stage embryos (D). In walking-stick (E) and cotyledonary-stage embryos (F), strong GFP expression was seen in epidermal cells located at the edges of cotyledons (c, indicated by white arrowheads) and in root caps (indicated by yellow arrowheads), weak GFP expression was observed in the hypocotyl (h). (E) and (F) were prepared by superimposing multiple scanned images. Scale bars=50 μm.

Fig. 4. Embryo culture in vitro did not rescue the defective cotyledon phenotype in embryos excised from pCLE19:CLE19_{G6T}:tCLE19 transgenic plants. Note the abnormal cotyledon establishment in embryos from pCLE19:CLE19_{G6T}:tCLE19 transgenic plants (B, C), as compared with the control embryo from the wild type (A). c, cotyledon; h, hypocotyl. Scale bars=50 μm.

Expression of CLE19_{G6T} in the ESR of the endosperm mimics the defective cotyledon phenotype in pCLE19:CLE19_{G6T}:tCLE19 transgenic plants

The promoter from ALE1, expressed in the ESR of the endosperm in Arabidopsis (Tanaka et al., 2001), was used to

To access the MEA expression pattern in the pCLE19:CLE19_{G6T}:tCLE19 transgenic plants, a pMEA:GUS reporter construct (Luo et al., 2000) was introduced into pCLE19:CLE19_{G6T}:tCLE19 transgenic plants by crossing. Similar GUS expression was observed in endosperms from the wild type and pCLE19:CLE19_{G6T}:tCLE19 transgenic plants in the first 5 DAP, and no GUS expression was detected in those wild-type ovules or wild-type-appearing ovules from pCLE19:CLE19_{G6T}:tCLE19 transgenic plants at 12 DAP (Fig. 7B, C). However, in those aborted seeds from the transgenic plants, GUS expression was often observed in the chalarzial region of the endosperm (Fig. 7D, E), suggesting a prolonged MEA expression in chalarzial endosperms in aborted seeds. This observation provides further evidence for delayed endosperm development in pCLE19:CLE19_{G6T}:tCLE19 plants.

Expression of CLE19_{G6T} in the ESR of the endosperm mimics the defective cotyledon phenotype in pCLE19:CLE19_{G6T}:tCLE19 transgenic plants

The promoter from ALE1, expressed in the ESR of the endosperm in Arabidopsis (Tanaka et al., 2001), was used to
Fig. 5. Expression of cotyledon-specific genes in arrested embryos from pCLE19:CLE19<sub>G6T</sub>:tCLE19 transgenic plants. (A–D) Confocal microscopic examinations of embryos excised from wild-type plants (WT) carrying pWOX1:SV40-3XGFP (A) or pWOX3:SV40-3XGFP marker constructs (C), or from plants carrying pWOX1:SV40-3XGFP:pCLE19:CLE19<sub>G6T</sub>:tCLE19 (B) or pWOX3:SV40-3XGFP:pCLE19:CLE19<sub>G6T</sub>:tCLE19 double constructs (D). Note that similar GFP expression, though with a reduced level, was observed in cotyledon primordia of arrested embryos from pCLE19:CLE19<sub>G6T</sub>:tCLE19 transgenic plants (CLE19<sub>G6T</sub>), as compared with embryos from plants carrying only marker constructs (A, C). Scale bars=50 μm. (E, F) qRT-PCR showed reduced levels of WOX1 (E) and WOX3 expression (F) in ovules from the wild type (WT) and pCLE19:CLE19<sub>G6T</sub>:tCLE19 transgenic plants (CLE19<sub>G6T</sub>). Data represent the mean ±SD from three independently extracted RNA samples. Asterisks indicate significant differences from the wild type (P< 0.01 by Student’s t-test).

Fig. 6. Delayed embryo sac and endosperm development in pCLE19:CLE19<sub>G6T</sub>:tCLE19 transgenic plants. (A) Smaller sizes of embryo sacs in pCLE19:CLE19<sub>G6T</sub>:tCLE19 transgenic plants (CLE19<sub>G6T</sub>) as compared with those in the wild type (WT). Box plots are used to show total embryo sac areas. The whole area occupied by the embryo and endosperm was measured. Upper and lower bars represent the largest and the smallest sizes of embryo sacs, respectively. Note that smaller embryo sac sizes were observed in transgenic plants at all four stages (globular, triangular, heart-shape, and torpedo) examined (n=30). (B) Lower numbers of endosperm nuclei in pCLE19:CLE19<sub>G6T</sub>:tCLE19 transgenic plants (CLE19<sub>G6T</sub>) compared with the wild type (WT) when counted at the triangular stage of embryo development (n=30). Significantly reduced endosperm nuclei numbers (P<0.05 by Welch’s t-test) were observed in transgenic plants (indicated by asterisks).

examine if ectopic expression of CLE19<sub>G6T</sub> in the endosperm can cause a defect in the embryo. To test the specificity of the ALE1 promoter, the ALE1 upstream region (1875 bp) was fused to the SV40-3XGFP reporter gene to create pALE1:SV40-3XGFP and transformed into Arabidopsis (Col-0). As expected, GFP expression was observed specifically in the ESR of endosperms, and no GFP expression was observed in embryos (Supplementary Fig. S1 at JXB online).

Next, a pALE1::CLE19<sub>G6T</sub> construct was made and transformed into Arabidopsis (Col-0). Among the 35 individual transgenic lines obtained, three (#1, #2, and #3) showed high ratios of seed abortion (indicated by arrowheads; Fig. 8A), with abortion frequencies of 33.3, 28.4, and 32.9%, respectively (Table 1). The phenotypes of these lines were similar to one another, and line #1 was selected for further analyses. Examination under a DIC microscope showed that embryo
Development in the aborted seeds was mostly arrested at the triangular stage (Fig. 8B, C), slightly earlier than observed in pCLE19:CLE19\textsubscript{G6T}:tCLE19 transgenic plants. These enlarged abnormal triangular embryos were often asymmetrical, most probably as a result of defective cotyledon development (indicated by arrowheads; Fig. 8C). Semi-thin sections...
in combination with PAS staining showed that endosperm cellularization occurred relatively normally in these aborted seeds (indicated by asterisks; Fig. 8D, E), while cotyledon development was severely arrested (indicated by arrowheads; Fig. 8C, E). The cell division pattern in the lower portion of the arrested embryos was normal, with a well-formed hypophysis and suspensor (Fig. 8E). It seems that expression of CLE19<sub>goT</sub> under the control of the ALE1 promoter led to an earlier defect in cotyledon establishment in embryos than that observed in pCLE19:CLE19<sub>goT</sub>:tCLE19 transgenic plants, and no evident defect in endosperm development. These data indicate that the CLE19<sub>goT</sub> expressed in the endosperm interferes with embryo development in a non-cell-autonomous manner.

**Discussion**

Genetic studies in *Arabidopsis* have identified a large number of embryo-defective (emb) mutants that exhibit a spectrum of embryo-lethal phenotypes, and endosperm development in these mutants is usually also defective (Liu and Meinke, 1998; McElver et al., 2001; Muralla et al., 2011), demonstrating indirectly that embryo and endosperm development are tightly linked. ALE1, ZOU, IKU2, and ESPI are only expressed in endosperms, not in embryos; mutations or down-regulation of these genes cause defects in embryos (Tanaka et al., 2001; Garcia et al., 2003; Xing et al., 2013; Yang et al., 2008; Costa et al., 2014), suggesting that endosperm-expressed genes are involved in plant embryogenesis. In contrast, there was no direct evidence to show that embryo-expressed genes regulate endosperm development. In this study, it was shown that CLE19, as an embryo-expressed CLE gene, regulates both embryo and endosperm development in *Arabidopsis*.

In recent years it has been shown that CLE genes function as small extracellular peptides to regulate a number of developmental processes in a non-cell-autonomous manner (Fletcher et al., 1999; Fiers et al., 2005; Murphy et al., 2012). Two CLE genes are implicated in embryo development. CLE8, which is expressed in the embryo proper and the endosperm, regulates suspensor divisions via activation of WOX8 expression (Fiume and Fletcher, 2012). *BnCLE19* was first identified in *B. napus* as a CLE gene expressed in cotyledon primordia during embryogenesis (Fiers et al., 2004). Overexpression of *BnCLE19* and treatment of wild-type *Arabidopsis* seedlings with 14 amino acid CLE19 peptides led to CLV2-dependent premature stem cell differentiation in the root meristem (Fiers et al., 2004, 2005). Since the T-DNA insertional *cle19* mutant in *Arabidopsis* showed no visible phenotype (Fiers et al., 2004), the function of the endogenous CLE19 remains unknown. Expression analyses performed in this study revealed that, in addition to those epidermal cells in cotyledon primordia as in *BnCLE19* (Fiers et al., 2004), *CLE19* in *Arabidopsis* was also expressed in epidermal cells of hypocotyls and root caps, while no expression was observed in the endosperm. Further studies showed that transgenic plants carrying the pCLE19:CLE19<sub>goT</sub>:tCLE19 construct exhibited a dominant seed abortion phenotype, with defective cotyledon establishment in embryos and compromised nuclear proliferation and cellularization in endosperms, suggesting a role for CLE19 in co-ordinating embryo and endosperm development. This hypothesis was further supported by the observation that the defective cotyledon establishment phenotype in pCLE19:CLE19<sub>goT</sub>:tCLE19 transgenic plants was mimicked when CLE19<sub>goT</sub> was expressed under the control of an endosperm-specific ALE1 promoter. It has been reported before that synthetic 14 amino acid CLE19 peptide applied to seedlings in *vitro* promotes stem cell differentiation in root meristems (Fiers et al., 2005; Tamaki et al., 2013). Most probably CLE19 peptides produced in embryos act as a differentiation-promoting signal to regulate cotyledon establishment in embryos, and to promote nuclear division and cellularization in endosperms. Further studies are needed to identify receptors involved in perceiving the CLE19 peptide.

The formation of cotyledons is a milestone event in embryo development, marking the initiation of a major organogenesis phase in the embryo. Auxin biosynthesis and transport are involved in cotyledon initiation, determining the emergence and the positions of cotyledon primordia (Liu et al., 1993; Furutani et al., 2004; Cheng et al., 2007). In addition, several WOX family transcription factors are expressed during cotyledon formation: WOX1 and WOX3 are expressed at positions where the two cotyledon primordia are formed (Haefcker et al., 2004), while WOX2 is expressed in the apical region of the globular stage embryos (Haefcker et al., 2004). Although the wox1/wox3 double mutant exhibited no defect in cotyledon formation, wox1/wox2/wox3 triple mutations enhanced the wox2 defect in cotyledon formation (Breuninger et al., 2008), suggesting a synergic interaction among these transcription factors. CUP-SHAPED COTYLEDON 1 (CUC1) and CUC2 are expressed in the boundary of two cotyledons and regulate the final patterning of cotyledons (Aida et al., 1997, 1999; Vroemen et al., 2003), while ASYMMETRIC LEAVES 1 (AS1) and AS2 regulate the adaxial–abaxial pattern of the cotyledons (Byrne et al., 2000; Lin et al., 2003). Data from this study showed that the primary defect of pCLE19:CLE19<sub>goT</sub>:tCLE19 transgenic plants is the establishment, not the initiation, of these cotyledons, suggesting a multilayered regulation of cotyledon formation.

It is also interesting to note that only a fraction of seeds produced in pCLE19:CLE19<sub>goT</sub>:tCLE19 and pALE1::CLE19<sub>goT</sub> transgenic plants showed defects in embryo development; the remaining embryos are able to form normal seeds. Such an incomplete penetration of phenotypes has been reported in overexpression or mutations of several CLE genes. For example, when *BnCLE19* was overexpressed under the control of the CaMV 35S promoter, only 17% of the transgenic plants obtained exhibited the short-root and pin-shaped pistil phenotypes (Fiers et al., 2004). In the homozygous *cle8-1* mutant (with a point mutation in the CLE motif), only 15% of embryos showed defects in embryogenesis (Fiume and Fletcher, 2012). It is believed that dosage effects, regulated most probably at the post-transcriptional peptide processing levels, are very important for peptide hormones.

There is no doubt that the antagonistic peptide technology provides a powerful *in vivo* tool to study the function of
CLE genes (Song et al., 2013). The technology has already been used to elucidate the role of CLE22 in root development (Song et al., 2013), and CLE45 in phloem differentiation (Rodriguez-Villalon et al., 2014). The phenotypic and genetic analyses of the CLE19 gene in Arabidopsis by expression of the CLE19<sub>G6T</sub> construct under control of the endogenous CLE19 promoter or the endosperm-specific ALE1 promoter in this work allowed the roles of CLE19 in co-ordinating embryo and endosperm development to be defined. When endogenous regulatory elements are used in this type of study, it is expected that the antagonistic peptides produced may act in situ to interfere with the signal transduction pathways involved. One study published in this issue showed that synthetic CLE peptides with a G6T substitution are less effective than wild-type peptides in seedling treatments in vitro (Czyzewicz et al., 2015). This is expected since it has been shown in CLV3 that the G6 residue is critical for the CLV3 function: (i) a clv3-1 mutant with a severe defect in SAM homeostasis was caused by a substitution in G6 (Fletcher et al., 1999); (ii) pCLV3:CLV3<sub>G6A</sub>; tCLV3 is one of the least effective constructs in complementing the clv3-2 mutant (Song et al., 2012); and (iii) no transgenic plants carrying the pCLV3:CLV3<sub>G6T</sub>; tCLV3 construct showed complete complementation of the clv3-2 phenotype (Song et al., 2013). Although it has been shown that treatment of wild-type Arabidopsis seedlings with CLV3<sub>G6T</sub> Peptide in vitro, with peptides being refreshed every day, caused a slightly enlarged SAM phenotype (a weak clv3-like phenotype; Song et al., 2013), whether the antagonistic peptide technology could be used effectively in vitro remains to be evaluated further.

In summary, the results obtained in this study suggest that the CLE19 expressed in cotyledon primordia of Arabidopsis may act as a peptide ligand that constitutes a diffusible signal to regulate cotyledon establishment in embryos and to promote the nuclear proliferation and cellularization in endosperms.

### Supplementary data

Supplementary data are available at JXB online

**Figure S1.** ALE1 promoter activity in seed development.

**Table S1.** Primers used in this study.

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