Arabidopsis PRC1 core component AtRING1 regulates stem cell-determining carpel development mainly through repression of class I KNOX genes

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Abstract

Background: Polycomb repressive complex 2 (PRC2)-catalyzed H3K27me3 marks are tightly associated with the WUS-AG negative feedback loop to terminate floral stem cell fate to promote carpel development, but the roles of Polycomb repressive complex 1 (PRC1) in this event remain largely uncharacterized.

Results: Here we show conspicuous variability in the morphology and number of carpels among individual flowers in the absence of the PRC1 core components AtRING1a and AtRING1b, which contrasts with the wild-type floral meristem consumed by uniform carpel production in Arabidopsis thaliana. Promoter-driven GUS reporter analysis showed that AtRING1a and AtRING1b display a largely similar expression pattern, except in the case of the exclusively maternal-preferred expression of AtRING1b, but not AtRING1a, in the endosperm. Indeterminate carpel development in the atring1a;atring1b double mutant is due to replum/ovule-to-carpel conversion in association with ectopic expression of class I KNOX (KNOX-I) genes. Moreover, AtRING1a and AtRING1b also play a critical role in ovule development, mainly through promoting the degeneration of non-functional megaspores and proper integument formation. Genetic interaction analysis indicates that the AtRING1a/b-regulated KNOX-I pathway acts largely in a complementary manner with the WUS-AG pathway in controlling floral stem cell maintenance and proper carpel development.

Conclusions: Our study uncovers a novel mechanistic pathway through which AtRING1a and AtRING1b repress KNOX-I expression to terminate floral stem cell activities and establish carpel cell fate identities.

Keywords: Polycomb, AtRING1, KNOX-I, Floral stem cell, Carpel development

Background

The development of both animals and plants relies on stem cells, which are defined by their ability to renew themselves and give rise to daughter cells that differentiate and contribute to tissue and organ formation. In higher plants, stem cells reside in meristems, and cell lineage is easily traceable due to the immobility of cells. The shoot apical meristem (SAM) initiates at the embryo stage, and continuously produces the aerial part of the plant during post-embryonic growth. Upon transition to the reproductive phase, SAM usually shifts to the fate of inflorescence meristem (IM) and subsequently generates floral meristems (FMs) from the IM flanks [1–4]. Distinct from the indeterminacy of SAM and IM, the determinate FM produces a fixed number of peripheral floral organs around a central population of stem cells that are consumed in the formation of carpels. The ovules emerge from the meristematic placenta within the carpel, undergo the production of the embryo sac (ES, the female gametophyte/megagametophyte), and upon double fertilization ultimately give rise to seeds [5, 6]. For female gametophyte development, firstly, a single and enlarged megaspore mother cell (MMC) differentiates...
from the archesporial cell at the tip of the ovule primordium and undergoes meiosis to develop a tetrad of four haploid megaspores (developmental stage FG1). Normally the chalazal-proximal one survives and becomes the functional megasporangium. This megasporangium undergoes three rounds of mitotic division and cellularization to give rise to an eight-nucleate/seven-celled female gametophyte, which comprises three antipodal cells, two synergid cells, one central cell containing two unfused polar nuclei, and one egg cell (developmental stage FG5) [7].

The class I KNOX (KNOX-I) family gene SHOOT MERISTEMLESS (STM) and the feedback loop formed by CLAVATA (CLV) and WUSCHEL (WUS) have independent but complementary functions in shoot stem cell maintenance. For instance, STM prevents stem cell differentiation, while WUS specifies stem cell identity (reviewed in [8]). The knockdown mutants stm and wus display very similar flower phenotypes, such as the absence of carpels and a reduced number of other floral organs. In addition to STM, the other KNOX-I family genes KNAT1/BREVIPEDICELLUS (BP), KNAT2, and KNAT6 may also have a role in carpel development because overexpression of either STM or KNAT2 can induce ectopic carpel formation and ovule-to-carpel homeotic conversion within the gynoecium [9]. Very importantly, AGAMOUS (AG) plays a key role in the termination of floral stem cell maintenance. At flower developmental stage 3, WUS together with LEAFY (LFY) activate AG, which in turn shuts off WUS expression at stage 6, leading to the termination of stem cell maintenance and the initiation of carpel primordia [10–14]. Either ag, displaying spatially restricted but delayed WUS extinction, or clv, displaying an enlarged WUS expression domain, is sufficient to induce FM indeterminacy [13–18]. Thus, AG combined with the CLV-WUS feedback loop regulates carpel development, conveniently named the WUS-AG pathway. Recent studies demonstrate that some Polycomb group (PcG) proteins play an essential role within the WUS-AG pathway to terminate floral stem cell fate [19, 20].

PcG proteins constitute two major types of complexes: Polycomb repressive complex 2 (PRC2), which catalyzes histone H3 lysine 27 trimethylation (H3K27me3) on target chromatin, and PRC1, which acts as both the H3K27me3 reader and the histone H2A lysine 11 monoubiquitination (H2A K11ub1) writer. Arabidopsis PRC2 components are able to form at least three different complexes involved in somatic cell fate determinacy, vegetative development maintenance, vernalization, flower timing regulation, and seed development (reviewed in [21]). Arabidopsis PRC1 core components, including LIKE HETEROCROMOTIN PROTEIN1 (LHP1), AtBMI1, and AtRING1, display different evolutionary conservation [22]. Though LHP1 can interact with AtRING1 and AtBMI1 in vitro [23], the mutant phenotype of lhp1 shows some degree of difference from that of the atrring1a atring1b or atbmi1a atbmi1b double mutant. Furthermore, LHP1 was recently reported to co-purify with the PRC2 complex in vivo [24, 25], indicating that LHP1 is more closely associated with PRC2 in this specific context than PRC1. Arabidopsis PRC1 RING finger proteins AtRING1 and AtBMI1 act as the most conserved components involved in preventing seed germination and development of somatic embryo traits [23, 26, 27], maintaining stem cell identity [28], and promoting floral transition [29]. Intriguingly, atrring1a atring1b mutants display abnormal flower developmental phenotypes, yet the underlying mechanisms remain to be investigated.

In this study, we show that AtRING1a and AtRING1b play an essential role in Arabidopsis floral stem cell maintenance and carpel development, primarily via repression of the KNOX-I family genes. Both AtRING1a and AtRING1b genes display very similar expression patterns throughout the whole plant life cycle, except for the imprinting expression of AtRING1b, but not AtRING1a, in the endosperm. Indeterminate carpel growth in the atrring1a atring1b mutant is associated with homeotic replum-to-carpel and ovule-to-carpel conversions. Further molecular and genetic analyses demonstrate that AtRING1a/b modulate floral stem cell activity and carpel development, mainly through repression of the KNOX-I pathway. Lastly, our analyses indicate that defective ovule development in the atrring1a atring1b mutant is essentially due to survival of non-functional megaspores, growth arrest of integuments, and overproliferation of the nucellus.

Results

AtRING1a and AtRING1b display overlapping as well as different tissue-specific expression patterns

Reverse transcription polymerase chain reaction (RT-PCR) detected broad expression of AtRING1a and AtRING1b in multiple types of plant organs [28]. A more detailed analysis using the AtRING1a:AtRING1a::GUS reporter line showed that AtRING1a is expressed in the SAM, root apical meristem (RAM), the junction between shoot and root, and young leaves [23]. Here we extend the AtRING1a:AtRING1a::GUS expression analysis in different reproductive floral organs as well as during embryogenesis. GUS staining showed that AtRING1a is strongly expressed in floral organ primordia of inflorescences (Fig. 1a), in gynoecia and ovules (Fig. 1b and c), and in embryos throughout diverse developmental stages (Fig. 1d–g). We further verified AtRING1a expression pattern by in situ hybridization analysis of endogenous gene transcripts in wild-type (WT) plants (Additional file 1: Figure S1). The data confirmed the expression patterns determined by GUS reporter analysis and also showed that AtRING1a transcripts are detectable in both microsporocytes and megasporocytes (Additional file 1: Figure S1E and G).
Fig. 1. AtRING1a and AtRING1b exhibit similarities yet some differences in expression pattern at the reproductive stage. 

**a-g** Expression pattern of AtRING1a in AtRING1a::AtRING1a-GUS transgenic lines. 

- **a** Inflorescence. Note strong GUS staining in sepal primordia.
- **b** Floral bud at stages 8 and 9 of flower development. Note strong GUS staining in early floral organs. Unless otherwise indicated, flower developmental stages are defined according to [60].
- **c** Emerging flower. Note strong GUS staining in ovules, but none in mature pollen.
- **d** Globular stage of embryo development.
- **e** Heart stage of embryo development.
- **f** Linear cotyledon stage of embryo development.
- **g** Mature green stage of embryo development.

**h-q** Expression pattern of AtRING1b detected in AtRING1b::AtRING1b-GUS transgenic lines.

- **h** Three-day-old seedling.
- **i** One-month-old seedling.
- **j** Inflorescence.
- **k** Flower bud at stage 8.
- **l** Mature ovule.
- **m** Mature pollen and filament.
- **n** Fertilized ovule at 2 days after pollination (DAP).
- **o** Globular stage of embryo development.
- **p** Heart stage.
- **q** Bending cotyledon stage. Bars = 100 μm, except 50 μm in **a, b, j, and k.** 1 mm in **h and i**
To study the tissue specificity of \textit{AtRING1b} expression, we constructed an \textit{AtRING1b:AtRING1b-GUS} reporter containing \textit{AtRING1b} full-length genomic DNA and a ~1.6-kb promoter upsteam of the translation start site. GUS staining was performed using three independent transgenic lines; similar expression patterns were observed across all lines. During vegetative development, expression of \textit{AtRING1b:AtRING1b-GUS} was detected at high levels in SAM and RAM (Fig. 1h) and at moderate levels in young leaves and vasculature (Fig. 1i). During reproductive development, the GUS signal was strong in inflorescence floral organ primordia (Fig. 1j), gynoecia and ovules (Fig. 1k and l), early endosperm (Fig. 1n–p), and in embryos throughout a variety of developmental stages (Fig. 1o–q). These data show that \textit{AtRING1a} and \textit{AtRING1b} have largely overlapping tissue-specific expression patterns. Yet, differences also exist between \textit{AtRING1a} and \textit{AtRING1b}. Most remarkably, strong expression of \textit{AtRING1b} was detected in mature pollen grains (Fig. 1m), whereas \textit{AtRING1a} expression was barely detectable (Fig. 1c). Likewise, strong expression of \textit{AtRING1b} was detected in the endosperm during early seed development until the globular embryo stage (Fig. 1n–p), whereas \textit{AtRING1a} expression was undetectable (Fig. 1d).

\textbf{AtRING1b expression in endosperm is maternally imprinted}

The intriguing endosperm expression pattern of \textit{AtRING1b:AtRING1b-GUS} prompted us to test whether \textit{AtRING1b} is a parentally imprinted gene. Based on reciprocal crosses, we found that GUS activity was detected in the endosperm when \textit{AtRING1b:AtRING1b-GUS} ovules were fertilized by WT pollen (Fig. 2a). In contrast, GUS activity was undetectable in the endosperm when WT ovules were fertilized by \textit{AtRING1b:AtRING1b-GUS} pollen (Fig. 2b). This result indicates that only the maternal, but not the paternal, \textit{AtRING1b} allele is actively expressed in endosperm cells. To investigate whether PcG silencing and/or DNA methylation is involved in parental imprinting of \textit{AtRING1b}, we performed reciprocal crosses using the PRC2 mutant \textit{fertilization independent seed 2 (fis2)} and the \textit{DNA METHYLTRANSFERASE1} mutant \textit{met1-3} [30, 31]. The \textit{fis2} mutant behaved similarly to WT and displayed GUS activity only when \textit{AtRING1b:AtRING1b-GUS} was maternally derived but not paternally derived (Fig. 2c). In contrast, \textit{met1-3} displayed a different pattern, since GUS activity was detected in endosperm cells when \textit{AtRING1b:AtRING1b-GUS} was either maternally or paternally derived (Fig. 2d).

The \textit{AtBMI1c} gene was identified as maternally expressed in previous studies based on single nucleotide polymorphism (SNP) and RT-PCR analyses of seeds derived from crosses of different Arabidopsis ecotypes [32, 33]. In this study, we generated and tested expression of an \textit{AtBMI1c:AtBMI1c-GUS} reporter construct containing \textit{AtBMI1c} full-length genomic DNA and its ~2.1-kb upstream promoter region. Weak expression of \textit{AtBMI1c:AtBMI1c-GUS} was detected in the root tip, at the junction between shoot and root, and in pollen grains (Fig. 2e and f). High levels of expression were observed in the embryo sac and endosperm (Fig. 2g and h). Consistent with previous RT-PCR data [23], expression of \textit{AtBMI1c:AtBMI1c-GUS} was drastically increased in several tissue types in the \textit{atring1a;atring1b} mutant (Fig. 2i–m). Based on reciprocal genetic crosses, we found that \textit{AtBMI1c:AtBMI1c-GUS} has an imprinted expression pattern (Fig. 2n–q) similar to that of \textit{AtRING1b:AtRING1b-GUS} (Fig. 2a–d). Thus, both \textit{AtRING1b} and \textit{AtBMI1c} show maternally imprinted expression in the endosperm, and their genomic imprinting is regulated by \textit{MET1}-dependent CG DNA methylation but not by PRC2 silencing.

\textbf{Loss of AtRING1 drastically affects gynoecium development}

Compared with the WT Arabidopsis gynoecium consisting of two fused carpels (Fig. 3a), the \textit{atring1a;atring1b} double mutant displays diversely modified gynoecium phenotypes ranging from bulged/supernumerary to unfused carpels (Fig. 3b–d) as revealed through scanning electron microscopy (SEM) examination. To obtain further insight into the cytological basis underlying the defective gynoecium development of the \textit{atring1a;atring1b} double mutant, we created paraffin sections of gynoecium. Within WT gynoecia, marginal tissues of the carpel fusion give rise to two medial ridges (replums) that grow toward each other and eventually meet and fuse to form the septum (Fig. 3e–h). The meristematic replum is the region which gives rise to the placenta, ovules, and septa [34]. On the other hand, ovule primordia emerge from the placenta at both flanks of the replum. Then, ovule develops through MMC differentiation, functional megaspore production, and mature embryo sac formation processes. Within the \textit{atring1a;atring1b} mutant pistil, most ovule primordia are seemingly initiated from the placental region of a high number-carpel fused gynoecium (Fig. 3i and j). But later on, the replum loses its determinacy of septum fate and constantly overproliferates, developing into carpel-like organs (Fig. 3k–m) which can still produce some ovules on the margins and stigma papilla-like structures at the apex (Fig. 3n). Sometimes, the replum develops carpeloid tissues outside the gynoecium (Fig. 3c), and stigmatic features can be observed on the top of some ovules (Fig. 3o and p), together showing an ectopic overproliferation of carpeloid tissues. Thus, we conclude that homeotic conversions of replum-carpel and ovule-carpel characterize the basis of defective carpel development in the \textit{atring1a;atring1b} double mutant.
AtRING1b displays maternally imprinted expression in endosperm. a–d AtRING1b expression was analyzed in the seeds after reciprocal crosses of AtRING1b:AtRING1b-GUS (Columbia, Col background) with Col, fis2, or met1-3 at 3 DAP. e AtRING1b:AtRING1b-GUS (♀) × Col (♂). Similar patterns were observed for AtRING1b:AtRING1b-GUS (♀) × fis2 (♂) and AtRING1b:AtRING1b-GUS (♀) × met1-3. b Col (♀) × AtRING1b:AtRING1b-GUS (♂). c fis2 (♀) × AtRING1b:AtRING1b-GUS (♂). d met1-3 (♀) × AtRING1b:AtRING1b-GUS (♂). e–h Expression pattern of AtBMI1c investigated by analysis of AtBMI1c:AtBMI1c-GUS transgenic lines. i Staining in RAM (inset) and junction between root and shoot of 1-week-old seedling. j Developing anther. k Fertilized ovule at 1 DAP. l Developing seed at globular stage. m Expression pattern of AtBMI1c in atring1a;atring1b mutant in AtBMI1c:AtBMI1c-GUS lines. n Embryo-like structure produced in 2-week-old atring1a;atring1b seedling. o Developing gynoecium (about stage 9). p Developing gynoecium (about stage 12) and young ovule (inset). q Increased levels of BMI1 transcripts in the atring1a;atring1b mutant detected by qRT-PCR (Student’s t test, *p < 0.05, **p < 0.01). Error bars represent SD for three biological replicates. n–q AtBMI1c expression analyzed in the AtBMI1c:AtBMI1c-GUS construct in seeds after reciprocal crosses, as described in a–d for AtRING1b:AtRING1b-GUS. Arrowheads indicate chalazal endosperm. Bars = 100 μm, except 500 μm in e and l, and 50 μm in f.
Loss of AtRING1 function leads to defective ovule and embryo sac development

In WT Arabidopsis, the ovule exhibits determinate growth, ultimately developing into a seven-celled and eight-nucleus ES enclosed by inner and outer integuments. But in the atring1a;atring1b mutant, ovule development occasionally adapts carpel fate (Fig. 3o and p) and becomes indeterminate. In order to dissect abnormalities of ovule and ES development in the atring1a;atring1b mutant, confocal laser scanning microscopy (CLSM) and differential interference contrast (DIC) observations were carried out. WT ovules show characteristics typical of different developmental stages: FG1 to FG6 (Fig. 4a; [7]). Defective mutant ovules fall into two major classes: class I (~8%, N = 145) displaying reduced ES with relatively normal nuclear division and integument development (Fig. 4b), and class II (~92%, N = 145) displaying arrested or no ES with abnormal integument formation (Fig. 4c). Different extents of integument or nucellus defects are observed. In normal WT ovule development, prior to anthesis the nucellus degenerates, leaving the ES in direct contact with the endothelium (integumentary tapetum), differentiated from the inner layer of the inner integument (FG1 and FG2-I, Fig. 4a; [35, 36]). In contrast, most atring1a;atring1b mutant ovules display an overproliferated nucellus growing out of outer and inner integuments (Fig. 4d and e). Compared with the double integuments growing to cover and enclose the nucellus during normal ovule development, outer integument expansion in some mutant ovules (~30%, N = 145) is severely inhibited. In WT ovules the outer integument primordium initiates and grows asymmetrically, with only its adaxial side extending significantly. In contrast, uniform extension of the integuments surrounding the nucellus occurs in about 6% of the
mutant ovules, resulting in symmetric integument growth lacking an S-shaped curvature. Furthermore, ~10% of the mutant ovules exhibit homeotic conversion of integument/nucellus-to-stigmatic papilla (Fig. 4f and g). In some ovules (~5%) two nucelli are enclosed within the same integuments (Fig. 4h and i). Occasionally, the outer integument in the mutant ovule is transformed into a blade-like structure (Fig. 4j), which is reminiscent of the supposed ancestral origin, a cupule with a leaf-like structure surrounding one or more ovules [37].

In spite of integument and nucellus growth defects, one MMC can differentiate within each nucellus of the atrin1a;atrin1b mutant ovule (Fig. 4k) as normally found in WT ovules. One MMC undergoes meiosis to produce four...
megaspores, from which three degenerate and one becomes a functional megaspore (FM, Fig. 4l). In most of the mutant ovules, megaspore degeneration is disturbed, leading to survival of more than one megaspore (Fig. 4m–o). Aniline blue staining [38] showed callose accumulation (Fig. 4p and q), suggesting that the surviving megaspores go through growth arrest in subsequent stages. Occasionally, one of the surviving megaspores can undergo one cycle of mitotic division, resulting in the coexistence of megaspores and FG2-stage ES with two nuclei (Fig. 4r). Nevertheless, these surviving megaspores or FG2-stage ES eventually undergo growth arrest and degenerate (Fig. 4s).

Taken together, our observations indicate that AtRING1a and AtRING1b regulate ES and ovule development through determination of ovule identity, promotion of accurate megaspore degeneration, and inhibition of nucellus overproliferation.

**AtRING1 is required for suppression of KNOX-I genes**

There are two major regulatory pathways involved in stem cell determination of carpel development: the **WUS-AG** pathway (mainly including *LFY, WUS*, and **AG**) and the **KNOX-I** pathway (mainly including **STM** and **KNAT2**) [9]. In addition, three **AG**-related MADS-box genes, **SHATTERPROOF1** (**SHP1**), **SHP2**, and **SEED-** **STICK** (**STK**), all redundantly control carpel and ovule identities [39, 40]. To gain insight into molecular mechanisms underlying defective carpel development in the *atring1a*/*atring1b* mutant, we performed quantitative RT-PCR (qRT-PCR) to investigate the expression profiles of these genes in the floral buds of *atring1a*/*atring1b* compared to WT. **LFY** showed a dramatic increase in expression, whereas the more downstream regulators **WUS** and **AG** were reduced in expression in the mutant (Fig. 5a). While **SHP1** and **SHP2** were unaffected, **STK** also showed reduced expression. In sharp contrast, **KNOX-I** pathway genes, including **STM**, **BP**, **KNAT2**, and **KNAT6**, all showed increased expression in the mutant (Fig. 5a).

To further dissect spatial and temporal expression patterns of some key regulatory genes, we introgressed the corresponding promoter::**GUS** reporters into the *atring1a*/*atring1b* mutant. Histological staining revealed that **LFY::GUS** is undetectable in WT flower sections (Fig. 5b) but is ectopically expressed in developing carpel and placenta, anthers, and additional organs between petal and carpel in the *atring1a*/*atring1b* mutant (Fig. 5c). The **WUS::GUS** reporter showed specific expression in the floral meristem and developing nucelli similar to WT (Fig. 5d) and *atring1a*/*atring1b* (Fig. 5e). The **AG::GUS** reporter showed weak expression in stamens, and very strong expression in the placenta and ovules inside the gynoecium in WT (Fig. 5f). In the *atring1a*/*atring1b* mutant, **AG::GUS** expression is drastically reduced in placenta and ovules, but still appears in ectopic stigma papillae of carpelloid sepals (Fig. 5g). It is known that **LFY** and **WUS** bind independently at the second intron of **AG** and cooperate to activate **AG** expression, but neither **LFY** nor **WUS** alone appears to be sufficient to activate **AG** [41]. Consistently, our data showed that a higher level of **LFY** but a lower level of **WUS** failed to elevate **AG** expression in the *atring1a*/*atring1b* mutant. To provide further insight, we analyzed the expression of **CLV3**, which is known to restrict **WUS** expression to the SAM and FR [42]. The **CLV3::GUS** reporter displayed a strict FM expression in WT (Fig. 5h) as expected. In the *atring1a*/*atring1b* mutant, however, **CLV3::GUS** showed a drastic increase of expression in FM as well as ectopic expression in carpel primordia, very young carpels, the placenta region, stigmatic tissue, and the nucellus of ovules (Fig. 5i). The high level of **CLV3** likely restricts **WUS** expression in the *atring1a*/*atring1b* mutant. Our interest further turns to **KNOX-I** genes. Examination of **KNOX-I** genes using **STM::GUS**, **KNAT2::GUS**, and **BP::GUS** reporters revealed that they are ectopically expressed at high levels broadly in young developing gynoecia and in placental tissues, ovules, and carpelloid tissues in the *atring1a*/*atring1b* mutant as compared to their highly restricted expression in WT (Fig. 5j–o).

Taken together, both our qRT-PCR and reporter gene analyses indicate that the **KNOX-I** genes, but neither **WUS** nor **AG**, are derepressed in *atring1a*/*atring1b*, and that the spatiotemporal pattern of ectopic **KNOX-I** gene expression correlates with the mutant carpel phenotype.

**Genetic evidence for a pivotal role of AtRING1 in KNOX-I suppression during carpel development**

To directly evaluate the role of **KNOX-I** genes in *atring1a*/*atring1b* mutant carpel phenotype determinacy, we generated an *atring1a*/*atring1b*/*stm-7* triple mutant (Additional file 2: Figure S2). The weak mutant allele **stm-7** contains a transfer DNA (T-DNA) insertion in the second intron of the **STM** locus (Additional file 2: Figure S2) and displays defective inflorescence development, a reduced number of outer floral organs, and no central carpel (~4.2 sepal, ~1 petal, ~1.5 stamen, and 0 carpels, n = 17) (Fig 6a and b, Additional file 2: Figure S2) [43]. Interestingly, we found that *atring1a*/*atring1b* partially rescues the morphological architecture and floral phenotype of **stm-7**. For instance, the *atring1a*/*atring1b*/*stm-7* triple mutant produces inflorescences without obvious whorled phyllotaxy (Fig. 6c) replacing the “aerial rosettes” phenotype with repeated “inflorescence-vegetative”-type development in **stm-7** (Fig. 6a, Additional file 2: Figure S2). The triple mutant flower (~4.2 sepal, ~4.4 petal, ~2.4 stamen, and 3 separate carpels, n = 17) with delayed fourth whorl development
frequently displays unfused carpels with few defective
ovules (Fig. 6d to f, h to j). More rarely, closed carpels
are observed in the central whorl (Fig. 6g). Additionally,
homeotic conversions occur frequently, with ~100%
of sepals showing carpelloid-like structures (Fig. 6k). It is
known that KNOX-I genes have redundant functions; for
instance, ectopic expression of KNAT2 and BP can sup-
press stm flower phenotypes to various extents [9].
Therefore, we compared by qRT-PCR the expression
levels of the other KNOX-I genes in floral buds between
the atr1a atr1b stm-7 triple mutant and the stm-7
single mutant. Our data showed that expression levels
of BP, KNAT2, and KNAT6 are elevated in the triple
mutant (Fig. 6l), suggesting that their derepression by
at1a at1b and their redundant function with
STM possibly accounts for some phenotypes observed in
the at1a at1b stm-7 triple mutant. Lastly, we
generated the at1a at1b bp-1 triple mutant, which displays
characteristics of the at1a at1b double mutant with the exception of downward-curved
pedicel growth similar to bp-1 (Additional file 3: Figure
S3), indicating a specific role of BP in determining
proper growth of floral pedicels.

It is previously known that the MYB-family transcrip-
tion factor AS1 represses the expression of BP, KNAT2,
and KNAT6 in a PRC1/PRC2-associated manner [44, 45].
Investigation of the *atring1a;atring1b* triple mutant during the reproductive stage (Additional file 4: Figure S4) revealed that the *as1-1* mutation further enhances the *atring1a;atring1b* phenotype (Fig. 6 m–u). Expansion of outer floral organs (except carpels) was severely inhibited, resulting in protruding gynoecia in *atring1a;atring1b;as1* flowers (Fig. 6n and o), which is in agreement with the higher expression of *BP* and *KNAT2* in the flower buds of *atring1a;atring1b;as1-1* as compared to *atring1a;atring1b* (Fig. 6v). All sepals from the *atring1a;atring1b;as1-1* triple mutant showed carpelloid-like structures (Fig. 6o) with some producing naked placenta-like structures from the inner position (Fig. 6p and q). This latter observation is consistent with strong expression of *ASI* at the inner surface of WT sepal primordia [46]; loss of *ASI* likely enhances expression of *KNOX-I* genes and indeterminate growth in *atring1a;atring1b;as1-1*. Development of whorl 4 in the *atring1a;atring1b;as1-1* triple mutant displayed severe pleiotropic phenotypes with indeterminate growth. For instance, several style-stigma structures extend from the flank of the central pistil (Fig. 6r), a secondary gynoecium grows outwards from within another unclosed one (Fig. 6s), and spiral reiterations of carpel-like structures margined by ovules are found along style-like structures topped by stigmatic tissues (Fig. 6t and u). Together, these data indicate that *as1-1* enhances flower and carpel phenotypes of the *atring1a;atring1b* mutant through further synergistic increases in *KNOX-I* gene expression. But...
an alternative explanation with a small possibility would be that AS1 might act on other unknown carpel-controlling genes which are competitively regulated by KNOX-I genes.

**Carpel development in the atring1a;atring1b mutant still requires WUS function**

It is well known that STM and WUS act independently but complementarily in the maintenance of proper shoot apical meristem activity [8]. Although neither WUS nor AG is repressed by ATRING1a/ATRING1b, it remains unclear whether the WUS-AG pathway has a role in determinacy of the atring1a;atring1b mutant phenotype. To investigate the role of WUS in the atring1a;atring1b mutant, we constructed an atring1a;atring1b;wus-8 triple mutant (Additional file 5: Figure S5). The wus-8 mutant displays a typical loss-of-function wus phenotype, as previously described [47], e.g., premature termination of shoot and floral meristem activities, absence of carpels, and reduced numbers of other floral organs (~2.6 sepals, ~0.1 stamen, and 0 carpels, n = 10) (Fig. 7a). The atring1a;atring1b;wus-8 triple mutant shows higher numbers of outer floral organs and the absence of central carpels (~5.3 sepals, ~13.2 petals, ~3.8 stamens/petaloid stamens, and 0 carpels, n = 10) (Fig. 7b and c), indicating that loss of ATRING1 fails to rescue wus-8 in carpel development. In this triple mutant flower, additional filamentous organs were observed at whorls interior to the sepals (Fig. 7c–e). Some of these organs can develop into carpel-like structures with branching filamentous structures that mimic ovules but lack nucellus and integument differentiation (Fig. 7f). These ascribed phenotypes of the atring1a;atring1b;wus-8 mutant flowers are closely similar to those previously reported for the flowers of the STM<sup>OE</sup>-wus plants that overexpress STM in the wus mutant [9].

To evaluate an effect of WUS overexpression in the atring1a;atring1b mutant, we constructed the atring1a;atring1b;clv3-2 triple mutant (Additional file 6: Figure S6). CLV3 polypeptide acts as a small secreted ligand, binding to CLV1 and CLV2-CORYNE (CRN) heteromeric receptors to restrict the domain of WUS expression (reviewed in [48]). clv mutants with increased WUS expression accumulate excess numbers of undifferentiated cells in both shoot and floral apices, leading to shoot fasciation, enlarged floral meristems, and supernumerous carpels (Fig. 7g, Additional file 6: Figure S6, [49]). Remarkably, as shown in Fig. 7h–j, the atring1a;atring1b;clv3-2 triple mutant displays sharply increased carpel numbers (~7.5, n = 37) as compared to either atring1a;atring1b (~3.2, n = 14) or clv3-2 (~4.4, n = 34). In addition, papillae overproliferation on stigma and inside gynoecia is also more severe in the triple mutant (Fig. 7i–k). Thus, loss of CLV3 releases WUS suppression, further enhancing carpel indeterminacy of the atring1a;atring1b mutant. Indeed, qRT-PCR analysis confirmed that WUS expression is higher in atring1a;atring1b;clv3-2 than in atring1a;atring1b (Fig. 7l).

Taken together, our data indicate that while derepression of KNOX-I genes in atring1a;atring1b induces carpel indeterminacy, central carpel development still depends on

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**Fig. 7** Phenotype analysis of atring1a;atring1b;wus-8 and atring1a;atring1b;clv3-2 triple mutants. a–f Phenotype of atring1a;atring1b;wus-8 triple mutant flower. a A typical wus flower showing reduced floral organs and absence of carpel. b A typical atring1a;atring1b;wus-8 triple mutant flower showing increased number of sepals and petals, but still lacking central carpel. c Absence of central carpel but production of filamentous extra organs in atring1a;atring1b;wus-8 triple mutant flower. d Filamentous organ produced from the base of sepal. e Filamentous organ with a long branch curled inside a carpeloid sepal. f Ectopic carpel-like structure developed from outer whorls of atring1a;atring1b;wus-8 triple mutant flower. Arrow indicates a branch mimicking an ovule outgrowth. g–k Gynoecium phenotype of atring1a;atring1b;clv3-2 triple mutant flower. g A typical clv3-2 gynoecium fused with four carpels. h Increased carpel number and i overproliferated stigmatic papilla-like structures in atring1a;atring1b;clv3-2 triple mutant. j Abundant carpel-like structures outgrown from inside of gynoecium. k Stigmatic papilla-like structures overproliferating at top of gynoecium. Bars = 1 mm, except 200 μm in c–f. I Elevated WUS expression in floral buds of atring1a;atring1b;clv3-2 triple mutant compared with segregated atring1a;atring1b sibling detected by qRT-PCR (Student’s t test, **p < 0.01). Error bars represent SD from three biological replicates.
**Discussion**

**PRC1 RING finger genes are broadly expressed, with AtRING1b and AtBMI1c specifically showing parental imprinting**

Loss of either AtRING1a or AtRING1b does not impact plant development, but the *atring1a;atring1b* double mutant displays pleiotropic phenotypes throughout vegetative and reproductive stages, indicating their redundant functions in plant development. Detailed expression analysis using GUS reporter constructs confirmed very similar and wide-ranging expression patterns of both AtRING1a and AtRING1b during the plant life cycle. In the early vegetative stage, embryo-like structures develop in the SAM and young leaves, and *pkl*-like taproot and twist rosette leaves are also found in *atring1a;atring1b*, which is in agreement with strong expression of both AtRING1a and AtRING1b found in SAM, RAM, young leaves, and vasculatures. Proceeding to the reproductive phase, AtRING1a and AtRING1b were first detected in the inflorescence apex and various floral organ primordia, which is consistent with the high number of floral organs and homeotic conversions observed in *atring1a;atring1b*. AtRING1a/AtRING1b expression gradually decreases or completely ceases as surrounding floral organs expand, suggesting the highest activity for AtRING1a/AtRING1b in proliferating/rapidly dividing tissues. Lastly, both AtRING1a and AtRING1b are expressed during various stages of carpel, ovule, and embryo development, which is consistent with the homeotic conversions of replum/ovule-to-carpel and defective ovule formation observed in *atring1a;atring1b* mutant gynoecia.

The major difference between AtRING1a and AtRING1b is the specific expression of AtRING1b, but not AtRING1a, in endosperm. So far, most known examples of imprinted genes are confined to the endosperm in higher plants. For instance, the PRC2 component genes FIS2 and MEA display maternally biased expression in the endosperm [31, 50, 51]. Moreover, all known plant genes with imprinted expression depend on differential DNA methylation, PRC2 activity, or both. Investigation based on reciprocal crosses of either the AtRING1b:AtRING1b-GUS reporter or the AtBMI1c:AtBMI1c-GUS reporter with WT, met1-3, or fis2 showed that they display preferentially maternal expression in the endosperm, and that both AtRING1b and AtBMI1c are regulated by CG DNA methylation independent of the FIS2-PRC2 complex. Loss of function of each FIS2-PRC2 component (i.e., MEA, FIE, FIS2, and MSI1) causes endosperm overproliferation without fertilization, embryo abortion, and seed lethality [21]. Neither *atbmi1c* nor *atring1b* single mutants show a visible phenotype during endosperm development, indicating that both have redundant functional homologs or that PRC1 might have only a minor effect and separate function from FIS2-PRC2 during endosperm development.

**Ovule development is impaired in the *atring1a;atring1b* mutant**

Both AtRING1a and AtRING1b display strong expression throughout ovule and ES development, indicating their potential importance in megasporogenesis and megagametogenesis. Indeed, the *atring1a;atring1b* mutant displays abnormal development during ovule development, ranging from ovule morphology and structure to ES formation. On one hand, AtRING1a and AtRING1b inhibit nucellus overproliferation, but indirectly promote outer integument growth. On the other hand, AtRING1a/b regulate ES development by ensuring degeneration of destined megaspores after meiosis. Thus, AtRING1a/b coordinate ovule development in both sporophytic and gametophytic phases. Many gametophytic ovule mutants have normal sporophytic tissue structures, but sporophytic ovule mutants usually have abnormal gametophyte development, suggesting that integument and ES development are interdependent processes and that accurate architecture of sporophytic tissue is necessary for successful development of a fully functional gametophyte [52]. For example, bell1 (*bel1*) ovules develop a single integument-like structure (ILS) taking the place of the two integuments, and fail to produce a normal ES [36, 53, 54]. In *short integuments1* (*sin1*) ovules, both integuments are too short to enclose the nucellus, and the ES does not develop [36, 55]. Therefore, continuous signaling from sporophytic tissue appears necessary to precisely direct gametophyte development. Here, in the *atring1a;atring1b* double mutant, defective outer integument and nucellus growth may cause the arrest of ES development. Alternatively, AtRING1a/b might control sporophytic tissue and ES development in a parallel manner. Further investigation through complementation experiments by introducing recombinant genes expressing AtRING1 under the control of integument-, nucellus-, or ES-specific promoters into the *atring1a;atring1b* mutant may be helpful to address these questions.

**AtRING1a and AtRING1b control carpel development mainly through repression of KNOX-I genes**

In Arabidopsis there are at least two independent and complementary pathways, the *WLIS-AG* pathway and the *KNOX-I* pathway, controlling stem cell activity and carpel development. Several lines of evidence indicate that AtRING1a and AtRING1b act mainly via repression of the *KNOX-I* but not the *WLIS-AG* pathway. Firstly, qRT-PCR showed that the expression of *KNOX-I* genes is significantly
increased, which is in contrast to the decreased expression of WUS and AG, in the atring1aatriing1b mutant. Notably LFY, an upstream regulator of the WUS-AG pathway, is drastically upregulated in floral buds of atring1aatriing1b, which might be associated with floral reversion such as the "flower-in-flower" phenotype observed in the mutant [28]. Secondly, GUS reporter analysis revealed that ectopic KNOX-I gene expression occurs in developing carpels, ovules, and carpel-like structures of the atring1aatriing1b mutant, whereas AG lines display weak GUS staining. Expression of WUS in secondary ovules and of AG in ectopic papillae of carpelloid-like sepals was observed in the atring1aatriing1b mutant, which likely reflects the requirement for WUS-AG in specifying ovule-carpel identity. Thirdly, ovule-to-carpel conversion observed in the atring1aatriing1b mutant is reminiscent of transgenic plants overexpressing STM or KNAT2 described in a previous study [9]. Finally, genetic analysis demonstrates that misexpression of KNOX-I genes is important for the defective carpel developmental phenotype observed in atring1aatriing1b. Loss of AtRING1 activities partially rescue stm architecture and flower phenotype due to release of the other KNOX-I genes in the atring1aatriing1bstm-7 triple mutant; this resembles as1 partial rescue of the stm phenotype in the as1;stm double mutant via upregulation of other KNOX-I genes [46]. Furthermore, we found that as1 can enhance atring1aatriing1b flower and carpel phenotypes due to a synergistic derepression of KNOX-I genes in the atring1aatriing1bas1-1 triple mutant. Thus, repression of KNOX-I genes constitutes an important regulatory mechanism in carpel and ovule development, and a dosage-dependent effect of KNOX-I genes likely explains the degree of severity on central carpel development defects observed across the mutants studied (Fig. 8a).

Our conclusion that the PRC1 core components AtRING1a and AtRING1b repress the KNOX-I but not the WUS-AG pathway is also in agreement with a previous study carried out using atring1aatriing1b mutant seedlings [28]. PRC1 RING finger proteins AtBMI1a and AtBMI1b are also not required for AG repression in seedlings [27]. In contrast, it is well known that AG is derepressed in lhp1 and PRC2-related mutant plants, indicating that LHP1 and PRC2 are involved in the developmental switch from SAM to FM. During the reproductive stage, PRC2-mediated H3K27me3 has been shown to play a critical role in repressing WUS directly via AG recruitment, or indirectly via competitive displacement by AG at the promoter of the WUS repressor KNLI; together these mechanisms account for floral stem cell termination and carpel development initiation ([19, 20]; Fig. 8b). Our finding that AtRING1 suppresses KNOX-I and CLV3 provides additional novel information to the gene networks controlling floral stem cell activity and carpel development (Fig. 8b). Stem cells are consumed upon carpel development, yet they are required at the initiation of carpel development; thus, disruption of the stem cell master regulator WUS or STM leads to the absence of central carpel development. Our analyses of atring1aatriing1b together with the atring1aatriing1bstm-7 and atring1aatriing1bas1-1 triple mutants clearly establish a key role of KNOX-I suppression in maintenance of carpel and ovule determinacy. Interestingly, derepression of KNOX-I alone is insufficient, and proper carpel initiation depends on the WUS-AG pathway as evidenced by the absence of carpel development in the atring1aatriing1b;wus-8 triple mutant and the polycarpous proliferation in the atring1aatriing1b;clv3-2 triple mutant. Although direct genetic interaction between AtRING1 and AG is unexamined so far, it is well established that CLV3 and WUS regulate carpel development through AG, which is the key regulator in determination of carpel identity [13, 14]. Thus, it is reasonable to conclude that the WUS-AG and KNOX-I pathways act independently and complementarily in regulation of carpel development. Similarly, it is well known that WUS and STM act independently and complementarily in maintaining vegetative shoot development [56]. These independent and complementary pathways, together with the finding that AtRING1 and PRC2 regulate various pathway genes with different specificity, may mean that it is advantageous for stem cells to integrate diverse developmental and environmental cues to cope with plant developmental plasticity. It is also reasonable to speculate that AtRING1-mediated suppression of CLV3 may provide a link coordinating the WUS-AG pathway with the KNOX-I pathway in the regulation of floral stem cell activity and carpel development (Fig. 8b). Meanwhile, PRC2-mediated H3K27me3 involvement in suppression of KNOX-I genes during vegetative growth is well described [57]. Future studies are necessary to determine to which extent PRC2 repression is involved in floral stem cell termination and carpel development and whether it is associated with AtRING1 function.

Conclusion
Our study provides important information about tissue-specific expression patterns and unravels a key role of the PRC1 core component genes AtRING1a and AtRING1b in suppression of KNOX-I genes, which independently but complementarily act together with the WUS-AG pathway to determine floral stem cell proliferation and termination during flower carpel development.

Methods
Plant materials
Mutant alleles fis2 (SALK_009910), met1-3 (CS16394), stm-7 (N409575, GK-100F11), wus-8 (SAIL_150_G06), as1-1 (CS146), clv3-2 (CS8066), and bp-1 (CS30) and GUS reporters LFY::GUS (N9776) and CLV3::GUS (N9610) were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA) or the Nottingham
Arabidopsis Stock Centre (NASC, Loughborough, UK). The atring1a atring1b double mutant and atring1a atring1b bas1-1 triple mutant have been described previously [23, 28]. WUS::GUS and KNOX::GUS (STM::GUS, BP::GUS, and KNAT2::GUS) reporters and the atring1a atring1b double mutant background have also been described previously [28]. Seeds were surface sterilized (70 and 95% ethanol for 10 min, respectively) and plated on Murashige and Skoog (MS) medium (MS salts, 0.9% sucrose, pH 5.7, 0.9% bactoagar). After stratification at 4 °C for 2 days in the dark, plates were incubated in a growth chamber at 22 °C under a 16-h light/8-h dark regime. After 10 days, the seedlings were transferred to soil and grown under the same conditions.

**Generation of mutant combinations**

To generate the atring1a atring1b stm triple mutant, a stm-7/+ heterozygote was crossed to an atring1a/+ atring1b/+ plant. The atring1a+/+ atring1b−/−stm−/+ triple mutant was obtained in the F2 generation by genotyping (the genotyping primers are listed in Additional file 7: Table S1), which produced four segregating phenotypes: WT, stm, atring1a atring1b, and the triple mutant phenotype in the F3 generation. Plants were analyzed by PCR to select the atring1a atring1b stm−7 homozygous mutant, which exhibits an atring1a atring1b−like phenotype in the vegetative stage and partially rescued stm−7 flower phenotype in the reproductive stage.

To generate the atring1a atring1b wus triple mutant, a wus−8/+ heterozygote was crossed to an atring1a+/+ atring1b−/+ plant. The atring1a−/+ atring1b+/−wus−/+ triple mutant was obtained in the F2 generation by genotyping (the primers are listed in Additional file 7: Table S1), which produced four segregated phenotypes: WT, wus, atring1a−/+ atring1b−/+, and the triple mutant phenotype in

![Fig. 8 Hypothetical model of AtRING1-mediated KNOX-I repression in carpel development. a Hypothetical dosage effect of KNOX-I explaining the varied severity of carpel developmental defects observed in the studied mutants. Because different KNOX-I genes regulate carpel development with an efficiency of STM > KNAT2 > BP, we propose an effective KNOX-I amount (Y-axis) by considering KNAT2 = 1/N STM and BP = 1/(N + X) STM, with N > 1, X > 0. T12 represents the amount for WT carpel development, and Tmin and Tmax indicate the minimum and maximum threshold, respectively, for allowing normal carpel development. Mutants with estimated range of effective KNOX-I levels and respective carpel phenotypes are indicated. b Hypothetical model of AtRING1 function within a gene network controlling floral stem cell activity and carpel development. AtRING1 as well as PRC2 (CLF) and LHP1 are colored. KNOX-I genes (including STM, KNAT2, and BP), CLV3, and the WUS-AG-KNU feedback loop are indicated. Arrows indicate promotion, and T-shaped bars indicate repression.](image-url)

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the F3 generation. Plants were analyzed by PCR to select the *atring1a*::*atring1b::*wus-wus-8* homozygous mutant, which exhibits an *atring1a*::*atring1b*-like phenotype in the vegetative stage and no central carpel development in the reproductive stage.

To generate the *atring1a*::*atring1b::*clv3-2* triple mutant, a clv3-2 homozygote was crossed to an *atring1a*+::*atring1b+-* plant. The *atring1a*+::*atring1b+-::*clv3-2-/-* triple mutant displaying the typical clv3 phenotype was obtained in the F2 generation by genotyping, and it produced three segregating phenotype populations: clv3, *atring1a*::*atring1b+*, and the triple phenotype in the F3 generation. Plants were analyzed by PCR to select the *atring1a*::*atring1b::*clv3-2* homozygous mutant, which exhibits an *atring1a*::*atring1b*-like phenotype in the vegetative stage and proliferated stigma in the reproductive stage.

Generation of *atring1a*::*atring1b*::*bp-1* was similar to that of *atring1a*::*atring1b+*::*bp-1*. Firstly, an *atring1a*+::*atring1b+-::*bp+-* triple mutant displaying the typical bp mutant phenotype was obtained in the F2 generation. Three phenotypes including *bp*, *atring1a*::*atring1b+*, and the triple mutant phenotype were segregated in the F3 generation. Genotyping PCR was performed to identify the homozygous triple mutant *atring1a*::*atring1b*::*bp-1*, which displays an *atring1a*::*atring1b*-like phenotype with a downward pedicel.

**Plasmid construction**

For *AtRING1b*::*AtRING1b-GUS* construction, the DNA fragment containing the upstream promoter and full genomic DNA region of the *AtRING1b* gene lacking the stop codon (−1586 to +2657 bp) was amplified from Arabidopsis genomic DNA using specific primers, digested using *SacI* and *BamHI*, and cloned into pBI101 to create a GUS reporter gene fusion. For *AtBMI1c*::*AtBMI1c-GUS* construction, the *AtBMI1c* genomic region without the stop codon plus its upstream promoter (−2126 to +2246 bp) was obtained using specific primers, digested, and cloned into pBI101, similar to *AtRING1b*::*AtRING1b-GUS* construction. These binary vectors were introduced into *Agrobacterium tumefaciens* GV3101 and transformed into Arabidopsis plants by the floral dip method [58]. Primers for plasmid construction are listed in Additional file 7: Table S1.

**RNA isolation and qRT-PCR**

Total RNA was isolated using the NucleoSpin RNA Plant kit (Macherey-Nagel, Düren, Germany). qRT-PCR was performed on a LightCycler 480II (Roche), as recommended by the manufacturer. Reaction volumes were scaled to 10 μl final volume and comprised 5 μl SYBR Green PCR master mix (Roche), 2 μl primer mix, and 1 μl template cDNA. All reactions were repeated in triplicate. Protein phosphatase 2 (PP2A) was used as an internal control. The primers for qRT-PCR are listed in Additional file 7: Table S1. Three biological replicates were performed; the original data together with the statistical analysis are given in Additional file 8: Table S2.

**Histochemical staining and imprinting analysis**

For GUS (β-glucuronidase) staining, seedlings or floral buds were submerged in 90% acetone for 30 min on ice, washed twice with 1 M sodium phosphate buffer (pH 7.2) for 15 min at room temperature, and subsequently incubated in X-Gluc solution at 37 °C for 1–12 h depending on the desired staining intensity. Thereafter, seedlings were incubated overnight in 70% ethanol at 4 °C. The standard X-Gluc solution contains 0.1 M sodium phosphate buffer (pH 7.2), 0.5 mM Fe(CN)₃, 0.5 mM Fe(CN)₆, 0.1% Tween-20, and 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). For imprinting analysis, GUS staining was performed using siliques collected at 3 days after pollination. All crosses in this study were performed at identical growth conditions and timings. Crossing success was confirmed in the F1 progeny by genotyping using GUS-specific primers and the respective mutant primes (Additional file 7: Table S1).

**Microscopy**

SEM images were taken using a Hitachi S-3400 N microscope (Hitachi High-Technologies Europe, Krefeld, Germany). Bright-field photographs of individual flowers were taken using a dissecting microscope (Leica, Germany). For DIC observation, dissected pistils were cleared and mounted in chloral hydrate:glycerol:H₂O (8:2:1, w:v:v) overnight and observed using a DIC microscope (Zeiss, Germany). CLSM observation of ovules was performed according to the method previously described [7] with slight modifications. Dissected pistils with exposed ovules were immersed in fixative (4% glutaraldehyde, 0.1 M phosphate-buffered saline (PBS), pH 7.0), vacuum infiltrated for 30 min, and then fixed overnight at room temperature. Following fixation, the tissue was dehydrated through a graded ethanol series with about 10–20 min per step. After dehydration, the tissue was cleared in benzyl benzoate:benzyl alcohol (2:1, v/v) for 1 or 2 days. Individual ovules were dissected, mounted with immersion oil (high viscosity), and observed using a Zeiss LSM 700 META laser scanning microscope (Zeiss, Germany) with a 488-nm argon laser and an LP 530 filter.

**Paraffin section and in situ hybridization**

For paraffin sectioning, samples were fixed in formaldehyde-glacial acetic acid: 70% ethanol (1:1:18, v:v:v) and dehydrated in a graded butanol/ethanol series. Tissues were embedded in paraffin (Leica, Germany) and microtome sections (10 μm) applied onto silane-coated slides. Sections were deparaffinized in xylene and dehydrated through a graded ethanol series before toluidine blue staining. Sections were observed under a Nikon Eclipse
Additional files

Additional file 1: Figure S1. Expression pattern of AtRING1a in the reproductive stage examined by in situ hybridization using a specific AtRING1a probe. (A) Leaf primordia. (B) and (C) SAM. (D) FM. (E) Young flower. (F) Developing ovule. (G) Developing ES. (H) Developing seed in globular stage. (I) Heart stage. (J) Mature green stage. Bars = 50 μm in (A)–(I). (JPG 951 kb)

Additional file 2: Figure S2. Plant architecture of atring1a;atring1b;bp-1/- mutant. (A) Adult plant of stm-7 mutant. (B) Adult plant of atring1a;atring1b;stm-7 mutant. Bars = 2 cm. (C) Schematic structure of the stm-7 mutant allele GK-100 F11 containing a transfer DNA (T-DNA) insertion in the second intron of STM. Gray box represents UTR, black box represents exon, and line represents intron. (JPG 623 kb)

Additional file 3: Figure S3. Phenotype analysis of atring1a;atring1b;bstm-1/- plant. (A) Adult bstm-1 plant (Let). (B) Adult bstm-1 control. (C) Adult atring1a;atring1b;bstm-1 plant. The control bstm-1 plant and bstm-1/- mutant (B) and atring1a;atring1b;bstm-1/- triple mutant (C) are derived from the same F2 generation of atring1a/+;atring1b/+;bstm-1/- mutant. Insets close the close-up view of downward branch related to corresponding mutant. Bars = 2 cm in (A)–(C) and 1 mm in the corresponding insets. (JPG 711 kb)

Additional file 4: Figure S4. Phenotype analysis of atring1a;atring1b;as1-1/- mutant. (A) Adult plant architecture of as1-1/- mutant. (B) Adult plant architecture of atring1a;atring1b;as1-1/- mutant. Bars = 2 cm. (JPG 665 kb)

Additional file 5: Figure S5. Phenotype analysis of atring1a;atring1b;wus-8/- triple mutant. (A) Adult plant of wus-8/- mutant. (B) Adult plant of atring1a;atring1b;wus-8/- triple mutant. (C) Close-up view of (B), showing the main florescence of atring1a;atring1b;wus-8/- triple mutant. Bars = 2 cm in (A) and (B) and 500 μm in (C). (D) The wus-8/- allele (SALK_150_G06) harboring a T-DNA insertion in the second intron of WUS. Gray box represents UTR, black box represents exon, and line represents intron. (JPG 599 kb)

Additional file 6: Figure S6. Phenotype analysis of atring1a;atring1b;tkn-2/- triple mutant. (A) Whole plant architecture of tkn-1/- mutant. (B) Whole plant architecture of atring1a;atring1b;tkn-2/- triple mutant. The control tkn-2/-/- mutant and atring1a;atring1b;tkn-2/- triple mutant are derived from the same F2 generation of atring1a/+;atring1b/+;tkn-2/-/- mutant. Bars = 2 cm. (JPG 521 kb)

Additional file 7: Table S1. Primers used for genotyping, plasmid construction, and qRT-PCR. (DOC 86 kb)

Additional file 8: Table S2. Original data and statistical analysis for qRT-PCR analyses. (XLSX 12 kb)

Abbreviations
AG: AGAMOUS; BP: BREVIPEDICELLUS; CLSM: confocal laser scanning microscopy; CLV: CLAVATA; DIC: differential interference contrast; ES: embryo sac; FIS2: FERTILIZATION INDEPENDENT SEED 2; FM: floral meristem; H3K27me3: histone H3 lysine 27 trimethylation; IIM: inflorescence meristem; KNU: KNUCKLES; LFP: LEAFY; LHP1: LIKE HETEROCROMOTIN PROTEIN1; MET1: DNA METHYLTRANSFERASE1; MMC: megaspore mother cell; Pcg: Polycystic polycystic; PRCl: Polycystic repressive complex; RAM: root apical meristem; SAM: shoot apical meristem; SEM: scanning electron microscopy; SHP1: SHATTERPROOF1; STK: SEEDSTICK; STM: SHOOT MERISTEMLESS; WUS: WUSCHEL

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Availability of data and materials
All relevant data are published within the paper and its supporting additional files. Refer to Additional file 8: Table S2 for raw data values.

Authors’ contributions
WHS and DC conceived and designed the experiments. DC, AM, and LX performed the experiments. DC analyzed the data. DC and WHS wrote the paper with help from all the other authors. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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