RESEARCH REPORT

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

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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 independence in confining nucellus and chalaza and for patterning of ovule morphology. The synergistic phenotype indicates that WUS regulation is key to defining the nucellus-chalaza boundary.

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 expressions are elevated in the chalaza, and thus we identify transcription factors necessary for boundary demarcation between the nucellus and chalaza and for patterning of WUS expression.
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.

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.

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 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,1 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 integment 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 integment 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 does not require 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.
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

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-GFP3 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.
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; Doyle, 2006), but such branched structures are not observed in extant angiosperms. If this hypothesis is correct, then BEL1 and HD-ZIPIIIIs 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.
Construction of transgenic fragments

The *amiRNA* fragment was synthesized following Web MicroRNA Designer 3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). *amiRNA* binds 607 to 627 nucleotides of *WUS* (Fig. S1). *amiRNA*, as well as *PCNA* (~4046 to ~319), were inserted into pMLBarT (Fig. S1) using the GeneArt Seamless PLUS Cloning and Assembly Kit (Life Technologies). *bell1-6/+ cua-2 pbh-13 phv-11 er-2* plants were transformed with the construct by the floral dip method (Clough and Bent, 1998).

To generate p*WUS: CNA*-6miRNA or p*WUS: CNA*, the *WUS* promoter and *CNA* cDNA sequences were obtained from Col-0 genomic DNA or cDNA by PCR, respectively. *CNA*-6miRNA 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 p*WUS:uidA* plants is described in the supplementary Materials and Methods.

### Microscopy

For scanning electron microscopy (SEM), ovules were fixed (McAbbe 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 *PP2A3* (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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