CORONA, PHABULOSA AND PHAVOLUTA collaborate with BELL1 to confine WUSCHEL expression to the nucellus in Arabidopsis ovules

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RESEARCH REPORT

CORONA, *PHABULOSA* and *PHAVOLUTA* collaborate with *BELL1* to confine *WUSCHEL* expression to the nucellus in *Arabidopsis* ovules

Toshihiro Yamada[1,*], Yusuke Sasaki[1], Kayo Hashimoto[2,3], Keiji Nakajima[2] and Charles S. Gasser[4]

ABSTRACT

Angiosperm ovules consist of three proximal-distal domains – the nucellus, chalaza and funiculus – demarcated by developmental fate and specific gene expression. Mutation in three paralogous class III homeodomain leucine zipper (HD-ZIPIII) genes leads to aberrations in ovule integument development. Expression of *WUSCHEL* (*WUS*) is normally confined to the nucellar domain, but in this triple mutant expression expands into the chalaza. MicroRNA-induced suppression of this expansion partially suppresses the effects of the HD-ZIPIII mutations on ovule development, implicating ectopic *WUS* expression as a component of the mutant phenotype. *bell1* (*bel1*) mutants produce aberrant structures in place of the integuments and *WUS* is ectopically expressed in these structures. Combination of *bel1* with the HD-ZIPIII triple mutant leads to a striking phenotype in which ectopic ovules emerge from nodes of ectopic *WUS* expression along the funiculi of the primary ovules. The synergistic phenotype indicates that *BEL1* and the HD-ZIPIII genes act in at least partial independence in confining *WUS* expression to the nucellus and maintaining ovule morphology. The branching ovules of the mutant resemble those of some fossil gymnosperms, implicating *BEL1* and HD-ZIPIII genes as players in the evolution of the unbranched ovule form in extant angiosperms.

KEY WORDS: Ovule, HD-ZIPIII, *WUS*, *BEL1*, Chalaza, Integument, *Arabidopsis thaliana*

INTRODUCTION

Ovules are the developmental precursors of seeds. In angiosperms, the ovule consists of three developmental domains; the nucellus, chalaza and funiculus (Fig. 1A) (Balasubramanian and Schneitz, 2000). The nucellus contains a megasporangium, where the female gametophyte develops. The inner and outer integuments form from the chalaza and enclose the nucellus. The funiculus is a stalk-like structure that connects the ovule to the ovary wall. For correct development of the ovules, it is essential to establish this proximal-distal patterning; hence, a shift in the boundary between domains results in aberrant ovule morphology (e.g. Balasubramanian and Schneitz, 2000; Gross-Hardt et al., 2002).

WUSCHEL (*WUS*) is a homeobox gene that characterizes the nucellus by its restricted expression in this domain, and this nucellar expression is necessary for initiation of the two integuments that develop from the chalaza (Gross-Hardt et al., 2002). *WUS* promotes the expression of an auxin efflux facilitator, *PIN-FORMED 1* (*PIN1*), via transcriptional activation of *SPOROCYTELESS* [*SPL*; also known as *NOZZLE* (*NZZ*)], and this mechanism is necessary for nucellus formation (Bencivenga et al., 2012). Extension of the integuments is incomplete when *WUS* expression is driven in the chalzal domain by the *AINTEGUMENTA* promoter (*pANT*) (Gross-Hardt et al., 2002). In *pANT≫WUS* plants additional putative outer integuments also emerge just below the extended *WUS* expression area (Gross-Hardt et al., 2002; Sieber et al., 2004), as if a new boundary is established between the nucellus and chalaza. These results indicate that *WUS* regulation is key to defining the nucellus-chalaza boundary.

A recent study showed that externally applied cytokinin expands *WUS* expression into the chalaza (Bencivenga et al., 2012). *WUS* expression is also altered in *bell1* (*bel1*) (Bencivenga et al., 2012; Brambilla et al., 2007) (see Fig. S2), but the change in the expression is not as profound, suggesting that other factors play roles in *WUS* regulation.

Class III homeodomain leucine zipper (HD-ZIPIII) genes establish the identity of the adaxial tissue of lateral organs (Emery et al., 2003; McConnell et al., 2001), as well as regulating *WUS* expression in shoot and floral apices in cooperation with *CLAVATA3* or *ERECTA* (*ER*) homologs (Green et al., 2005; Landau et al., 2015; Lee and Clark, 2015; Mandel et al., 2014). In ovules, HD-ZIPIII genes are involved in the development of the integuments; thus, in most ovules of loss-of-function HD-ZIPIII gene mutants [i.e. *corona phabulosa phavoluta* (*cna phb phv*)] the integuments are absent, reduced or malformed (Kelley et al., 2009). The mechanism of these effects remains unclear.

Here, we show that *CNA*, *PHB* and *PHV* cooperatively repress *WUS* expression in the chalaza, and that this repression is important for ovule development. A combination of these mutations with *bel1* leads to additional misexpression of *WUS* outside of the nucellus and a novel phenotype not seen in either class of mutant. Thus, we identify transcription factors necessary for boundary demarcation between the nucellus and chalaza and for patterning of *WUS* expression.

RESULTS AND DISCUSSION

WUS expression extends into the chalaza in *cna phb phv*

In *cna phb phv* carpels with ovules at stage 2 (for stages, see Schneitz et al., 1995), *WUS* expression was ~1.8-fold higher than in wild-type (WT) carpels at the same stage (Fig. S1). Since gynoecium formation is almost completed in *cna phb phv* at this stage, we hypothesize that the increase results from elevated *WUS* expression in developing ovules.

In contrast to WT, where *WUS* expression was confined to the nucellus (Fig. S2), in most *cna phb phv* ovules at stage 2-II, *WUS*

[1]School of Natural System, College of Science and Engineering, Kanazawa University, Kanazawa 920-1192, Japan. [2]Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan. [3]Graduate School of Humanities and Sciences, Nara Women's University, Nara 630-8506, Japan. [4]Department of Molecular and Cellular Biology, University of California, Davis, CA 95616, USA.

*Author for correspondence (ptilo@mb.infoweb.ne.jp)

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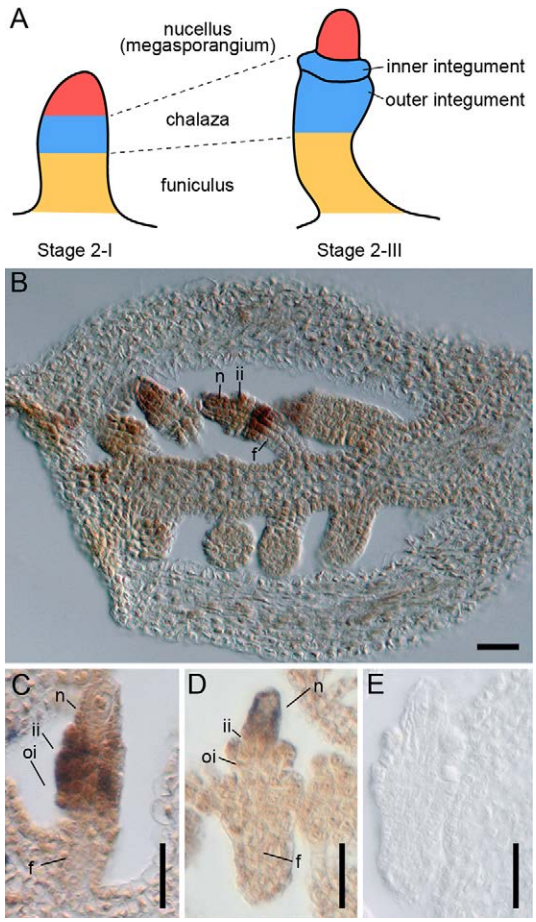

Fig. 1. Patterning of ovules and *WUS* expression in *cna phb phv*. (A) Three developmental domains in *Arabidopsis* ovules. (B-E) *WUS* expression in *cna phb phv* at stage 2-I (B) and stage 2-III (C-E). (E) Negative control hybridized with sense probe. f, funiculus; n, nucellus; ii, inner integument; oi, outer integument. Scale bars: 25 µm.

expression extended into the chalaza, including the integuments (Fig. 1B). Misexpression of *WUS* in the chalaza was still observed in ovules at stage 2-III, whereas expression in the nucellus decreased (Fig. 1C). A subset of ovules did, however, exhibit WT-like expression of *WUS* (Fig. 1D). In *cna phb phv* some ovules do develop normally, although most exhibit aberrant integument development (Kelley et al., 2009). Coexistence of ovules with normal and abnormal *WUS* expression is thus consistent with the ovule phenotypes.

In summary, *CNA*, *PHB* and *PHV* are required for preventing *WUS* expression in the chalaza. However, the sporadic WT-like phenotype in *cna phb phv* ovules implies that other gene(s) are also involved in the regulation of *WUS*.

Ovule defects in *cna phb phv* are suppressed by *pCNA:amiRWUS*

Growth of the integuments was disturbed in *pANT≫WUS* plants (Gross-Hardt et al., 2002). Thus, the misexpression of *WUS* could account for the aberrant shape of the integuments in *cna phb phv*.

The ectopically expressed *WUS* in *cna phb phv* was knocked down using an artificial microRNA for *WUS* (*amiRWUS*). Since the *CNA* promoter (*pCNA*) drives gene transcription throughout the chalaza, including the two integuments, we utilized *pCNA* as the driver of *amiRWUS* expression (Fig. S1). In gynoecia of *cna phb phv pCNA:amiRWUS* at stage 2, *amiRWUS* reduced *WUS* expression to a level that averaged 60% of that observed in *cna phb phv* (Fig. 2A). Suppression of *WUS* expression was also evaluated using a *gWUS-GFP*$_3$ transgene (Tucker et al., 2008) (Fig. S2). This gene showed an expanded GFP signal in *cna phb phv*, but the expanded signal was absent from the chalaza in almost all ovules of *cna phb phv pCNA:amiRWUS* (compare Fig. 2C,E with 2D,F; Fig. S2).

Development of the integuments was restored in most ovules of *cna phb phv pCNA:amiRWUS* (compare Fig. 2G,I with 2H,J). The percentage of normal ovules per carpel significantly increased in *cna phb phv pCNA:amiRWUS* as compared with *cna phb phv* (Fig. 2B). Therefore, the misexpression of *WUS* partly accounts for the aberrant shape of the integuments in *cna phb phv*.

Similar to phenotypes of loss-of-function *cna phb phv* mutants, ovules in HD-ZIPIII gain-of-function mutants, such as *phb-1d* or *phv-1d*, have aberrant integuments (Kelley et al., 2009). However, in *phb-1d*, *WUS* expression did not differ from WT (Sieber et al., 2004), suggesting that *PHB* has effects on integument growth that are independent of *WUS* repression.

As in lateral organ primordia, *CNA*, *PHB* and *PHV* are expressed in the adaxial tissue of the inner integument. Thus, it is suggested that polarity establishment is also required for inner integument expansion (Kelley et al., 2009). The aberrant ovules in *cna phb phv pCNA:amiRWUS* suggest that *CNA*, *PHB* and *PHV* promote integument growth by other mechanisms, such as establishing adaxial-abaxial polarity, as well as by repressing *WUS*. Alternatively, these aberrant ovules might be attributed to variability in expression of the *amiRWUS* transgene.

HD-ZIPIII expression is not sufficient to repress *WUS*

Seeds are formed even when either *CNA*, *PHB* or *PHV* is constitutively expressed by the CaMV 35S promoter (Prigge et al., 2005), in contrast to the seedless phenotype of *wus* (Gross-Hardt et al., 2002), suggesting that HD-ZIPIII factors do not directly repress *WUS* expression. We drove expression of *CNA* under the control of the *WUS* promoter to corroborate these results. Since HD-ZIPIII transcripts are post-transcriptionally targeted for degradation by microRNA (miR) 165/166 (Emery et al., 2003), we used *CNA-δmiRNA*, in which the miRNA binding site is modified to be insensitive to miR165/166, in addition to WT *CNA*.

Both in *pWUS:CNA-δmiRNA* (Fig. 2L,O) and *pWUS:CNA* (Fig. 2M,P), ovules did not obviously differ from those of WT (Fig. 2K,N), suggesting that *CNA* requires other factors to regulate *WUS* in the chalaza.

Pattern of cytokinin regulation is not altered in *cna phb phv*

WUS expression extends into the chalazal domain when cytokinin is externally applied to the gynoecium (Bencivenga et al., 2012). Therefore, misexpression of *WUS* in *cna phb phv* might result from cytokinin upregulation. We compared cytokinin responses between WT and *cna phb phv* ovules using a *TCS:GFP* marker (Müller and Sheen, 2008).

In WT ovules, cytokinin response is observed in the chalaza and funiculus at stage 2-III and later (Fig. 3A,B) (Bencivenga et al., 2012). The same pattern is observed in *cna phb phv* ovules with aberrant (Fig. 3C,D) or normal (Fig. 3E,F) integuments, but expression levels are reduced, compared with WT (Fig. 3G). These data indicate that *CNA*, *PHB* and *PHV* regulation of *WUS* expression is not by means of cytokinin upregulation. In addition, ovule morphology is not affected when cytokinin degradation is promoted by constitutive expression of Cytokinin oxidase/dehydrogenase genes (Werner et al., 2003), supporting the conclusion that the *cna phb phv* phenotype is independent of cytokinin regulation.

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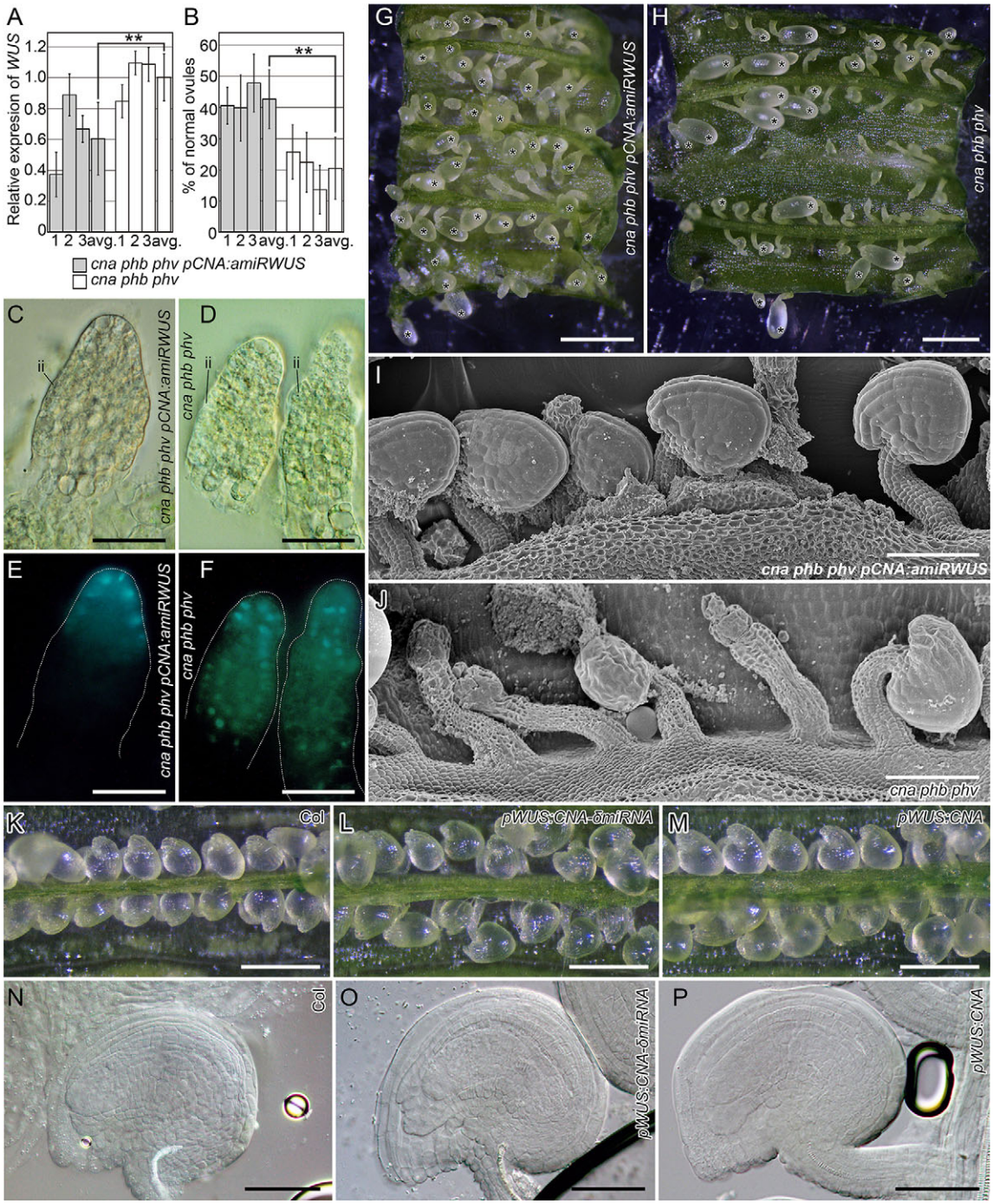

Fig. 2. Phenotypes of *cna phb phv*, *cna phb phv pCNA:amiRWUS*, *pWUS:CNA-δmiRNA* and *pWUS:CNA*. (A,B) Relative expression of *WUS* quantified by qRT-PCR with three biological replicates (A), and percentage of normal ovules per gynoecium (n=15) (B). Three independent lines were examined. **P<0.01, Student's t-test. Error bars indicate s.d. (C-F) DIC images of stage 2-II ovules (C,D) and expression of *WUS* monitored by the *gWUS-GFP*$_3$ transgene (E,F) in *cna phb phv amiRWUS* (C,E) and *cna phb phv* (D,F). (G-J) Ovule phenotypes of *cna phb phv amiRWUS* (G,I) or *cna phb phv* (H,J) observed by stereomicroscope (G, H) or SEM (I,J). Asterisks indicate normal ovules. (K-P) Ovules of WT (Col) (K,N), *pWUS:CNA-δmiRNA* (L,O) and *pWUS:CNA* (M,P) at stage 4-I. ii, inner integument. Scale bars: 25 µm in C-F; 50 µm in N-P; 100 µm in I,J; 0.25 mm in K-M; 0.5 mm in G,H.

bel1 and *cna phb phv* exhibit synergistic phenotypes

In *bel1* an amorphous structure forms in place of the integuments and can convert to a carpelloid organ (e.g. Robinson-Beers et al., 1992; Ray et al., 1994). In *bel1* ovules *WUS* expression is shifted downward into the boundary region between the nucellus and chalaza (Bencivenga et al., 2012) (Fig. S2). Thus, we crossed *cna phb phv* with *bel1* to evaluate the interaction of the genes.

bel1 cna phb phv ovules form the amorphous structure seen in *bel1*, but the primary ovulate axis was observed to branch through the formation of ectopic ovule primordia (Fig. 3H,I). These extra primordia were formed directly on the funiculus below the chalaza, in contrast to the extra putative nucelli of *bel1*, which are born on the adaxial side of the amorphous organ (Robinson-Beers et al., 1992). The ectopic ovules form amorphous organs typical of *bel1* mutants in place of integuments (Fig. 3I). Formation

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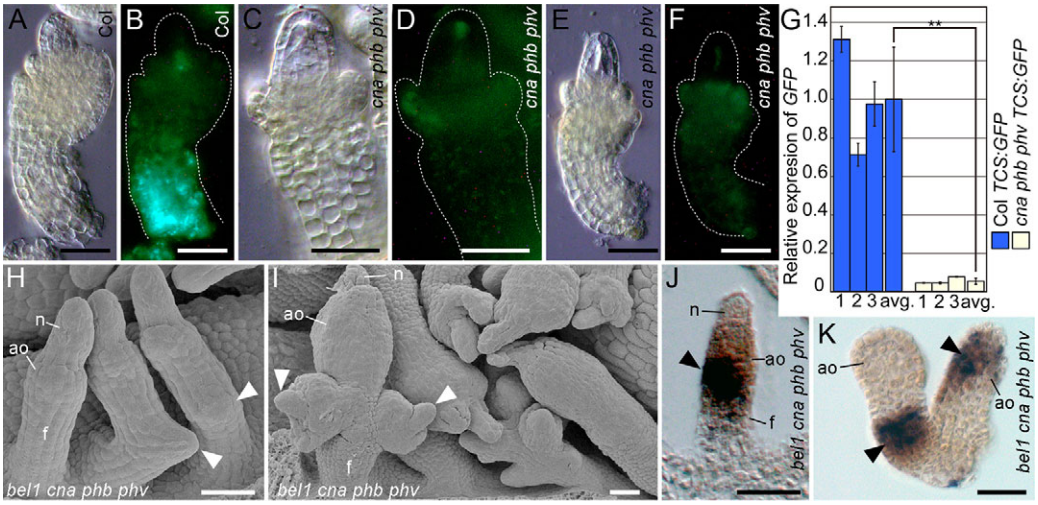

Fig. 3. Cytokinin responses in *cna phb phv* and phenotypes of *bel1 cna phb phv*. (A,C,E) DIC images of stage 2-III ovules. (B,D,F) GFP expression from the *TCS:GFP* transgene. (A,B) WT (Col). (C,D) *cna phb phv* ovule with aberrant integuments. (E,F) *cna phb phv* with normal integuments. (G) Relative expression of *GFP* quantified by qRT-PCR with three biological replicates. ***P*<0.01, Student's *t*-test. Error bars indicate s.d. (H-K) SEM images (H,I) and *WUS* expression (J, K) in *bel1 cna phb phv* ovules. Ectopic ovule primordia (H,I) or ectopic nodes of *WUS* expression where ectopic ovules will form (J,K) are indicated by arrowheads. f, funiculus; n, nucellus; ao, amorphous organ. Scale bars: 25 µm.

of ectopic ovules is preceded by nodes of ectopic *WUS* expression in the funiculus (Fig. 3J,K). *WUS* is expressed in the nucellus and chalaza of *bel1 cna phb phv* ovules (Fig. 3J), as in *cna phb phv* ovules (Fig. 1B,C). However, stronger expression was detected in the nucelli of the ectopic ovules at the same stage in their development (Fig. 3J). As the amorphous organ enlarged, *WUS* expression decreased in the nucellus and chalaza of the primary ovulate axis, whereas expression persisted in the ectopic ovules (Fig. 3K).

The ovulate axis does not branch in *cna phb phv* (Kelley et al., 2009) nor in *bel1* (Robinson-Beers et al., 1992). Consistent with the novel phenotype of *bel1 cna phb phv*, the *WUS* expression pattern in this quadruple mutant is different from that observed in *bel1* (Fig. S2) or in *cna phb phv* (Fig. 1B,C). These synergistic phenotypes suggest that *CNA*, *PHB* and *PHV* act in a different pathway from *BEL1* to regulate *WUS*. The combination of the two classes of mutations appears to allow for *WUS* expression further down the ovule axis than is observed in either single class. The formation of ectopic ovules from the funiculus implies that cells in the funiculus manifest placenta-like properties in the quadruple mutant.

CNA, *PHB* and *PHV* establish the boundary between nucellus and chalaza in *Arabidopsis* ovules

We show that *CNA*, *PHB* and *PHV* play a major role in the negative regulation of *WUS* in the chalaza. This regulation contributes to the establishment of the boundary between the nucellus and chalaza and promotes the proper development of two integuments. *CNA*, *PHB* and *PHV* repress *WUS* expression independently from *BEL1* or cytokinin upregulation (Fig. 4A).

Integument growth is normal in *cna*, *phb* and *phv* single mutants (Kelley et al., 2009), suggesting that they redundantly repress *WUS* expression. In stage 2 ovules, expression of *CNA*, *PHB* and *PHV* does not completely overlap (Kelley et al., 2009; Sieber et al., 2004) (Fig. 4B), whereas *WUS* is misexpressed throughout the chalaza in *cna phb phv*, a much broader area than the sum of the *CNA*, *PHB* and *PHV* expression areas. This discrepancy would imply that *CNA*, *PHB* and *PHV* regulate the expression of *WUS* through their repression of a diffusing factor or an action of the factor (X in Fig. 4). Auxin is a possible candidate for this factor because it is predicted to flow through the chalaza (Bencivenga et al., 2012; Kelley et al., 2012), and alterations in the pattern of auxin response correlate with ectopic *WUS* expression seen in *bel1* mutants (Bencivenga et al., 2012). Alternatively, *CNA* or *PHV* might have broader expression areas in stage I ovules, as actually reported for *PHB* (Sieber et al., 2004), and failure in suppression at this stage could affect the expression patterns of *WUS* at subsequent stages.

The striking synergistic branched ovule phenotype observed in *bel1 cna phv phb* mutants implies that the two classes of genes act in relative independence in their suppression of *WUS* activity outside of the nucellus. Angiosperm ovules are hypothesized to be homologous to the cupulate organs of some extinct gymnosperms, each of which were born on the apices of a branched axis (e.g. Doyle, 2006), but such branched structures are not observed in extant angiosperms. If this hypothesis is correct, then *BEL1* and HD-ZIPIIIs could have played a role in the evolution of the unbranched ovule form seen in all extant angiosperms.

MATERIALS AND METHODS

Growth conditions and plant materials

Arabidopsis plants were grown under continuous light at 23°C on soil. Seeds were sourced as described in the supplementary Materials and Methods.

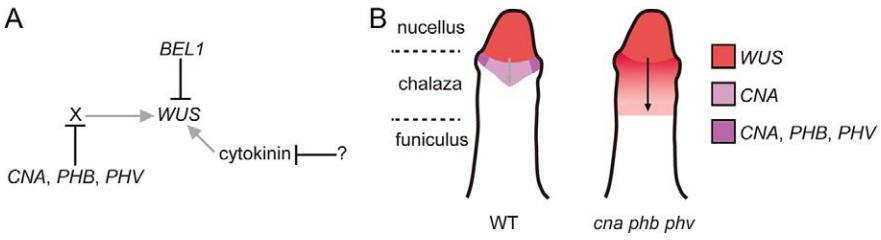

Fig. 4. Model of *WUS* regulation in chalaza. (A) Pathways repressing *WUS* expression in chalaza. X, a putative diffusing activator. The question mark represents an as yet unidentified inhibitor of cytokinin. (B) *WUS* and HD-ZIPIII expression areas in stage 2-II ovule. Movement of a factor is indicated by arrows in B.

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Construction of transgenic lines

The *amiRWUS* fragment was synthesized following Web MicroRNA Designer 3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). *amiRWUS* binds 607 to 627 nucleotides of *WUS* (Fig. S1). *amiRWUS*, as well as *pCNA* (−4046 to −319), were inserted into pMLBarT (Fig. S1) using the GeneArt Seamless PLUS Cloning and Assembly Kit (Life Technologies). *bel1-6/+ cna-2 phb-13 phv-11 er-2* plants were transformed with the construct by the floral dip method (Clough and Bent, 1998).

To generate *pWUS:CNA-δmiRNA* or *pWUS:CNA*, the *WUS* promoter and *CNA* cDNA sequences were obtained from Col-0 genomic DNA or cDNA by PCR, respectively. *CNA-δmiRNA* sequence was synthesized by overlap PCR as previously described (Emery et al., 2003). These fragments were cloned into pMLBarT as described above.

Further details of constructs and genotyping are given in the supplementary Materials and Methods and Table S1.

In situ hybridization and GUS staining

Fixation, embedding of tissue and *in situ* hybridization were performed as previously described (Mayer et al., 1998). GUS staining of *pWUS>>uidA* plants is described in the supplementary Materials and Methods.

Microscopy

For scanning electron microscopy (SEM), ovules were fixed (McAbee et al., 2006) or epoxy molds were made (Williams et al., 1987). Fluorescence images of GFP were taken with excitation and emission wavelengths of 470/20 nm and 505-530 nm, respectively, using an Axio Scope 2 Plus (Carl Zeiss).

qRT-PCR analysis

Total RNAs were extracted from gynoecia containing stage 2 ovules and contaminating DNA was digested with DNase. qRT-PCR analyses were performed using the One Step SYBR PrimeScript RT-PCR Kit (Takara). *WUS* and *GFP* expression levels were normalized to those of *PP2AA3* (At1g13320) (Czechowski et al., 2005). For further details, see the supplementary Materials and Methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

T.Y. and C.S.G. designed the study. T.Y. and Y.S. performed most experiments. K.H. and K.N. made constructs. T.Y. and C.S.G. wrote the manuscript. All authors commented on the manuscript.

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Supplementary information

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