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The Initiation of Embryogenesis in Rice

By

HUI REN

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

Venkatesan Sundaresan, Chair

John Harada

Bo Liu

Committee in Charge

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Abstract

In flowering plants, double fertilization involves the fusion of one sperm cell with the egg cell to form the embryo and the other with the central cell to form the endosperm. Early embryogenesis is predominantly maternal in origin, but there are a few transcription factors that are expressed from the male genome in rice. The transcription factor *BABY BOOM 1* (*OsBBM1*), which is initially expressed from the paternal genome, plays a pivotal role in initiating embryogenesis. Ectopic expression of *OsBBM1* in the egg cell is sufficient to bypass fertilization and induce parthenogenesis in rice.

This dissertation reveals that the WOX-family transcription factor *WUSCHEL-related homeobox 9A* (*OsWOX9A*), another paternally expressed transcription factor in rice zygotes, acts as a strong enhancer of *OsBBM1*-mediated parthenogenesis. Co-expression of *OsWOX9A* and *OsBBM1* in egg cells results in 86-91% parthenogenesis, representing 4- to 15-fold increases over *OsBBM1* alone. *OsWOX9A* and its paralog *OsWOX9C* together are required for normal embryo patterning and development. These findings suggest that embryo initiation is promoted by the synergistic action of paternal-genome-expressed transcription factors.

A notable outcome of this process is the occurrence of polyembryony, particularly the formation of the twin progeny at high frequencies. While the phenomenon has been observed, its underlying mechanisms remain largely unexplored.

We provide a brief description of the phenomenon and propose two possible models for the twinning events. The two proposed models are independent, but they can operate in combination. By evaluating the available evidence, we conclude that both models are required to

account for the diversity of twins and triplets observed. These insights not only inform basic plant developmental biology but also provide practical guidance for reducing polyembryony when incorporating synthetic apomixis into crop plants such as maize, where uniform single-embryo seeds are desired.

Parthenogenesis is an integral component of synthetic apomixis, a technology that enables clonal propagation of hybrids and the preservation of heterosis across generations. While previous studies have established parthenogenesis in diploid plants by ectopic expression of the *BABY BOOM* family of transcription factors, this dissertation extends synthetic apomixis to triploid rice. Triploid hybrids can achieve enhanced hybrid vigor compared to diploid hybrids. We demonstrated that triploid rice can be fertile through synthetic apomixis, thus it has the potential to be applied in triploid hybrids to enhance the agricultural traits.

This dissertation deepens our understanding of rice early embryogenesis and uncovers *OsWOX9A* as a synergistic regulator of *OsBBM1*-induced parthenogenesis. It highlights key challenges such as polyembryony and provides a framework for future research to further investigate rice embryogenesis and optimize synthetic apomixis for crop improvement.

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Chapter 1 Introduction

1.1 Double fertilization in flowering plants

In flowering plants, the alternation of generations is the predominant type of life cycle, which involves two distinct multicellular stages: the diploid sporophytic generation and the haploid gametophytic generation. The diploid sporophyte represents the dominant vegetative form of flowering plants, while the haploid gametophyte is specialized for sexual reproduction of the flowering plant. Gametes, egg cells and sperm cells, are produced by the multicellular haploid gametophyte. In the male gametophyte, two sperm cells are produced, while the female gametophyte typically consists of seven cells and eight nuclei, including the egg cell and central cell containing two polar nuclei[1].

Upon pollination, when a pollen grain lands on the stigma, it germinates and forms a pollen tube that grows towards the ovary, often entering through the micropyle. The pollen tube penetrates the cytoplasm of the degenerated synergid, releasing the two sperm cells into the embryo sac[2]. Embryogenesis starts with the fertilization of the egg cell by one of the sperm cells, which forms the zygote. The other sperm cell simultaneously fertilizes the central cell to form the triploid endosperm. These two events together comprise the process of double fertilization, which ensures coordinated development of the embryo and the endosperm and is unique to angiosperms[2]. The zygote gives rise to an embryo after multiple rounds of mitotic cell division, and the endosperm serves as a nutrient reserve for the developing embryo with other developmental functions.

1.2 Zygotic transition and early embryogenesis

The molecular mechanisms involved in the activation of embryonic development in the newly formed zygote are not well understood in plants. In animals, the initiation of embryogenesis is orchestrated by maternal factors deposited in the egg cytoplasm before fertilization and later by *de novo*-synthesized zygotic factors, a developmental shift referred to as the maternal-to-zygotic transition (MZT)[3,4]. This transition combines two interdependent events: zygotic genome activation (ZGA), followed by maternal RNA degradation[3–5]. The MZT is the first major developmental transition in the life cycle and also represents a critical regulatory switch during early embryogenesis in both animals and plants.

These concepts have been extended to MZT in plants. For maternal RNA degradation, the global clearance of these maternal transcripts occurs shortly after fertilization in plant zygotes[6]. The other major component of the MZT is the ZGA, which also occurs rapidly. In animals, the timing of ZGA varies among species. In terms of the embryonic mitotic cell cycles, human (*Homo sapiens*) and mouse (*Mus musculus*) have the ZGA earliest, with the first zygotic transcripts being detected at the one-cell stage[4]. While in *C.elegans*, zebrafish (*Danio rerio*), and *Drosophila*, zygotic transcription was first detected in cell cycles 2, 6, and 8, respectively[4]. Genome-wide gene expression analysis has shown that ZGA reflects a period over which transcription is gradually activated in animals[4]. In plants, however, the *de novo* zygotic gene transcription occurs shortly after fertilization and even before the first cell division in species such as tobacco[7], Arabidopsis[6], and maize[8,9]. In Arabidopsis, approximately 13.7% of genes in the genome exhibit transcriptional activation in the zygote, indicating the global ZGA in the early zygote[6]. In rice, there are hundreds of genes differentially expressed in the early zygote and thousands are activated in the late zygote,

and the magnitude changes seen here are observed only in later stages in animals, when the major embryonic genome activation in human early embryos occurred during the transition of the 4- to 8-cell stage[5,10]. Collectively, these findings suggest that ZGA in plants occurs rapidly after fertilization and globally even before the first zygotic division in plants.

1.3 Parent of origin effects

Both maternal and paternal factors have been shown to contribute to embryo initiation. Although early studies reported minimal or undetectable paternal gene activity, suggesting that the activity of many genes during early embryo and endosperm formation could depend solely on transcription of the maternally inherited allele before or after fertilization[11], subsequent findings challenge this notion. For instance, early activity of the paternally derived *AtRPS5A*::GUS transgene was reported[12]. By crossing a double-mutant genotype, *knolle keule*, characterized by defective cytokinesis, which results in multinucleate single-celled embryos, with wild-type, it was demonstrated that the introduction of functional paternal alleles from wild-type plants is sufficient to rescue embryonic development from the earliest zygotic stage[12]. These findings collectively suggest that while the maternal genome might play a major role in early embryogenesis in plants, the paternal genome is also functionally essential and indispensable, underscoring the biparental control of zygotic genome activation and early developmental programming.

The initiation of embryonic development in flowering plants is a highly coordinated process involving contributions from both maternal and paternal genomes. There is a diverse set of maternal transcripts present in the egg cell prior to fertilization. During the MZT, these maternal transcripts are selectively degraded, and the initiation of zygotic transcription takes place[5,6]. A few specific paternal transcripts are delivered by the sperm cell, some of which act as triggers for

embryo initiation[5]. Maternal and paternal effects on early embryogenesis may also arise from gene imprinting, in which one parental allele is preferentially expressed over the other[13].

In Arabidopsis, a large-scale analysis of maternal and paternal contributions during early embryogenesis has shown that, for many genes expressed in early embryos, both parental genomes contribute, but the maternal allele initially plays a more important role than the paternal allele[14]. This maternal contribution likely involves both maternal transcripts inherited from the egg cell, as well as newly synthesized zygotic maternal transcripts[14]. The coordination between maternal and paternal factors has been demonstrated in the regulation of zygote development. For example, in the *YODA* pathway in Arabidopsis, the sperm cell delivers the *SHORT SUSPENSOR* (*SSP*) transcripts, which are translated after fertilization[13]. Simultaneously, the egg cell expresses *HOMEODOMAIN GLABROUS11 (HDG11)* and *HDG12*[13]. *SSP* acts upstream of *YODA*, transducing a signal that results in the phosphorylation of *WRKY2*, which then activates the *WUSCHEL HOMEOBOX 8* (*WOX8*) and *WOX9* in conjunction with *HDG11* and *HDG12* to promote the asymmetric division of the Arabidopsis zygote[13,15]. Additionally, the maternal effect *EMBRYO SURROUNDING FACTOR1* (*ESF1*) also acts upstream of *YODA* to promote suspensor identity[13].

In rice, early zygote transcriptome profiling has revealed a strong maternal bias, with only a few paternally biased genes identified by characterizing hybrid rice zygote transcriptomes and comparing them with the gametic transcriptomes[5,16]. Differential gene expression analysis in hybrid zygotes derived from *Japonica* and *Indica* demonstrated highly asymmetric parental contribution, but also indicated a direct involvement of the paternal genome in the initiation of embryogenesis through expression of key transcription factors (TFs) after fertilization[5]. One such

TF identified was the paternally expressed *BABY BOOM 1* (*OsBBM1)*, which plays a pivotal role in initiating embryo development[5,17]. The *OsYUC* genes, *OsYUC6*, *OsYUC7*, and *OsYUC9*, which are downstream targets of *OsBBM1*, are involved in auxin biosynthesis and are expressed *de novo* from the maternal genome in the zygote[18]. These indicate that the initiation of the zygote development program involves a paternally expressed factor (*OsBBM1*) that then activates a maternally expressed developmental program for auxin biosynthesis, cell cycle progression, and other basic cellular processes. This highlights a coordinated mechanism of parental genome-specific gene expression that ensures the successful initiation and progression of zygote development in rice.

1.4 *BBM*-mediated and *PAR*-mediated parthenogenesis

Although the early zygote transcriptome in rice is predominantly maternal in origin, unexpectedly, a subset of TFs predicted to promote embryogenesis in both Arabidopsis and rice are expressed *de novo* from the paternal genome after fertilization[5,16]. Among these, *OsBBM1*, the rice ortholog of Arabidopsis *BABY BOOM (AtBBM)*, has been identified as a key regulator[5]. Egg cell-specific expression of *OsBBM1* (*OsBBM1*-ee) is sufficient to initiate embryo development without fertilization, a phenomenon known as parthenogenesis, leading to the formation of haploid plants comprising only the maternal genome[17]. These findings indicate that the fertilization requirement for initiation of embryogenesis in rice is primarily mediated by the paternal expression of *OsBBM1*.

BBM genes are found in most angiosperms. Orthologs such as *AtBBM*, *BnBBM1,* and *BnBBM2* in the *Brassica* family have been shown to induce somatic embryogenesis in dicots by overexpressing them[19], which indicates their potential in inducing parthenogenesis. The *PsASGR*-

BABY BOOM-like (*PsASGR-BBML*) genes of apomictic *Pennisetum squamulatum*, which are located within the Apospory-Specific Genomic Region (ASGR) that confers both apomeiosis and parthenogenesis, share high homology with *AtBBM*[20–22]. Ectopic expression of *PsASGR-BBML* in egg cells of maize, rice, tobacco, and sexual pearl millet can induce parthenogenesis and generate haploid offspring[21–23]. However, expression of *PsASGR-BBML* or *AtBBM* in Arabidopsis egg cells was not sufficient to induce parthenogenesis, as evidenced by previous studies and unpublished data from our lab (see Appendix I)[22].

A recent study demonstrated that expressing *AtBBM* in Arabidopsis egg cells is challenging. The embryo-specific activity of *AtBBM*, as well as the presence of transcriptional repressors that inhibit its expression in egg cells, combine to limit its ability to induce parthenogenesis[24]. To overcome these limitations, a chimeric construct incorporating a partial replacement of *AtBBM* with *OsBBM1* was used, which successfully enhanced the expression of *AtBBM* in Arabidopsis egg cells and led to the formation of parthenogenetic embryos, although no haploid progeny was obtained[24]. The transcription factor *AtRKD5*, a member of RWP-RK domain-containing (RKD) family, binds to the 3′ end of *AtBBM* and functions as an inhibitory regulator of *AtBBM* expression in the egg cell. In the *rkd5* mutant background, the enhanced *AtBBM* expression in the egg cell was sufficient to generate haploid progeny[24]. These results demonstrate the latent capacity of *AtBBM* to induce parthenogenesis and provide a foundation for elucidating the mechanisms underlying *BBM*-mediated embryo initiation in Arabidopsis.

In addition to the *BBM*-mediated parthenogenesis pathway, a natural parthenogenesis allele, the *PARTHENOGENESIS* (*PAR*) gene, has recently been identified from apomictic dandelion (*Taraxacum officinale*)[25]. *PAR* encodes a K2-2 zinc finger, EAR-domain protein. In the apomictic

dandelion, the dominant *PAR* allele is expressed in egg cells and triggers embryo development in unfertilized egg cells.[25] The recessive sexual *par* allele is highly expressed in mature pollen, suggesting that the gene product may cause a block on embryogenesis after fertilization in sexual species. When dandelion *PAR* was ectopically expressed in the egg cell of the related sexual crop lettuce (*Lactuca sativa*) using an egg-cell-specific promoter, it induced the development of ectopic cell division and formation of haploid embryo-like structures without fertilization, which aborted at later developmental stages and did not result in viable seeds due to a lack of sexual triploid endosperm[25].

Arabidopsis possesses two homologs of *PAR*: *DAZ3* (DUO1 activated zinc finger 3 (ZF3)) and *DAZ3like/TREE1* (transcriptional repressor of EIN3-dependent ethylene-response 1). The identification and functional characterization of *PAR* and its homologs open new avenues for exploring alternative pathways of parthenogenesis and zygotic reprogramming in Arabidopsis, as well as other plant species, thereby providing a comparative framework for studying early embryogenesis across plant species.

1.5 *BBM* and other TFs that initiate zygotic embryogenesis

OsBBM1 is a paternally expressed gene, and its ectopic expression in egg cells is sufficient to bypass the requirement of fertilization in initiating embryogenesis[17]. In rice, three *BBM* genes (*OsBBM1, OsBBM2, OsBBM3*) are redundantly required for embryogenesis. The viability of the *Osbbm1 Osbbm2 Osbbm3* triple mutant seeds was severely affected, and the seed viability from the *OsBBM1/Osbbm1 Osbbm2/Osbbm2 Osbbm3/Osbbm3* mother plant depends on the presence of a functional *OsBBM1* allele inherited from the male parent[17], highlighting the essential and male-specific contribution of *OsBBM1* to early zygotic development. There is a fourth *BBM* gene,

OsBBM4, present in rice, which is expressed in sperm cells but not detectable in egg cells or zygotes[5,16]. When *OsBBM4* was further knocked out, about 50% of the resulting seeds from a heterozygous *OsBBM1/Osbbm1 Osbbm2/Osbbm2 Osbbm3/Osbbm3 Osbbm4/Osbbm4* mother plant were not viable, half of which were heterozygous for *OsBBM1 (OsBBM1/Osbbm1)*. These results are consistent with the male-specific expression of *OsBBM1* in zygotes[18].

OsBBM1-induced parthenogenesis in rice initially demonstrated an efficiency of approximately 29%[17]. There are other transcription factors that could have evolved to become male genome-expressed in order to enhance or coordinate with the action of *BBM* to improve parthenogenesis. Among these, members of the *WUSCHEL-related homeobox* (*WOX*) transcription factor family have emerged as key regulators of both somatic and zygotic embryo development. A promising candidate gene involved in early embryogenesis is *WOX9* (*WUSCHEL-related Homeobox 9*). *WOX9* is expressed in the zygote across multiple species, including both dicots (Arabidopsis, tobacco) and monocots (rice, maize)[5,26–28]. In Arabidopsis, *AtWOX9* exhibits *de novo* expression from the paternal genome after fertilization[6], a pattern reminiscent of *BBM* genes. In *Medicago truncatula*, the promoter of *MtWOX9-1* is active in somatic embryos, and overexpression of *MtWOX9-1* leads to an increase in embryogenic capacity, as well as to alter the expression of two embryogenesis-related MADS-box genes, which are *AGL15* and *AGL8* homologs[29]. There are three *WOX9* orthologs in rice, which are *OsWOX9A*, *OsWOX9B*, and *OsWOX9C*[30]. Among them, *OsWOX9A* is a paternally expressed gene and has *de novo* expression from the male genome in the zygote, similar to *OsBBM1*[5], suggesting *OsWOX9A* may have functional parallels with *OsBBM1* and have the potential to be an enhancer or a co-regulator of *BBM*-mediated parthenogenesis in rice.

In this dissertation, I have investigated the role of *OsWOX9A* in early embryogenesis and demonstrated that, together with its paralog *OsWOX9C*, it is required for normal embryo patterning and development. Furthermore, *OsWOX9A* acts synergistically with *OsBBM1* to promote embryogenesis in rice. I also explored the potential of extending *BBM*-mediated parthenogenesis to triploid rice and showed that synthetic apomixis can overcome the barrier of triploid sterility, enabling the production of fertile triploid rice.

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Chapter 2 Synergistic induction of fertilization-independent embryogenesis in rice egg cells by paternal genome-expressed transcription factors

Hui Ren[1], Kyle Shankle[1], Myeong-Je Cho[2], Michelle Tjahjadi[2], Imtiyaz Khanday[2, 3, *], Venkatesan Sundaresan[1, 2, 3, *]

1 Department of Plant Biology, University of California, Davis, CA 95616, USA

2 Innovative Genomics Institute, Berkeley, CA 94704, USA.

3 Department of Plant Sciences, University of California, Davis, CA 95616, USA

* Correspondence: khanday@ucdavis.edu; sundar@ucdavis.edu

2.1 Abstract

In flowering plants, rapid activation of the zygotic genome occurs after fertilization, but there is limited knowledge of the molecular pathways underlying embryo initiation. In rice, a key role is played by the transcription factor *BABY BOOM 1 (OsBBM1)*, initially expressed from the paternal genome. Ectopic *OsBBM1* expression in the egg cell can override the fertilization requirement, giving rise to parthenogenetic progeny. Here we show that the WOX-family transcription factor *DWARF TILLER1 (OsDWT1)/WUSCHEL-LIKE HOMEODOMAIN 9*

(OsWOX9A), another gene paternally expressed in zygotes, is a strong enhancer of embryo initiation by *OsBBM1*. Co-expression of *OsWOX9A* and *OsBBM1* in egg cells results in 86-91% parthenogenesis, representing 4- to 15-fold increases over *OsBBM1* alone. These results suggest that embryo initiation is promoted by the synergistic action of paternal genome-expressed transcription factors in the fertilized egg cell. These findings can be utilized for efficient production of haploids, as well as clonal hybrid seeds in crop plants.

Author contribution

V.S., I.K. and H.R. designed the study. I.K. and K.S. made the constructs for *OsWOX9A* ectopic expression. M-J.C. and M.T. performed the rice transformations. H.R. performed all other experiments, and analyzed the data with V.S. and I.K. H.R., V.S. and I.K wrote the manuscript.

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Synergistic induction of fertilization-independent embryogenesis in rice egg cells by paternal-genome-expressed transcription factors

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Hui Ren[1], Kyle Shankle[1], Myeong-Je Cho[2], Michelle Tjahjadi[2], Imtiyaz Khanday[2,3] ✉ & Venkatesan Sundaresan[1,2,3] ✉

In flowering plants, rapid activation of the zygotic genome occurs after fertilization[1–3], but there is limited knowledge of the molecular pathways underlying embryo initiation[4]. In rice, a key role is played by the transcription factor *BABY BOOM 1* (Os*BBM1*), initially expressed from the paternal genome[1]. Ectopic Os*BBM1* expression in the egg cell can override the fertilization requirement, giving rise to parthenogenetic progeny[5]. Here we show that the WOX-family transcription factor *DWARF TILLER1* (Os*DWT1*)/*WUSCHEL-LIKE HOMEODOMAIN 9* (Os*WOX9A*)[6], another gene paternally expressed in zygotes, is a strong enhancer of embryo initiation by Os*BBM1*. Co-expression of Os*WOX9A* and Os*BBM1* in egg cells results in 86–91% parthenogenesis, representing 4- to 15-fold increases over Os*BBM1* alone. These results suggest that embryo initiation is promoted by the synergistic action of paternal-genome-expressed transcription factors in the fertilized egg cell. These findings can be utilized for the efficient production of haploids, as well as clonal hybrid seeds in crop plants[7,8].

In a previous study comparing the transcriptomes of zygotes and egg cells of rice (*Oryza sativa*), de novo expression of transcription factors known to promote somatic embryogenesis was observed in zygotes after karyogamy[1,9]. Unexpectedly, a few of these transcription factors, including *BABY BOOM 1* (Os*BBM1*: Os11g19060) and *DWARF TILLER1*/*WUSCHEL-LIKE HOMEODOMAIN 9* (Os*WOX9A*: Os01g47710), belonging to the APETALA 2/ETHYLENE RESPONSE FACTOR[10] and WUSCHEL-related homeobox (WOX) families[6], respectively, were found to be initially expressed only from the paternal genome[1]. Subsequently, it was found that ectopic egg cell expression of Os*BBM1* is sufficient to induce parthenogenesis (fertilization-independent embryogenesis), resulting in haploid rice plants comprising only the maternal genome[5]. The fertilization requirement for the initiation of embryogenesis in rice is thus mediated by the paternal allele of Os*BBM1* delivered by the sperm cell. However, the induction of parthenogenesis was incomplete, with the highest efficiency obtained corresponding to ~29% haploid progeny. This incomplete action of Os*BBM1* led us to consider whether other transcription factors might be participating in embryo initiation after fertilization, acting either in parallel or as enhancers of Os*BBM1*. A prime candidate for this role would be Os*WOX9A* (also known as Os*DWT1*), which also exhibits paternal-allele-specific de novo expression in the fertilized egg cell, though it differs from Os*BBM1* in that it is undetectable in sperm cells (Fig. 1a).

To test the possibility that Os*WOX9A* might be an additional factor promoting the zygotic transition, we ectopically expressed Os*WOX9A* under the *Arabidopsis* egg-cell-specific promoter *EC1.2* (ref. 11), which has previously been shown to function in rice[5] (Fig. 1b). We screened the progeny of eight T_0 primary transformant lines for haploid plants

[1]Department of Plant Biology, University of California, Davis, CA, USA. [2]Innovative Genomics Institute, Berkeley, CA, USA. [3]Department of Plant Sciences, University of California, Davis, CA, USA. ✉e-mail: khanday@ucdavis.edu; sundar@ucdavis.edu

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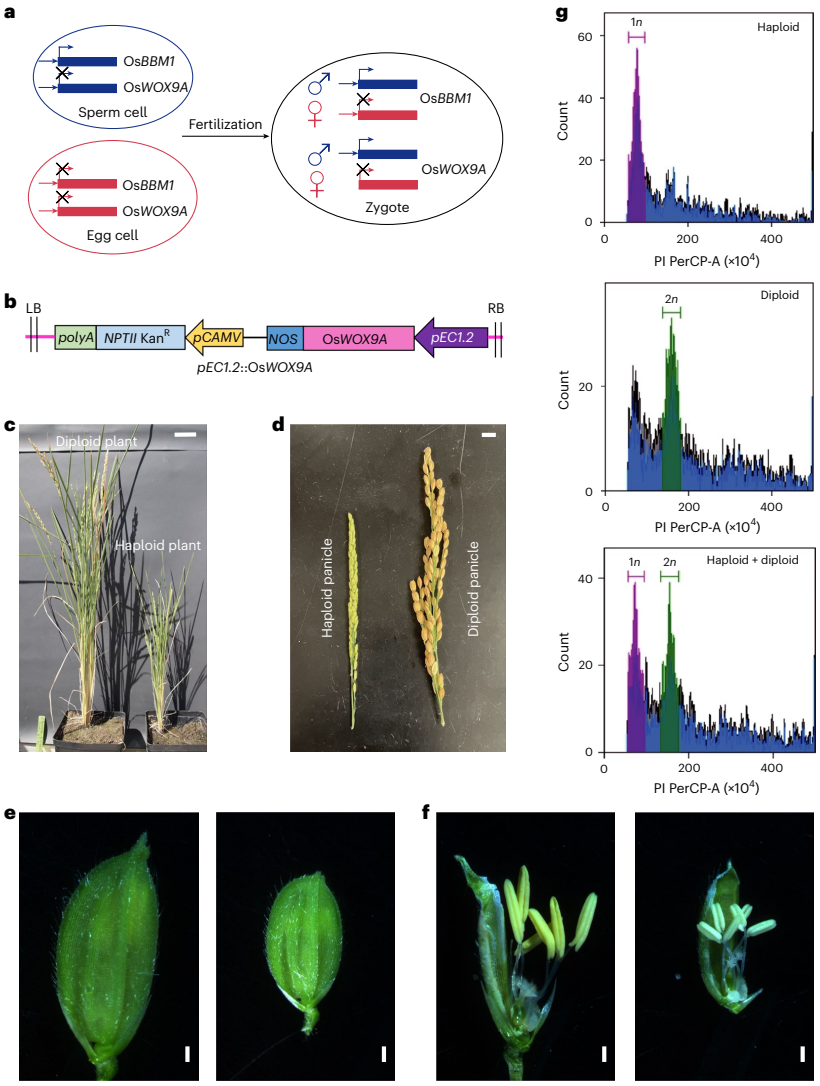


Fig. 1 | Os*DWT1*/Os*WOX9A* is an efficient enhancer of Os*BBM1*-mediated parthenogenesis. a, Schematic illustration of Os*BBM1* and Os*WOX9A* expression in gametes and zygotes. Blue indicates paternal alleles; red indicates maternal alleles. **b**, Schematic of the *pEC1.2*::Os*WOX9A* binary vector for egg cell expression of Os*WOX9A*. LB, left border; RB, right border. Details of the construct are provided under Methods. **c**, T_2 diploid and haploid Os*BBM1-ee* + Os*WOX9A-ee* progenies. The plant sizes differ between diploid (left) and haploid (right) siblings. Scale bar, 5 cm. **d**, Difference in panicle size between haploid and diploid plants. Panicles are smaller in haploids (left) than in diploids (right). Scale bar, 2 cm. **e**, Differences in floret morphology between haploids and diploids. Left: Os*BBM1-ee* + Os*WOX9A-ee* diploid. Right: Os*BBM1-ee* + Os*WOX9A-ee* haploid. Scale bars, 0.5 mm. **f**, Differences in floral organ sizes between haploids and diploids. Left: Os*BBM1-ee* + Os*WOX9A-ee* diploid. Right: Os*BBM1-ee* + Os*WOX9A-ee* haploid. Scale bars, 0.5 mm. **g**, Flow cytometry analysis for ploidy determination. Top: a haploid plant sample showing a haploid (1*n*) peak. Middle: a diploid plant sample showing a diploid (2*n*) peak. Bottom: peaks from a mixed sample of haploid and diploid plants. PI PerCP-A, propidium iodide and Peridinin Chlorophyll Protein-A refer to the fluorescence channel.

that would be produced by parthenogenesis. No haploid plant was found in these screens (0/540 total progeny scored), indicating that Os*WOX9A* expression in rice egg cells is insufficient for the production of parthenogenic haploid progeny, and therefore Os*WOX9A* is unlikely to be capable of embryo initiation independent of Os*BBM1*.

We next investigated whether Os*WOX9A* might function as an enhancer of Os*BBM1* to promote embryogenesis. To test this hypothesis, we transformed the *pEC1.2*::Os*WOX9A* egg cell expression construct (henceforth Os*WOX9A-ee*) into a line homozygous for the *pEC1.2*::Os*BBM1* transgene. The parthenogenesis frequencies arising

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from lines that carried only *pEC1.2*::Os*BBM1* were in the range of 10–29% haploid progeny[5]. We used *pEC1.2*::Os*BBM1* line #8c (henceforth Os*BBM1-ee*), which was previously found to have the highest frequency (29%) of parthenogenesis[5]. Ten primary transformants from seven events were obtained and screened for haploid plants in the progeny to determine the frequency of parthenogenesis when both Os*BBM1* and Os*WOX9A* are co-expressed in egg cells. Seven of ten T_0 lines produced haploid progeny, with frequencies that varied from 3.70% to 48.21% (Supplementary Table 1). The variable haploid frequencies of the transformants and the decrease in haploid frequency in some lines relative to that of the progenitor line used for transformation (29%) are probably the result of regeneration, a process known to affect gene expression in rice[12]. The haploid plants were initially identified by their dwarfed appearance (Fig. 1c) and by their sterile flowers (Fig. 1d–f). Flow cytometry was performed to confirm the ploidy of haploid and diploid progenies (Fig. 1g).

For further characterization, we selected two primary transformants, #12b and #7d, that showed the highest frequencies of haploid progeny—45.78% and 48.21%, respectively (Supplementary Table 1). Since the primary transformants (T_0) are assumed to have only one copy of the *pEC1.2*::Os*WOX9A* transgene, only half the egg cells in the T_0 plants would have received the transgene. To identify homozygotes for both Os*WOX9A-ee* and Os*BBM1-ee* in the T_1 plants, we performed genotyping of the T_2 seeds for the *pEC1.2*::Os*WOX9A* transgene (Supplementary Table 2). This allowed us to identify the plants homozygous and hemizygous for Os*WOX9A-ee* in the T_1 generation. As noted above, the transformation was performed in a line already homozygous for Os*BBM1-ee*; therefore, all progeny had inherited the *pEC1.2*::Os*BBM1* transgene.

Analysis of the T_2 progeny from genotyped T_1 parents of line #12b revealed a haploid frequency (measured by percentage of haploid T_2 progeny) of ~91.04% from homozygous parents, while the hemizygous T_1 parents had an average 42.31% haploid frequency, which correlates with the lower copy number of *pEC1.2*::Os*WOX9A* (Table 1a and Supplementary Tables 3a and 4a). Importantly, plants from line #12b in which the *pEC1.2*::Os*WOX9A* transgene was absent (azygous) due to segregation had a much reduced average haploid frequency of 20.14%. This basal frequency of haploid progeny is attributable to the *pEC1.2*::Os*BBM1* transgene, which is homozygous in the T_0 generation and therefore not segregating in the T_1 parents. There is thus a 4.5-fold increase in haploid frequency (from ~20% to ~90%) arising from the presence of the *pEC1.2*::Os*WOX9A* transgene. Similar results were obtained with the other high-parthenogenesis-efficiency line, #7d (Table 1a and Supplementary Table 4a). For this line, the haploid frequency increased by 15-fold in plants homozygous for the *pEC1.2*::Os*WOX9A* transgene compared with plants lacking the transgene. The average haploid frequencies were 86.36% for plants homozygous for *pEC1.2*::Os*WOX9A*, 35.96% for hemizygous plants and 5.45% for plants without the *pEC1.2*::Os*WOX9A* transgene but still homozygous for *pEC1.2*::Os*BBM1* (Table 1a). We also examined a third line, #2, from a primary transformant that exhibited a somewhat lower parthenogenesis efficiency (21.74%) (Supplementary Table 1). Here also, the presence of the *pEC1.2*::Os*WOX9A* transgene was correlated with a higher haploid frequency of 29.55% for hemizygous plants, versus 13.00% for plants without the *pEC1.2*::Os*WOX9A* transgene but still homozygous for *pEC1.2*::Os*BBM1* (Table 1a and Supplementary Table 4a). Thus, in all three independent events examined, higher efficiencies of haploid progeny were correlated with the number of copies of the segregating *pEC1.2*::Os*WOX9A* transgene, which confers Os*WOX9A* expression in the egg cell (Supplementary Table 3a). Similar analyses using T_3 progeny from T_2 genotyped plants of the two best-performing lines revealed haploid frequencies of ~90% for plants homozygous for *pEC1.2*::Os*WOX9A*, whereas the haploid frequencies for plants without this transgene were ~10% (Table 1b and Supplementary Tables 3b and 4b). Limited analysis using available T_4 progeny of *pEC1.2*::Os*WOX9A* homozygotes from line #7d plants found a haploid frequency of >95% (Supplementary Table 4c). The high efficiencies of haploid progeny in the presence of *pEC1.2*::Os*WOX9A* therefore appear to be stably maintained through multiple generations.

Table 1 | Frequency of haploids in the T_2 and T_3 generations from independent transformants carrying *pEC1.2*::Os*WOX9A* in a homozygous *pEC1.2*::Os*BBM1* background

(a) Haploid ratio in the T_2 generation

Line	Parent genotype	Diploid progeny (*n*)	Haploid progeny (*n*)	Haploid frequency (%)
#12b	*pEC*::Os*WOX9A*/*pEC*::Os*WOX9A*	12	122	91.04
	pEC::Os*WOX9A*/–	90	66	42.31
	–/–	115	29	20.14
#7d	*pEC*::Os*WOX9A*/*pEC*::Os*WOX9A*	12	76	86.36
	pEC::Os*WOX9A*/–	73	41	35.96
	–/–	156	9	5.45
#2	*pEC*:: Os*WOX9A*/–	31	13	29.55
	–/–	87	13	13.00

(b) Haploid ratio in the T_3 generation

Line	Parent genotype	Diploid progeny (*n*)	Haploid progeny (*n*)	Haploid frequency (%)
#12b	*pEC*::Os*WOX9A*/*pEC*::Os*WOX9A*	10	107	91.45
	pEC::Os*WOX9A*/–	35	28	44.44
	–/–	121	12	9.02
#7d	*pEC*::Os*WOX9A*/*pEC*::Os*WOX9A*	11	95	89.62
	pEC::Os*WOX9A*/–	46	33	41.77
	–/–	137	17	11.04

For each transformant line, the frequencies of haploid progeny are significantly higher with increasing copies of the *pEC1.2*::Os*WOX9A* transgene. Statistical significance was determined for all pairwise comparisons using two-sided Fisher's exact tests. The detailed list of *P* values is provided in Supplementary Table 3.

An associated phenotype we observed is that of twin rice plants arising from a single seed, at high frequencies in all parthenogenetic lines. These twins appeared as two seedlings arising from the same single seed that were either haploid-haploid or haploid-diploid (Extended Data Fig. 1c,d). For the calculation of haploid frequencies, haploid-haploid twins from a single seed were counted as single haploid plants (Supplementary Tables 1 and 4). The occurrence of twins was previously observed with parthenogenesis induction using Os*BBM1* in rice[13] and Zm*BBM1* in maize[14], but at much lower frequencies than with the Os*BBM1-ee* + Os*WOX9A-ee* combination reported here. The possible origins of twins, such as premature divisions of the zygote or changes in the cell fate of synergids to egg cells, have been discussed previously[13,14]. Further investigation is required to understand the mechanisms that result in twins from parthenogenesis induction in sexual plants.

Os*WOX9A* encodes a WOX transcription factor (Extended Data Fig. 2a), and the cognate gene Os01g47710 has been annotated variously as Os*WOX7* (RAP-db[15,16] and MSU Rice genome databases[17]) and Os*WOX12/13* (MSU[17]). It has now been redesignated as Os*WOX9A*[18], because its closest *Arabidopsis* homologue is *STIMPY*/At*WOX9* (ref. 6), a gene that functions in both embryo and vegetative development[19,20] (Extended Data Fig. 2b). In rice, *dwt1*/*wox9a* mutant plants develop main shoots with normal height and larger panicles, but dwarf tillers bearing smaller panicles than those of the wild type[6]. Weak mutant alleles of *DWT-LIKE 2* (Os*DWL2*, also known as Os*WOX9C*: Os05g48990),

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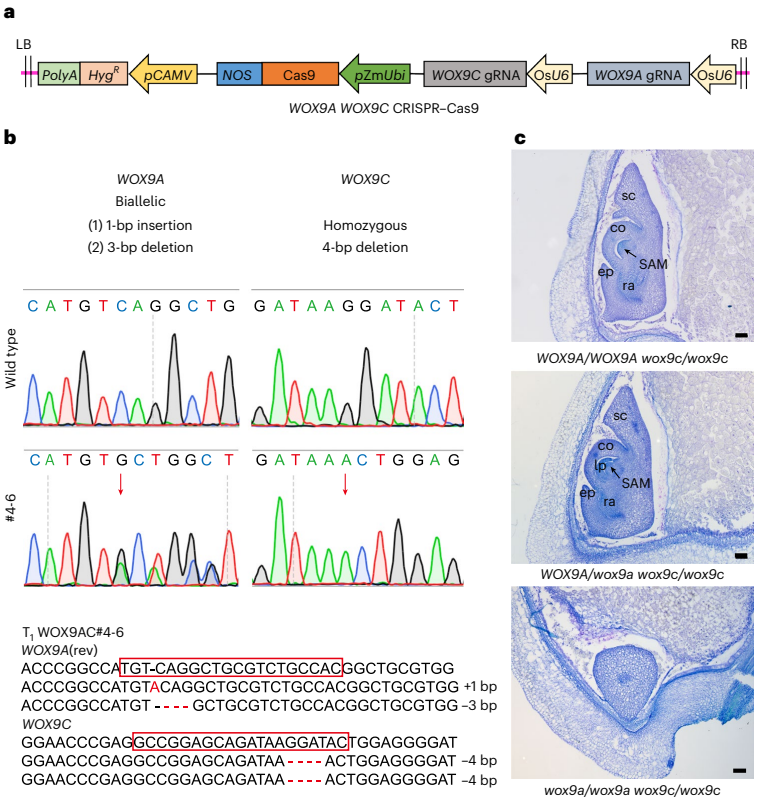


Fig. 2 | Os*WOX9A* and Os*WOX9C* are redundantly required for embryogenesis in rice. a, Schematic of the CRISPR–Cas9 construct to create the *wox9a wox9c* double mutant. **b**, Sequence chromatograms at mutation sites and mutant DNA sequences of Os*WOX9A* and Os*WOX9C* compared to wild-type sequences. *WOX9A*/*wox9a wox9c*/*wox9c* line #4-6 is biallelic for Os*WOX9A* with a 1-bp insertion allele and a 3-bp in-frame deletion allele. For Os*WOX9C*, this line has a homozygous 4-bp deletion. The sequences in red boxes are protospacers of single guide RNAs. The reverse primer was used for the Os*WOX9A* sequencing results presented. **c**, Histological analysis of embryos from segregating seeds of the *WOX9A*/*wox9a wox9c*/*wox9c* WOX9AC#4-6 line at 7 DAP. All seeds were genotyped as described in the Methods. *WOX9A*/*WOX9A wox9c*/*wox9c* (top, $n = 6/6$) and *WOX9A*/*wox9a wox9c*/*wox9c* (middle, $n = 21/21$) embryos show normal development. *wox9a*/*wox9a wox9c*/*wox9c* double-mutant (bottom, $n = 11/11$) embryos show arrested development with no organ morphogenesis. Arrested embryos appear to have no radicle, coleoptile, shoot apical meristem, scutellum or other structures. Instead, these embryos have only an epidermal layer and the isotropic cells surrounding a core of denser, smaller cells inside. co, coleoptile; ep, epiblast; lp, leaf primordia; ra, radicle; SAM, shoot apical meristem; sc, scutellum. Scale bars, 200 µm.

a rice gene that is paralogous to Os*WOX9A*, enhance the dwarfism and tiller phenotypes of the *dwt1* mutant[18]. Homozygous null mutants of *dwt1* are viable, indicating that Os*WOX9A* is not essential for embryo or seedling development. However, null *dwt1 dwl2* mutant plants could not be recovered, due to failure of seed germination, suggesting the possibility of embryo lethality[18].

Since Os*WOX9A* is a strong enhancer of parthenogenesis when expressed in the egg cell, we investigated its possible function in normal embryogenesis in rice by targeting both Os*WOX9A* and Os*WOX9C* for knockout mutants using a CRISPR construct[21] (Fig. 2a). The T_1 progeny of the T_0 transformants were characterized to identify plants that carried null alleles of both mutations and had lost the original CRISPR T-DNA by segregation. As an example, plant WOX9AC#4-6 carries a null mutation in Os*WOX9A* and a presumptively functional Os*WOX9A* allele with an in-frame 3-bp deletion, and is homozygous for a null mutation for Os*WOX9C* (Fig. 2b). Although the seeds harvested from the plant looked normal in size and shape, the seed germination rate was just ~75% versus the expected >97% for wild-type controls. We genotyped the non-germinated seeds as well as all the germinated seedlings obtained from this plant. All 88 seedlings from germinated seeds had at least one copy of the putatively functional 3-bp allele, whereas 24 of the 28 non-germinated seeds contained *wox9a wox9c* double-knockout embryos (Table 2). This analysis was subsequently repeated with two other primary transformants carrying predicted null alleles for both genes (Extended Data Fig. 3). We genotyped the germinated seedlings and non-germinated seeds in the progeny, and again all the *wox9a wox9c* double-knockout seeds failed to germinate in these two independently derived lines (Table 2). All the non-germinated rice seeds from these mutants had an appearance and size indicative of normal endosperm development. Therefore, the failure to germinate probably arises from defective embryogenesis.

Histological analysis of the embryos at different time points after fertilization, starting at three days after pollination (DAP), revealed that embryos genotyped as *wox9a wox9c* double mutants showed early

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Table 2 | Germination rates of seeds from CRISPR-generated *wox9a wox9c* double mutants

Line	Seed genotypes	Germinated (*n*)	Non-germinated (*n*)	*P*
#w3	*WOX9A*/*WOX9A* (15) + *WOX9A*/*wox9a* (37)	51	1 (1 het)	8.5×10^{-13}
	wox9a/*wox9a* (13)	0	13	
#4-6	*WOX9A*/*WOX9A* (28) + *WOX9A*/*wox9a* (64)	88	4 (3 +/+, 1 het)	4.6×10^{-21}
	wox9a/*wox9a* (24)	0	24	
#501a	*WOX9A*/*WOX9A* (21) + *WOX9A*/*wox9a* (24)	43	2 (1 +/+, 1 het)	3.3×10^{-11}
	wox9a/*wox9a* (13)	0	13	

All lines were biallelic or homozygous null for *wox9c* and segregating for *wox9a* null mutations. The total numbers of seeds for each genotype are indicated in parentheses. In all three independent lines, the *P* values for the association between *wox9a*/*wox9a* homozygotes and the failure of germination were determined by two-sided Fisher's exact tests. +/+, *WOX9A*/*WOX9A*; het, *WOX9A*/*wox9a*.

abnormal development and arrest compared with sibling embryos that carried a functional Os*WOX9A* allele (Fig. 2c and Extended Data Fig. 4). In all of the genotyped homozygous *WOX9A*/*WOX9A wox9c*/*wox9c* and heterozygous *WOX9A*/*wox9a wox9c*/*wox9c* seeds, the embryos resembled wild-type embryos and had normal organ formation during embryo development (Fig. 2c and Extended Data Fig. 4). In contrast, the genotyped *wox9a*/*wox9a wox9c*/*wox9c* mutant embryos were smaller and showed delayed growth compared with wild-type embryos from 3 DAP, then underwent cell proliferation without corresponding developmental patterning at 5 DAP and were developmentally arrested when observed at the later stages of 7 DAP and 10 DAP (Fig. 2c and Extended Data Fig. 4). Due to technical limitations of non-destructive genotyping, embryos earlier than 3 DAP were not analysed. These results indicate that Os*WOX9A* and Os*WOX9C* together are required for normal embryo patterning and development as early as the 3-DAP (~150-cell) stage. Due to the presence of a third paralogous gene Os*WOX9B* (also known as Os*DWL1*: Os07g34880), which is still functional in these mutants, it is possible that triple mutants might result in additional embryo phenotypes. While this manuscript was under revision, embryonic defects arising from double knockouts of Os*WOX9A* and Os*WOX9C* were independently reported[22]. Those results are generally consistent with the observations of embryo developmental defects at different times after pollination in this work. However, direct comparisons cannot be made since that study did not perform genotyping for embryos, and the penetrance of embryo defects in double mutants and in heterozygotes was therefore undetermined. Our data show complete penetrance of the early-arrest phenotype in the double mutants. Additionally, as extensively documented in *Arabidopsis*, viable heterozygous embryos can display transient defects and delayed growth, presumptively due to differential activation of the parental alleles[23]. Here we can exclude parental effects for the stages examined, since both heterozygous and wild-type embryos were genotyped and found to be histologically indistinguishable. We cannot exclude the possibility of parental effects at very early stages (<3 DAP), but any such effects are likely to be transient. Interestingly, null mutants of tomato Sl*WOX9* also appear to be embryo lethal, suggesting that *WOX9* genes might also be required for embryogenesis in many dicot plants[24].

Os*WOX9A* is not expressed in unfertilized egg cells, and after fertilization only the paternal allele of Os*WOX9A* is initially expressed in zygotes[1]. Involvement of the paternal genome in the initiation of embryogenesis has been previously proposed on the basis of analysis of the zygote transcriptome[1,25]. Support for this concept has been provided by the demonstration that both in sexual rice plants and in apomictic dandelion, genes expressed in sperm cells—Os*BBM1* and *PAR*, respectively—can trigger embryo initiation in egg cells[5,8]. Our new results suggest an extension of this concept to a model in which fertilization provides multiple pluripotency factors (Os*BBM1* and Os*WOX9A*) expressed from the paternal genome to the quiescent egg cell, which act synergistically to initiate the embryonic program. However, the modes of action of Os*BBM1* and Os*WOX9A* differ in important respects. Os*BBM1* is sperm-cell-expressed, ectopic expression of Os*BBM1* in unfertilized egg cells is sufficient to initiate embryogenesis[5] and there is an absolute requirement for the male allele of Os*BBM1* (ref. 26). Os*WOX9A* is not expressed in either male or female gametes, and ectopic expression of Os*WOX9A* in the egg cell alone does not initiate embryogenesis without fertilization. It is possible that they operate in distinct pathways to promote embryo initiation and establishment, with Os*BBM1* acting as the initial trigger and Os*WOX9A* acting in subsequent steps promoting the stable establishment of the zygotic state. This could explain why there is more efficient induction of zygotes from egg cells when both are co-expressed. Embryo expression of Os*WOX9A* continues through the 3-DAP to 10-DAP stages[15,22], accompanied by Os*WOX9C* expression[27], during which Os*BBM1* is also expressed[5].

Os*BBM1* is also capable of inducing somatic embryogenesis from heterologous tissues[5,26]. The activation of a dexamethasone-inducible form of Os*BBM1* (Os*BBM1–GR*) in germinating seeds induces callus formation from scutellar tissues[26] (Extended Data Fig. 5a–c and Supplementary Fig. 1). In contrast, we found that induction of Os*WOX9A* using an Os*WOX9A–GR* fusion does not induce callus formation, and fully differentiated seedlings are observed (Extended Data Fig. 5c, panel 2). However, co-induction of Os*WOX9A–GR* with Os*BBM1–GR* enhances the callus induction capacity of Os*BBM1–GR*, again demonstrating a synergistic action of the two transcription factors (Extended Data Fig. 5c, panels 3 and 4, and Supplementary Fig. 1). Notably, the co-expression of Os*WOX9A–GR* and Os*BBM1–GR* additionally suppresses shoot growth, indicating that Os*WOX9A* further promotes the establishment of an undifferentiated fate (Extended Data Fig. 5c, panel 4, and Supplementary Fig. 1). The calli after co-expression of Os*BBM1* and Os*WOX9A* are similar in appearance to the embryogenic calli induced by Os*BBM1* alone—that is, compact, pale and nodular—and phenotypically distinct from non-embryogenic calli[26]. This assessment was confirmed by the increased expression of the rice homeobox gene Os*OSH1*, which is expressed in early embryos and associated with the maintenance of undifferentiated cells[28,29] (Extended Data Fig. 5d). It was further confirmed by a large reduction in the expression of Os*AMY1* (*Alpha-Amylase 1*), a marker gene for non-pluripotent calli that is downregulated in calli that have acquired pluripotency[30] (Extended Data Fig. 5d).

Unlike mutants that are heterozygous for Os*BBM1*, which result in embryo arrest when the null allele is inherited from the male[5,26], embryo lethality of Os*WOX9A* heterozygotes (*wox9a*/*WOX9A wox9c*/*wox9c*) was not significant on the basis of germination frequencies (Table 2), implying that the maternal Os*WOX9A* allele is functional despite its delayed expression in zygotes. This is consistent with the absence of phenotypes observed in the histology of heterozygous *wox9a*/*WOX9A* embryos at 3 DAP and later stages (Fig. 2c and Extended Data Fig. 4). These observations suggest that Os*WOX9A* might be acting at a later step than Os*BBM1* in the conversion of an egg cell to a zygote. Os*WOX9A* was not detected as a direct target of Os*BBM1* (ref. 26), consistent with its absence of transcription in wild-type sperm cells, which are already expressing Os*BBM1*. The absence of haploids in Os*WOX9-ee* plants implies that Os*BBM1* is also unlikely to be a direct target of Os*WOX9A*, since Os*BBM1* activation by Os*WOX9-ee* would be expected result in parthenogenesis. Because Os*WOX9A* is expressed only after fertilization, it is possible that it is activated by factors present in the egg cell. In this model, the maternal allele of Os*WOX9A* would be epigenetically silenced in both the egg cell and the early zygote, and initially, only the paternal allele would be capable of activation. In *Arabidopsis*, the

At*WOX8* gene is expressed in the egg cell and subsequently regulated by a combination of maternal and paternal factors[31]. Interestingly, transcriptome data from *Arabidopsis* zygotes show that expression of the At*WOX9* gene is similar to that of rice Os*WOX9A*—that is, At*WOX9* is expressed de novo in early zygotes, and also only from the male allele[3]. Whether At*WOX9* can also function in promoting embryo initiation in *Arabidopsis*, singly or in combination with other factors, is presently unknown but may merit future investigation. *WOX9* genes are members of a gene family related to *WUSCHEL*, a gene involved in the maintenance of the shoot stem cell niche[32], and *BBM* genes are members of the *PLETHORA* gene family that act as pluripotency factors in root stem cells[33]. Since Os*WOX9A* and Os*BBM1* do not appear to be direct targets of each other, it is likely that they act in different gene networks, but with a subset of direct or indirect downstream targets involved in embryo initiation. An example of a convergence of independent gene networks in plant development can be found in the *STM* and *WUS* transcription factors that promote shoot meristem maintenance[34]. More generally, considering that parthenogenesis involves the conversion of the egg cell into a totipotent zygote, there are interesting parallels between the synergistic action of *BBM1* and *WOX9A* demonstrated here and the induction of pluripotent stem cells in mammals by the four "Yamanaka factors" (Oct4, Sox2, Klf4 and c-Myc)[35]. Of these, only the germline-specific factor Oct4 is capable of inducing pluripotency singly, albeit with much lower efficiency[36], leading to a model of Oct4 acting stochastically to promote a cellular state that is then stabilized by the other independently regulated factors[37]. Thus, in both cases, the efficient induction of the cellular transition to pluripotency involves an essential factor (Oct4 in mouse, *BBM1* in rice) to initially trigger the transition, acting synergistically with other transcription factors that may further promote or establish the reprogramming.

In conclusion, Os*WOX9A* is an efficient enhancer of Os*BBM1*-mediated parthenogenesis, and consistent with an embryogenesis function, knockouts of Os*WOX9A* and Os*WOX9C* exhibit embryo arrest. Specifically, egg cell expression of Os*WOX9A* can boost the parthenogenesis efficiency of Os*BBM1* by several fold, to over 90% average haploid frequency, making this system suitable for translation to plant breeding and agriculture. Parthenogenesis has direct applicability to the production of haploid plants for accelerated plant breeding. An especially exciting application is in synthetic apomixis[5], when further combined with the elimination of meiosis to produce clonal progeny, simplifying the large-scale production of hybrid seeds with high yields in major food crops[7,8]. Although we previously found that changing the architecture of the T-DNA construct can result in higher frequencies of apomixis with Os*BBM1* (ref. 13), the relevant *cis* elements for the effect remain unknown, and therefore those results are not easily replicated. Here we have demonstrated by genetic segregation a strong enhancement by Os*WOX9A* acting in *trans*, which is generalizable and easy to incorporate into any construct design. The Os*BBM4* gene has also been used to induce parthenogenesis in rice[38]. It is likely that parthenogenesis enhancement by co-expression of Os*WOX9A* will also be applicable to Os*BBM4*, given its functional redundancy with Os*BBM1* (ref. 26). For the practical application of these findings, the promoters used to drive Os*WOX9A* expression in the egg cell will require optimization. Although the *pEC1.2* promoter is egg cell specific in *Arabidopsis*[11], we observed that all *pEC1.2*::Os*WOX9A* lines showed vegetative phenotypes, including shorter plants and narrow leaves (Extended Data Fig. 1a,b), which are typical characteristics of Os*WOX9A* overexpression plants[39], suggestive of leaky vegetative expression of *pEC1.2*::Os*WOX9A* in these plants. It is likely that these pleiotropic vegetative effects also affect overall fertility, because the fertility of plants carrying this transgene appeared to be consistently reduced relative to wild-type plants (Supplementary Table 5). The recent report that rice plants with overexpression of Os*WOX9A* using a vegetatively expressed promoter are sterile or have very low seed setting supports this interpretation[39]. We did not observe any vegetative phenotypes in plants carrying *pEC1.2*::Os*BBM1* alone, suggesting that rice plants may have a higher tolerance for leaky somatic expression of Os*BBM1* than for Os*WOX9A*. Although further improvements to achieve specific expression will be required for translation of these findings, this study provides a new route to obtain highly efficient parthenogenesis in rice, and probably in other cereals, by using a combination of the two zygote-expressed transcription factors under optimized promoters.

Methods

Plant materials and growth conditions

Rice cultivar Kitaake (*Oryza sativa* subsp. *japonica*) was used in this study. Wild-type, mutant and transgenic seeds were germinated on half-strength Murashige and Skoog medium[40] containing 1% sucrose and 0.3% phytagel in a growth chamber for 12 days, under a 14 h light/10 h dark cycle with 28/25 °C day/night temperatures and relative humidity >65%. The seedlings were then transferred to the greenhouse and grown under natural light conditions at 80% relative humidity and 28/20 °C day/night temperatures in Davis, California. Supplemental light and heat were used during the winter months.

T-DNA vector constructs

The full-length coding sequence (CDS) of Os*DWT1*/Os*WOX9A* was cloned by combining two fragments together. The 5′ 1,255-bp fragment was PCR amplified from calli complementary DNAs[26] using the primer combination *WOX9* F and *WOX9* R3 (ref. 41). The highly GC-rich 3′ 367-bp fragment was synthesized using GenScript (https://www.genscript.com). The two fragments were fused together at a unique NheI restriction enzyme site to complete the open reading frame. The CDS was initially cloned in a pUC57 vector and then moved to a *pCAMBIA2300*-based vector containing *NPTII*, a plant selection marker gene, in which the At*EC1.2* (*DD45*)[11] promoter and the *NOPALINE SYNTHASE* (*NOS*) gene terminator were already cloned for ectopic egg cell expression.

The construction of *p*Zm*UBI1*::Os*BBM1–GR* (Extended Data Fig. 5a) is described in Khanday et al.[5]. The *p*Zm*UBI1*::Os*WOX9A–GR* construct was generated by moving a Zm*UBIQUITIN*::*GR*::*NOSt* expression cassette into a *pCAMBIA2300* vector and then cloning the Os*WOX9A* gene downstream of the Zm*UBIQUITIN1* promoter (Extended Data Fig. 5a). The transformation of *p*Zm*UBI1*::Os*WOX9A–GR* was performed into wild-type cultivar Kitaake and separately into transgenic Kitaake carrying *p*Zm*UBI1*::Os*BBM1–GR* for the induction of both genes. Twelve independent lines were generated for each construct with similar results, and the lines with the best seed sets from the primary transformants were used for more detailed characterization.

For genome editing of the Os*WOX9A* and Os*WOX9C* genes, single guide RNA (sgRNA) sequences (*WOX9A* gRNA and *WOX9C* gRNA, respectively) were designed using the web tool http://omap.org/crispr/. The assembly of the CRISPR–Cas9 gene editing constructs was done as described previously[5]. Briefly, the sgRNAs were cloned in a *pENTR–sgRNA* entry vector, and each gRNA was driven by an Os*U6* promoter[42]. The *pCAMBIA1300*-based binary vector for plant transformations was constructed by Gateway LR clonase (Life Technologies) recombination. *pCAMBIA1300 WOX9A + WOX9C*–CRISPR (*WOX9A WOX9C* CRISPR–Cas9) was transformed into embryogenic calli derived from wild-type Kitaake seeds. Rice transformations were carried out at the plant transformation facility at the University of California, Davis, as well as the Innovative Genome Institute at the University of California, Berkeley. T_0 plants were grown in the greenhouse in Davis and screened for Os*WOX9A* and Os*WOX9C* mutations.

Genotyping

Genotyping of the *pEC1.2*::Os*WOX9A* transgene was carried out by PCR-amplifying the DNA fragment at the intersection of the *pEC1.2* promoter to the Os*WOX9A* CDS with the primers *pEC1.2* Seq F and *WOX9A* Seq R (the primer sequences are provided in Supplementary Table 6). The PCR amplicons were run on a 1.5% agarose gel, and the

fragment size of the amplicon (539 bp) was confirmed by comparison with the DNA marker (Extended Data Fig. 6a). *RICE FLORICAULA LEAFY*[43] (LOC_Os04g51000), a single-copy gene, was used as the internal control.

Genotyping of the *pEC1.2*::Os*BBM1* transgene was carried out by PCR-amplifying the DNA fragment at the intersection of the *pEC1.2* promoter to the Os*BBM1* CDS with the primers *pEC1.2* Seq F and *BBM1* Seq R (the primer sequences are provided in Supplementary Table 6). The PCR products were run on a 1.5% agarose gel, and the fragment size of the amplicon (514 bp) was confirmed by comparison with the DNA marker.

Genotyping of Os*WOX9A* and Os*WOX9C* mutants was carried out by PCR-amplifying DNA at the mutation site with the primers *WOX9A* Seq F and *WOX9A* Seq R, and *WOX9C* Seq F and *WOX9C* Seq R. The primer sequences are listed in Supplementary Table 6. Sanger sequencing of the two PCR amplicons of 406 bp and 368 bp, respectively, was performed. To select transgene-free T_1 azygous plants, PCR was conducted to amplify the plant selection marker, the *HYGROMYCIN PHOSPHOTRANSFERASE* gene, using the primers Hyg Seq F and Hyg Seq R (Extended Data Fig. 6b and Supplementary Table 6). For genotyping of developing seeds, endosperm from 3, 5, 7 and 10 DAP seeds was used, and embryos were collected for histological analysis as described previously[5].

Histological analysis

For histological analysis, 3, 5, 7 and 10 DAP embryos were collected and fixed in FAA (10% formaldehyde, 5% acetic acid and 50% ethanol). Tissue embedding and sectioning was performed as described previously[44]. For toluidine blue staining, after rehydration, sections crosslinked to glass slides were stained with 0.01% toluidine blue for two minutes as per Khanday et al.[5]. Microscopic images were acquired with Zeiss AxioVision v.4.8.2.

Flow cytometry

Nuclei for fluorescence-activated cell sorting analysis were isolated using a leaf-chopping method[45]. The isolated nuclei were stained with propidium iodide at 40 µg ml^{-1} in Galbraith's buffer. Fluorescence-activated cell sorting analysis and DNA-content estimation were carried out using a Cytoflex flow cytometer and CytExpert v.2.6 software.

RT-PCR and RT-qPCR

RNA extractions for all samples were performed using the RNAqueous micro kit (Invitrogen), followed by DNase treatment (included in the kit).

The cDNAs used to amplify the Os*WOX9A* CDS and for quantitative reverse transcription polymerase chain reaction (RT-qPCR) were synthesized using the iScript cDNA synthesis kit (BioRad) according to the manufacturer's instructions; further details can be found in Khanday et al.[5]. cDNA was quantified on the Nanodrop ND 1000 Spectrophotometer (Thermo Fisher Scientific). RT-qPCR was performed with iTaq universal SYBR Green supermix (BioRad) using a CFX96 Touch real-time PCR system (BioRad), and the data were collected with Bio-Rad CFX Manager v.3.1 software. *UBIQUITIN5* (Os03g13170) was used as the internal control, and fold changes in the relative abundance of transcripts were calculated as described previously[46]. For RT-qPCR, amplifications for each gene were performed in three biological replicates, and each biological replicate was repeated in three technical replicates for each sample. All the primers used for RT-PCR and RT-qPCR are listed in Supplementary Table 6.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information. The genetic materials are available from the corresponding authors upon request. The rice reference genome sequence is publicly available at http://rice.uga.edu/. Source data are provided with this paper.

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Author contributions

V.S., I.K. and H.R. designed the study. I.K. and K.S. made the constructs for Os*WOX9A* ectopic expression. M.-J.C. and M.T. performed the rice transformations. H.R. performed all other experiments. H.R. analysed the data with V.S. and I.K. H.R., V.S. and I.K. wrote the paper.

Competing interests

The University of California, Davis, has filed a patent application on efficient induction of parthenogenesis in crop plants (PCT/US2023/034142) arising from this work. The authors declare no other competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Imtiyaz Khanday or Venkatesan Sundaresan.

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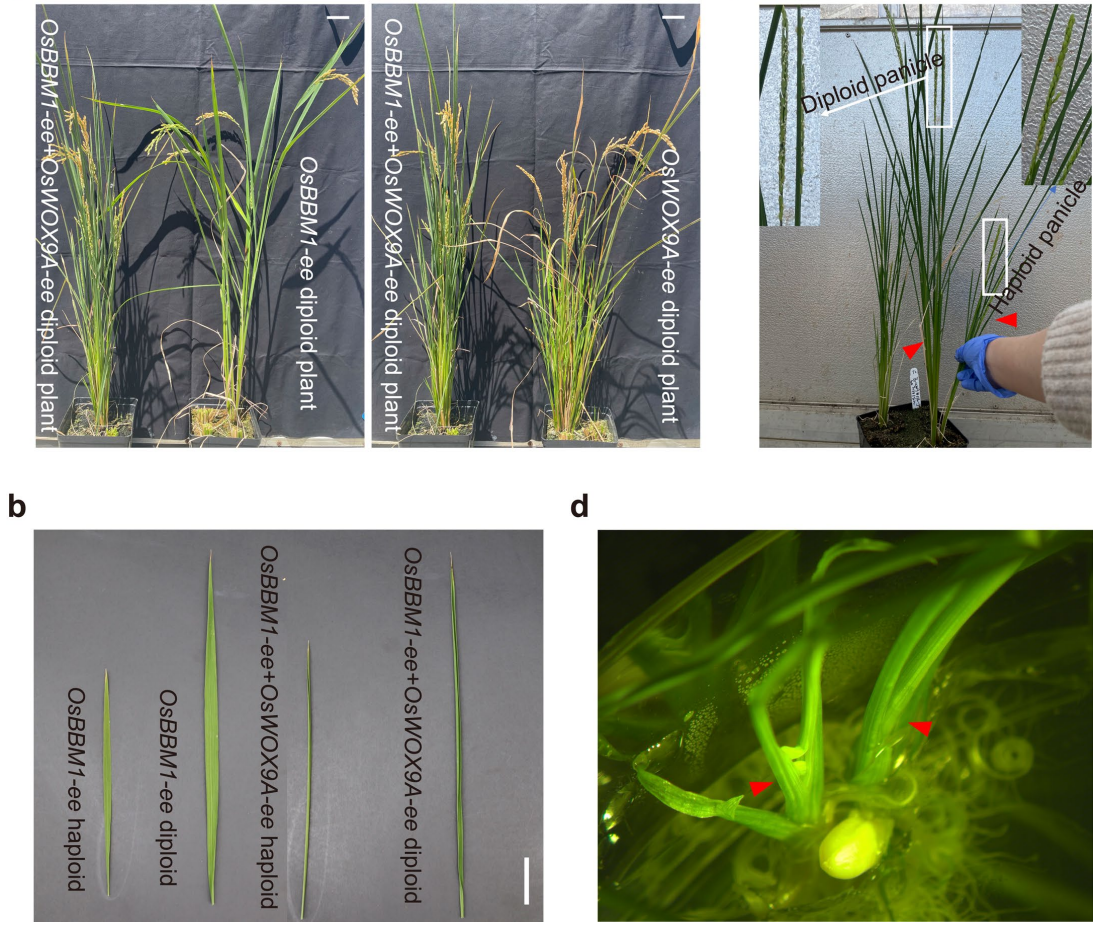
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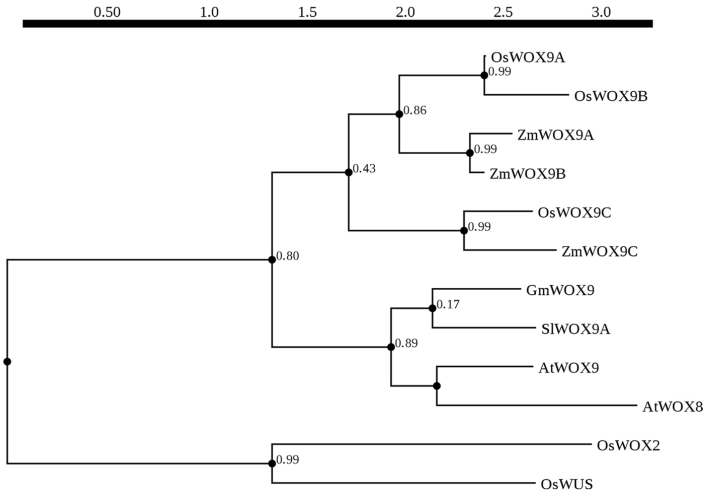
Extended Data Fig. 1 | Phenotypic effects of *pEC1.2::OsWOX9A* expression and appearance of twin plants after haploid induction. a. Expression of *pEC1.2::OsWOX9A* transgene resulted in smaller plants, whether in wild-type or in *OsBBM1-ee* background, suggestive of leaky vegetative expression from the *pEC1.2* promoter. Left, *OsWOX9A-ee* + *OsBBM1-ee* diploid plant *vs.* *OsBBM1-ee* only diploid plant, *OsWOX9A-ee* + *OsBBM1-ee* plants are smaller compared to those with only the *OsBBM1-ee* transgene; right, *OsWOX9A-ee* + *OsBBM1-ee* diploid plant *vs.* *OsWOX9A-ee* only diploid plant, *OsWOX9A-ee* plant in *OsBBM1-ee* background has similar size compared to that in wild-type background. Scale bar, 5 cm. **b**. *OsWOX9A*-ee + *OsBBM1-ee* plants have narrower leaves compared to *OsBBM1-ee* only plants. **c**-**d**, The occurrence of twin progenies in parthenogenetic plants. Red arrowheads point to each single seedling. **c**. Twin plants at flowering stage. Left, haploid-haploid twin; right, haploid-diploid twin. **d**. Twin seedlings arising from a single seed.

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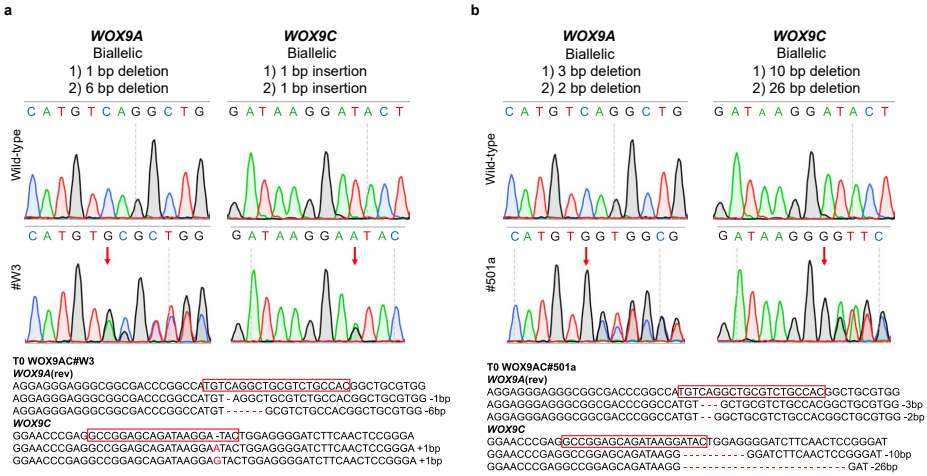
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GmWOX9      PEPKPRWNPKPEQIRILEAI-FNSGMVNPPRDEIRKIRVQLQEYGQVGDANVFYWFQNRKSRSKHKLR
SlWOX9A     PEPKPRWNPRPEQIRILEAI-FNSGMVNPPRDEIRKIRAKLQEYGQVGDANVFYWFQNRKSRSKHKQR
ZmWOX9A     PDPKPRWNPRPEQIRILEAI-FNSGMVNPPRDEIPRIRMRLQEYGQVGDANVFYWFQNRKSRSKNKQR
ZmWOX9B     PDPKPRWNPRPEQIRILEAI-FNSGMVNPPRDEIPRIRMRLQQYGQVGDANVFYWFQNRKSRSKNKLR
OsWOX9A     PDPKPRWNPRPEQIRILEAI-FNSGMVNPPRDEIPRIRMQLQEYGQVGDANVFYWFQNRKSRSKNKLR
OsWOX9B     PDPKPRRNPRPEQIRILEAI-FNSGMVNPPRDEIPRIRMQLQEYGQVGDANVFYWFQNRKSRSKNKLR
OsWOX9C     PEPKPRWNPRPEQIRILEGI-FNSGMVNPPRDEIRRIRLQLQEYGQVGDANVFYWFQNRKSRTKNKLR
ZmWOX9C     PEPRPRWNPRPEQIRILEGI-FNSGMVNPPRDEIRRIRLQLQEYGPVGDANVFYWFQNRKSRTKHKLR
OsWUS       RPSGTRWTPTTEQIKILRELYYSCGIRSPNSEQIQRIAAMLRQYGRIEGKNVFYWFQNHKARERQKKR
OsWOX2      ALANARWTPTKEQIAVLEGL-YRQGLRTPTAEQIQQITARLREHGHIEGKNVFYWFQNHKARQRQKQK
                  * .*  *** :*. : :  *  .*  ::* :*   *:::* : . ********:*:* ::* :
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b



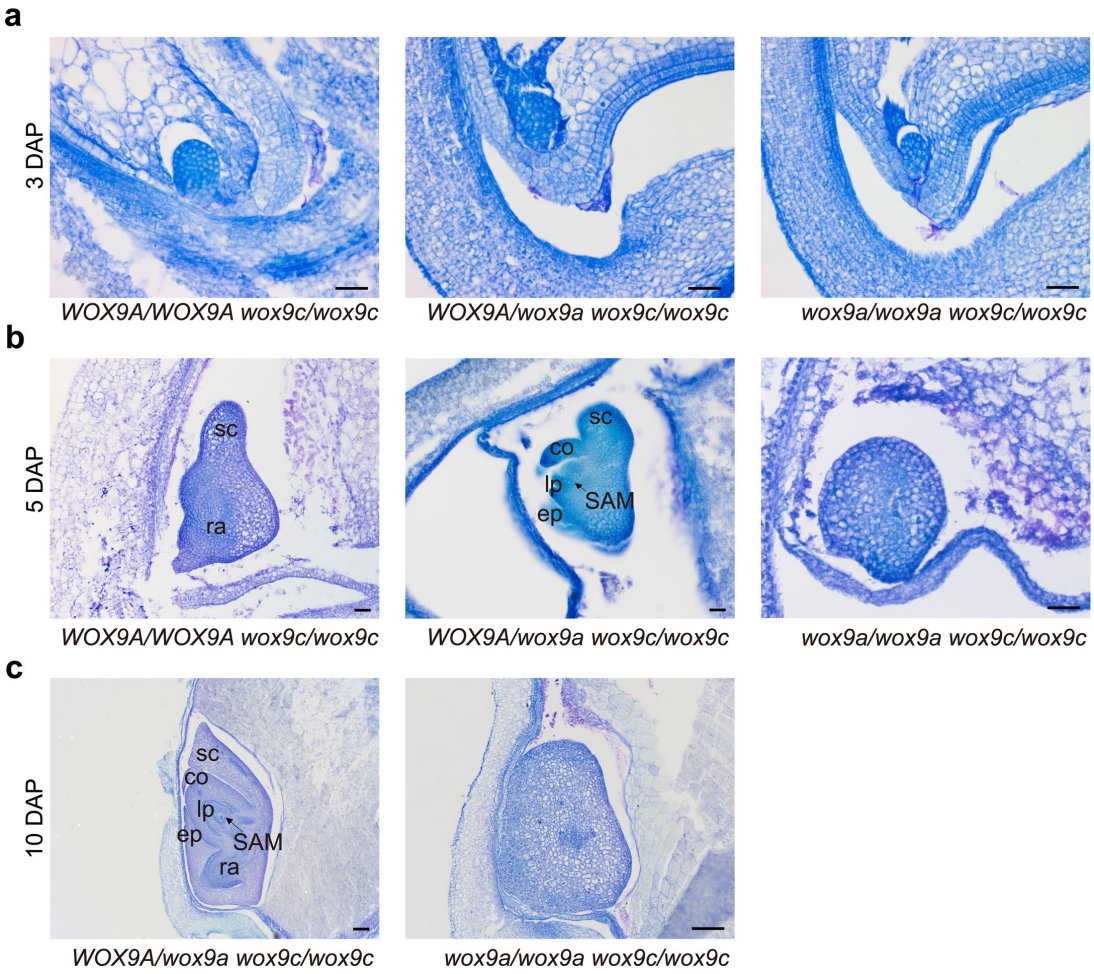
Extended Data Fig. 2 | Evolutionary relationship of *WOX9* family proteins. **a**. Alignment of a highly conserved 67 amino acid peptide domain of *WOX9* family proteins, corresponding to amino acid residues 64 to 130 of rice *OsWOX9A*. This region corresponds to a subdomain of the homeobox originally identified in *WUS*, the first member of this protein family to be characterized. For reference, the alignment of *OsWUS* and *OsWOX2* in this subdomain is shown in the bottom two rows. The red outlined box indicates the short peptide NVFYWFQN found in all the WUSCHEL-related proteins. However, the full 67 amino acid homeodomain is relatively poorly conserved in *OsWUS*, *OsWOX2* and other WUSCHEL-related proteins families that are not *WOX9* orthologs. **b**. A phylogenetic tree summarizing the evolutionary relationships between *WOX9* family proteins constructed using PhyML3.0 (ref. 47). *WOX9* gene duplications occurred independently in the cereal and Brassicaceae lineages.

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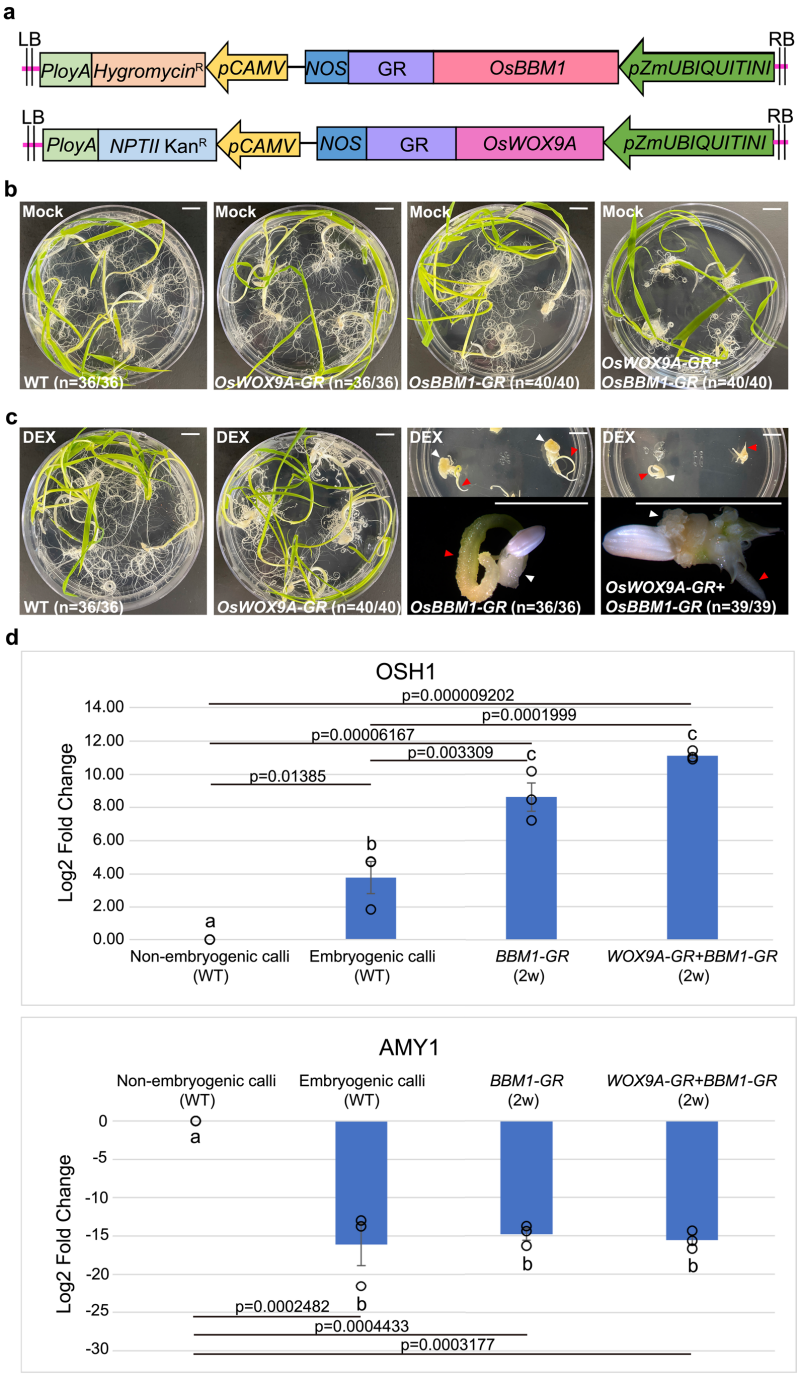
Extended Data Fig. 3 | Genotype of additional *wox9awox9c* mutant lines. **a**. Sequence chromatograms at mutation sites and mutant DNA sequences of *OsWOX9A* and *OsWOX9C* in transgenic line WOX9AC#W3 compared to wild-type sequences. This line is biallelic for *OsWOX9A* with a 1 bp deletion null allele and a 6 bp in-frame deletion allele. For *OsWOX9C*, this line is biallelic with both alleles as 1 bp insertions. **b**. Sequence chromatograms and mutant DNA sequences at mutation sites in the *OsWOX9A* and *OsWOX9C* genes of transgenic line WOX9AC#501a, compared to wild-type sequences. Line WOX9AC#501a is biallelic for *OsWOX9A* with a 3 bp in-frame deletion allele and a 2 bp deletion null allele. For *OsWOX9C*, this line is biallelic with 10 bp and 26 bp deletion alleles. SgRNA protospacer sequences are shown in red boxes. The reverse primer was used for *OsWOX9A* sequencing results presented.

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Extended Data Fig. 4 | Histological analysis of T2 progeny embryos from T1 line WOX9AC#4-6, segregating for *WOX9A* but homozygous mutant for *WOX9C*. **a**. Embryos from 3 DAP timepoint, before organogenesis stage. *WOX9A/WOX9A wox9c/wox9c* (left, n = 5/5) and *WOX9A/wox9a wox9c/wox9c* (middle, n = 6/6) embryos show normal development, while homozygous double mutant, *wox9a/wox9a wox9c/wox9c* (right, n = 6/6) embryos exhibit delayed development, showing smaller embryos with fewer cells. Scale bars, 100µm. **b**. Embryos from 5 DAP timepoint, after organ formation. *WOX9A/WOX9A wox9c/wox9c* (left, n = 4/4) and *WOX9A/wox9a wox9c/wox9c* (middle, n = 4/4) embryos show normal development, while *wox9a/wox9a wox9c/wox9c* (right, n = 2/2) embryos show arrested development. Co, coleoptile; ep, epiblast; lp, leaf primordia; ra, radicle; SAM, shoot apical meristem; sc, scutellum. Scale bars, 100µm. Arrested embryos appear to have no radicle, coleoptile, SAM, scutellum, or other structures. Instead, these embryos have only an epidermal layer, and the isotropic cells surrounding a core of more dense smaller cells inside. **c**. Embryos at 10 DAP, mature embryo developmental stage. *WOX9A/wox9a wox9c/wox9c* (left, n = 2/2) embryo shows normal development, while *wox9a/wox9a wox9c/wox9c* (right, n = 2/2) embryo shows arrested development. Scale bars, 400 µm.

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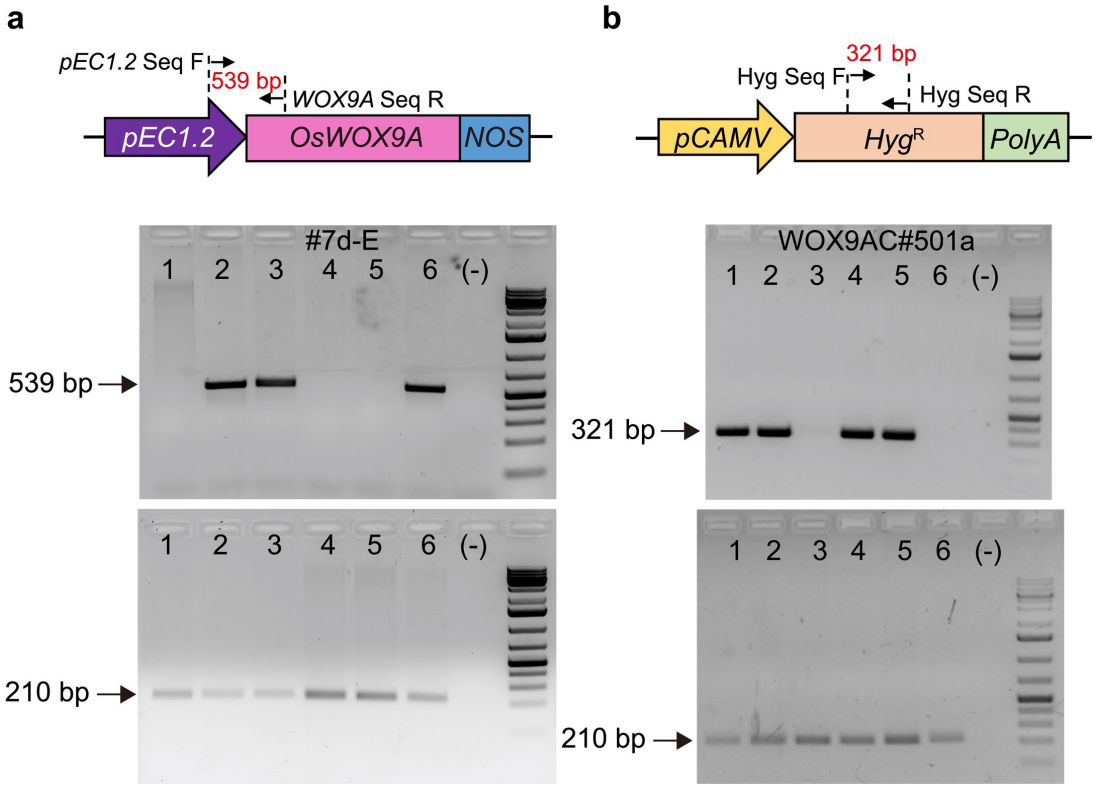


Extended Data Fig. 5 | See next page for caption.

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Extended Data Fig. 5 | Co-expression of *OsBBM1* and *OsWOX9A* using dexamethasone inducible glucocorticoid (GR) induction system. **a**. Schematics of *OsBBM1-GR* and *OsWOX9A-GR* constructs. (**b, c**). Phenotype of seeds germinating on ½ MS media mock treated **(b)**, or containing 10μM dexamethasone **(c)** (no other hormone added), for 2 weeks. From left to right, Wild-type (WT); *OsWOX9A-GR*; *OsBBM1-GR*; *OsWOX9A-GR* + *OsBBM1-GR*. Scale bars, 1 cm. Wild-type and *OsWOX9A-GR* germinated normally on dexamethasone (DEX) containing media, while *OsBBM1-GR* and *OsWOX9A-GR* + *OsBBM1-GR* formed calli from scutellum. Red arrowheads indicate shoots emerging from the seeds and white arrowheads point to calli. **d**. Expression of early embryo marker genes. RT-qPCR log2 fold changes in *OSH1* and *AMY1* transcripts is shown in *OsBBM1-GR* calli or *OsWOX9A-GR* + *OsBBM1-GR* calli treated for 2 weeks with 10μM dexamethasone. Embryogenic and non-embryogenic calli from scutellum of wild-type seeds were generated by callus induction media as previously described[26]. The log2 fold change is normalized to wild-type non-embryogenic calli. The height of columns represents mean values of the log2 fold changes, and error bars represent mean values ± SEM, calculated from three independent biological replicates and each data point represents the average of three technical replicates. Two-sided Tukey's HSD test was used for the statistical analysis, and P values for each comparison are displayed in the graphs. Samples from left to right are: Wild type non-embryogenic calli; wild type embryogenic calli; *OsBBM1-GR; OsWOX9A-GR* + *OsBBM1-GR*.

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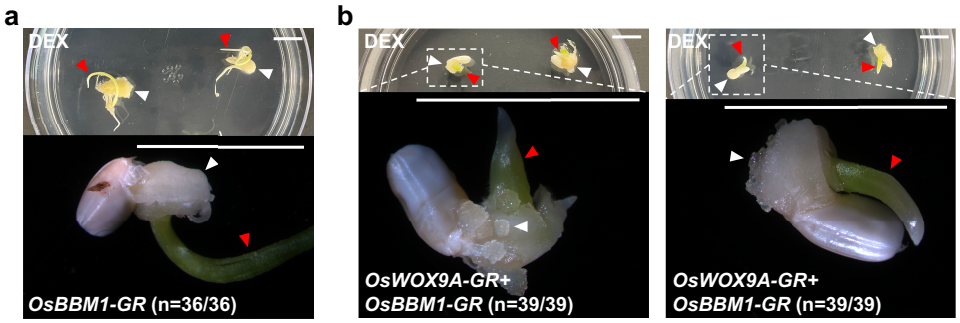
Extended Data Fig. 6 | Genotyping for *pEC1.2::OsWOX9A* and *WOX9A WOX9C* CRISPR-Cas9 segregating transgenic lines. a. PCRs amplifying transgene-specific DNA fragment at the intersection of *pEC1.2* promoter to *OsWOX9A* CDS with primers *pEC1.2* Seq F and *WOX9A* Seq R were performed to genotype the parthenogenetic lines. *RICE FLORICAULA LEAFY* (*RFL*)[43] (LOC_Os04g51000), a single copy gene, was used as the internal control. The fragment sizes of the amplicons - 539 bp for *pEC1.2::OsWOX9A* and 210 bp for *RFL*, respectively - were confirmed by comparison with the DNA marker. **b**. PCRs amplifying DNA fragment at *HYGROMYCIN PHOSPHOTRANSFERASE* (*HPTII*) gene were performed to confirm the presence of *WOX9A WOX9C* CRISPR construct, and *RFL* gene was used as an internal control. The fragment sizes of the amplicons - 321 bp for the CRISPR construct and 210 bp for *RFL*, respectively - were confirmed by comparison with the DNA marker. Primer sequences are provided in the Supplementary Table 6.

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Supplementary information https://doi.org/10.1038/s41477-024-01848-z

Synergistic induction of fertilization-independent embryogenesis in rice egg cells by paternal-genome-expressed transcription factors

In the format provided by the authors and unedited



Supplementary Fig.1: Additional examples of calli induction from co-expression of *OsBBM1* and *OsWOX9A* using dexamethasone inducible glucocorticoid (GR) induction system. More examples of phenotypes of germinating seeds from the experiment in Extended Data Fig. 5. Seeds germinated on ½ MS media containing 10μM dexamethasone (no other hormone added), for 2 weeks. **a**. *OsBBM1-GR*; **b.** *OsWOX9A-GR+OsBBM1-GR*. *OsBBM1-GR* and *OsWOX9A-GR+OsBBM1-GR* formed calli from scutellum. Suppression of shoot growth was observed with *OsWOX9A-GR+OsBBM1-GR* seeds but not with *OsBBM1-GR* seeds. Dotted boxes and lines indicate that the enlarged seed is the same one as outlined within the box on the plate. Scale bars, 1 cm. Red arrowheads indicate shoots emerging from the seeds and white arrowheads point to calli. Note that the seeds were removed from the plate for microscopy, so their orientations in the magnified images will differ from that on the plates.

T0 event	T1 progenies			
	Diploid (n=)	Haploid Twins(n=)	Haploid (n=)	% Haploid
#1a	26	1	0	3.70
#1b	23	4	0	14.81
#2	18	5	0	21.74
#3	22	0	3	12.00
#7d	29	25	2	48.21
#12a	23	6	3	28.13
#12b	45	34	4	45.78

Supplementary Table 1: Haploid induction and twin formation in *pEC1.2::OsWOX9A* plants in *OsBBM1-ee* background. Seeds from ten T0 events were germinated and tested. Three events showed transgene silencing as determined by failure of germination on hygromycin and G418 containing media. These were not analyzed further for haploidy induction. Parthenogenetic induction by *OsBBM1* egg cell expression results in twin embryo formation. Twins are two seedlings produced from one seed, and they always have at least one haploid seedling, confirmed by flow cytometry. Each pair of haploid twins is counted as one haploid plant. Haploid ratio = (haploid twins + haploid)/ (diploid+ haploid twins + haploid).

T0 event	T1 plants	T2 progenies		*pEC1.2::OsWOX9A* genotype
		pEC1.2::OsWOX9A +/+ or +/- (n=)	*pEC1.2::OsWOX9A* -/- (n=)	
#12b	12b-P	20	0	+/+
	12b-t1	24	0	
	12b-9	22	0	
	12b-O	16	8	+/-
	12b-t4	13	10	
	12b-t3	12	10	
#7d	7d-C	29	0	+/+
	7d-H	20	0	
	7d-2	12	9	+/-
	7d-E	10	11	
	7d-58	13	4	
#2	2-B	10	2	+/-
	2-C	8	4	
	2-D	8	4	

Supplementary Table 2: Genotyping data for T2 progenies of parthenogenetic lines. PCR amplifications to confirm the presence/absence of *pEC1.2::OsWOX9A* transgene were performed using primer *pEC1.2* Seq F and *WOX9A* Seq R. PCR amplifications of *RFL* gene were used as an internal control. Analyses are shown for progenies from three independent T0 lines.

a. Statistical analysis for haploids in T2 progeny				
Line#	Parent genotype	Diploid progeny (n=)	Haploid progeny (n=)	P values
#12b	*pEC::OsWOX9A/pEC::OsWOX9A*	12	122	1.9E-19
	pEC:: OsWOX9A/-	90	66	
	pEC:: OsWOX9A/-	90	66	3.9E-5
	-/-	115	29	
#7d	*pEC::OsWOX9A/pEC::OsWOX9A*	12	76	1.7E-13
	pEC:: OsWOX9A/-	73	41	
	pEC:: OsWOX9A/-	73	41	6.9E-11
	-/-	156	9	
#2	*pEC:: OsWOX9A*/-	31	13	0.0322
	-/-	87	13	

b. Statistical analysis for haploids in T3 progeny				
Line#	Parent genotype	Diploid progeny (n=)	Haploid progeny (n=)	P values
#12b	*pEC::OsWOX9A/pEC::OsWOX9A*	10	107	9.7E-12
	pEC:: OsWOX9A/-	35	28	
	pEC:: OsWOX9A/-	35	28	5.2E-8
	-/-	121	12	
#7d	*pEC::OsWOX9A/pEC::OsWOX9A*	11	95	2.3E-12
	pEC:: OsWOX9A/-	46	33	
	pEC:: OsWOX9A/-	46	33	7.9E-9
	-/-	137	17	

Supplementary Table 3: Statistical analysis for P value significance. Frequencies of haploids in the T2 and T3 progeny of *pEC1.2::OsWOX9A* in *OsBBM1-ee* background were analyzed by pairwise comparisons between siblings carrying different copy numbers of the segregating *pEC::WOX9A* transgene. P values were determined using two-sided Fisher's exact test. **a.** Statistical analysis for haploids in T2 progeny. **b.** Statistical analysis for haploids in T3 progeny. For each line, the frequencies of haploid progeny are significantly higher with increasing copies of the *pEC1.2::OsWOX9A* transgene.

a. Ploidy of T2 progenies							
T0 event	T1 plants	*pEC1.2::OsWOX9A* genotype	T2 progenies				
			Diploid (n=)	H-H Twins (n=)	H-D Twins (n=)	Haploid (n=)	% Haploid
#12b	12b-P	+/+	7	37	21	9	90.54
	12b-t1		1	29	9	6	97.78
	12b-9		4	3	8	0	73.33
	12b-O	+/-	45	12	7	2	31.82
	12b-t4		35	16	6	6	44.44
	12b-t3		10	11	3	3	62.96
	12b-s16	-/-	71	4	7	9	21.98
	12b-s18		44	2	3	4	16.98
#7d	7d-C	+/+	4	14	6	5	86.21
	7d-H		8	27	20	4	86.44
	7d-2	+/-	28	12	6	3	42.86
	7d-E		37	7	6	3	30.19
	7d-58		8	4	0	0	33.33
	7d-s2	-/-	82	2	4	1	7.87
	7d-s6		74	1	0	1	2.63
#2	2-B	+/-	7	0	3	1	36.36
	2-C		9	1	1	1	25.00
	2-D		15	4	2	0	28.57
	2-1	-/-	33	0	5	0	13.16
	2-2		54	2	4	2	12.90

b. Ploidy of T3 progenies							
T0 event	T2 plants	*pEC1.2::OsWOX9A* genotype	T3 progenies				
			Diploid (n=)	H-H Twins (n=)	H-D Twins (n=)	Haploid (n=)	% Haploid
#12b	12b-P-B5	+/+	8	30	25	13	89.47
	12b-P-C1		2	25	11	3	95.12
	12b-O-C4	+/-	31	15	5	2	41.51
	12b-O-C5		4	2	0	4	60.00
	12b-s16-1	-/-	65	2	2	4	10.96
	12b-s18-1		56	1	3	0	6.67
#7d	7d-H-6	+/+	6	22	5	28	90.16
	7d-H-7		5	21	9	10	88.89
	7d-E-A2	+/-	40	23	2	3	41.18
	7d-E-A3		6	4	0	1	45.45
	7d-s2-1	-/-	67	3	6	1	12.99
	7d-s6-3		70	1	5	1	9.09

c. Ploidy of T4 progenies							
T0 event	T3 plants	*pEC1.2::OsWOX9A* genotype	T4 progenies				
			Diploid (n=)	H-H Twins (n=)	H-D Twins (n=)	Haploid (n=)	% Haploid
#7d	7d-H-6-1	+/+	1	15	6	7	96.55
	7d-H-6-3		1	21	9	6	97.30

Supplementary Table 4: Detailed ploidy distributions from T2, T3 and T4 generations for selected lines of *pEC1.2::OsWOX9A* transformants in *OsBBM1-ee* background. a. Ploidy of T2 progenies from segregating T1 plants**. b.** Ploidy of T3 progenies from segregating T2 plants. **c.** Ploidy of T4 progenies from T3 *pEC1.2:: OsWOX9A* homozygotes. Haploid twins are plants with two seedlings arising from one seed, and they are either haploid-haploid twins (H-H; two haploid seedlings arising from one seed) or haploid-diploid twins (H-D; one haploid and one diploid seedling arising from one seed). Ploidy was confirmed by flow cytometry in all cases. Each pair of haploid twins is counted as one haploid plant. Haploid ratio = (H-H twins + H-D twins + haploid)/ (diploid+ H-H twins + H-D twins + haploid).

Line#	Parent genotype	Flowers	Filled Seeds	Fertility
#12b	*pEC::OsWOX9A/pEC::OsWOX9A*	363	177	0.4876
	pEC:: OsWOX9A/-	377	201	0.5332
	-/-	412	304	0.7379
#7d	*pEC::OsWOX9A/pEC::OsWOX9A*	284	118	0.4155
	pEC:: OsWOX9A/-	298	187	0.6275
	-/-	457	359	0.7856

Supplementary Table 5: Fertility of T3 plants from independent transformants carrying *pEC1.2::OsWOX9A* in a homozygous *OsBBM1-ee* background. The first three panicles from each plant and two plants per line per genotype were used to determine the fertility . Fertility = Number of filled seeds/total number of flowers.

Primer name	Sequence (5'->3')	Purpose
WOX9 F	5'-ATG GCG TCG TCG AAC AGG CAC-3'	Amplify *WOX9A* CDS
WOX9 R3	5'-CAGTCAGGCTAGCGGTGGC-3'	
WOX9A gRNA	5'-GTGGCAGACGCAGCCTGACA-3'	Crispr for *WOX9A* *WOX9C* mutant
WOX9C gRNA	5'-GCCGGAGCAGATAAGGATAC-3'	
pEC1.2 Seq F	5'- CTT CGT CTC CAA TAG GAG CG -3'	Genotyping
WOX9A Seq R	5'- GAG TTG AAG ATC GCC TCC AGG -3'	
WOX9A Seq F	5'- ATG GCG TCG TCG AAC AGG -3'	
WOX9C Seq F	5'- AGC TCG TCC TCT TCC TTC C -3'	
WOX9C Seq R	5'- ACT TGC GGT TCT GGA ACC AG -3'	
Hyg Seq R	5'- CTG CTC CAT ACA AGC CAA CCA C -3'	
Hyg Seq F	5'- CCG CAA GGA ATC GGT CAA TAC AC -3'	
RFL F4	5'- AAG ATG CGG CAC TAC GTG C -3'	
RFL R4	5'- CAC GTA CCA GAT GGC GAG G -3'	
BBM1 Seq R	5'- CAT GCC GAG GAA GTC CTC C -3'	
UBIQ5 F	5'- AGG ACA AGA TGA TCT GCC GC -3'	RT-qPCR for DEX-GR experiment
UBIQ5 R	5'- TTC TTC TTG GGC CTC AGC TG -3'	
AMY1 F	5'- ATG GTC GAC GAG AGG GTC AT -3'	
AMY1 R	5'- GCC GAC CTT CTG GTT CTG -3'	
OSH1 F	5'- GAG ATT GAT GCA CAT GGT GTG -3'	
OSH1 R	5'- CGA GGG GTA AGG CCA TTT GTA -3'	

Supplementary Table 6: Primers used in this study.

Chapter 3 Twin embryo formation by induced parthenogenesis

Hannah Peha*[1], Hui Ren*[1], Debra Skinner[1], Venkatesan Sundaresan[1,2]

[1]Department of Plant Biology, University of California-Davis, Davis, California, USA

[2]Department of Plant Science, University of California-Davis, Davis, California, USA

*These authors contributed equally

corresponding author: Venkatesan Sundaresan (sundar@ucdavis.edu)

orcid IDs: https://orcid.org/0009-0008-8793-6069, https://orcid.org/0009-0002-4095-619X, https://orcid.org/0000-0003-4715-670X, https://orcid.org/0000-0002-4670-0630

3.1 Abstract

Synthetic apomixis has been achieved through the induction of parthenogenesis by ectopic expression of the Baby Boom family of transcription factors. An associated phenomenon from this process is the formation of polyembryony including twin progeny at high frequencies, but the underlying mechanisms have not been explored. Here, we provide a brief description of the phenomenon, discuss potential mechanisms for twin formation in flowering plants, propose two possible models for their occurrence, and evaluate the available evidence from both dizygotic and monozygotic twins in relation to these models. The two proposed models are independent, but they can operate in combination. We conclude that both models are required to explain the types of

twins and triplets that we and others have observed. These models provide future directions for basic research, as well as suggest possible approaches towards reducing polyembryony when incorporating synthetic apomixis into crop plants such as maize where twinning is not desirable.

Author contributions

HP and HR contributed equally as co- first authors. Conceptualization, HP, VS; Data, HR, DS; Images, HR, DS; Writing, HP, VS; editing, VS, DS, HR. All authors have read and agreed to the manuscript.

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OPINION

Twin embryo formation by induced parthenogenesis

Hannah Peha[1] · Hui Ren[1] · Debra Skinner[1] · Venkatesan Sundaresan[1,2]

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Key message **Induction of parthenogenesis (embryo formation from unfertilized egg cells) by embryogenic transcription factors is associated with twin formation at high frequencies, and involves two distinct mechanisms.**

Abstract Synthetic apomixis has been achieved through the induction of parthenogenesis by ectopic expression of the Baby Boom family of transcription factors. An associated phenomenon from this process is the formation of polyembryony including twin progeny at high frequencies, but the underlying mechanisms have not been explored. Here, we provide a brief description of the phenomenon, discuss potential mechanisms for twin formation in flowering plants, propose two possible models for their occurrence, and evaluate the available evidence from both dizygotic and monozygotic twins in relation to these models. The two proposed models are independent, but they can operate in combination. We conclude that both models are required to explain the types of twins and triplets that we and others have observed. These models provide future directions for basic research, as well as suggest possible approaches towards reducing polyembryony when incorporating synthetic apomixis into crop plants such as maize where twinning is not desirable.

Keywords Apomixis · Zygote · Polyembryony · Monozygotic · Dizygotic

Introduction

Synthetic apomixis has the potential to be a revolutionary breeding tool for generating clonal hybrids. The successful introduction of synthetic apomixis in rice, a sexual plant and a major crop (Khanday et al. 2019), and the subsequent adaptation of this method to generate clonal hybrid rice have made the practical application of this approach to agriculture a realistic possibility (reviewed in Mahlandt et al. 2023). Inducing parthenogenesis has been a key step in achieving synthetic apomixis through this method. The paternally expressed transcription factor *BABY BOOM1* (*BBM1*) drives the egg-to-zygote transition in rice (Khanday et al. 2019). *BBM1* and its orthologs in other cereals, as well as the related *ASGR-BBML1* gene from apomictic pearl millet, can induce parthenogenesis when ectopically expressed under an egg cell specific promoter (Conner et al. 2017; Khanday et al. 2019; Skinner et al. 2023).

An unexpected aspect of this induced parthenogenesis is the occurrence of polyembryony, especially the formation of twins observed in several crop and model plants. The examples shown in Fig. 1. illustrate the formation of dizygotic twins, defined as originating from separate eggs (Fig. 2a) and of monozygotic twins, defined as originating from a single egg that later splits as a zygote (Fig. 2b). Twin seedlings arising from egg cell expression of *BBM1* genes have been reported in *Oryza sativa* (Conner et al. 2017; Sidhu et al. 2022; Vernet et al. 2022; Dan et al. 2024) *Zea mays* (Skinner et al. 2023) and the dicot *Arabidopsis thaliana* (Liu et al. 2024); and in *O. sativa,* triplets have also been reported (Dan et al. 2024). In some studies, from 33–100% of the parthenogenic events resulted in twins (Sidhu et al. 2022; Ren et al. 2024). Naturally occurring twins are rare in flowering plants, occurring at less than 0.02% (Nettancourt and Stokes 1960; Vernon and Meinke 1994), except in some natural apomicts. Twin embryos have been observed up to 8% in the gametophytic apomict *Elymus rectisetus* (Cai et al. 1997), and 13–50% in accessions of the apomict maize relative *Tripsacum dactyloides* (Farquharson 1955; Bantin et al. 2001). Polyembryony has also been reported in apomictic

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Hannah Peha and Hui Ren have contributed equally to this work.

✉ Venkatesan Sundaresan
sundar@ucdavis.edu

[1] Department of Plant Biology, University of California, Davis, CA, USA

[2] Department of Plant Sciences, University of California, Davis, CA, USA

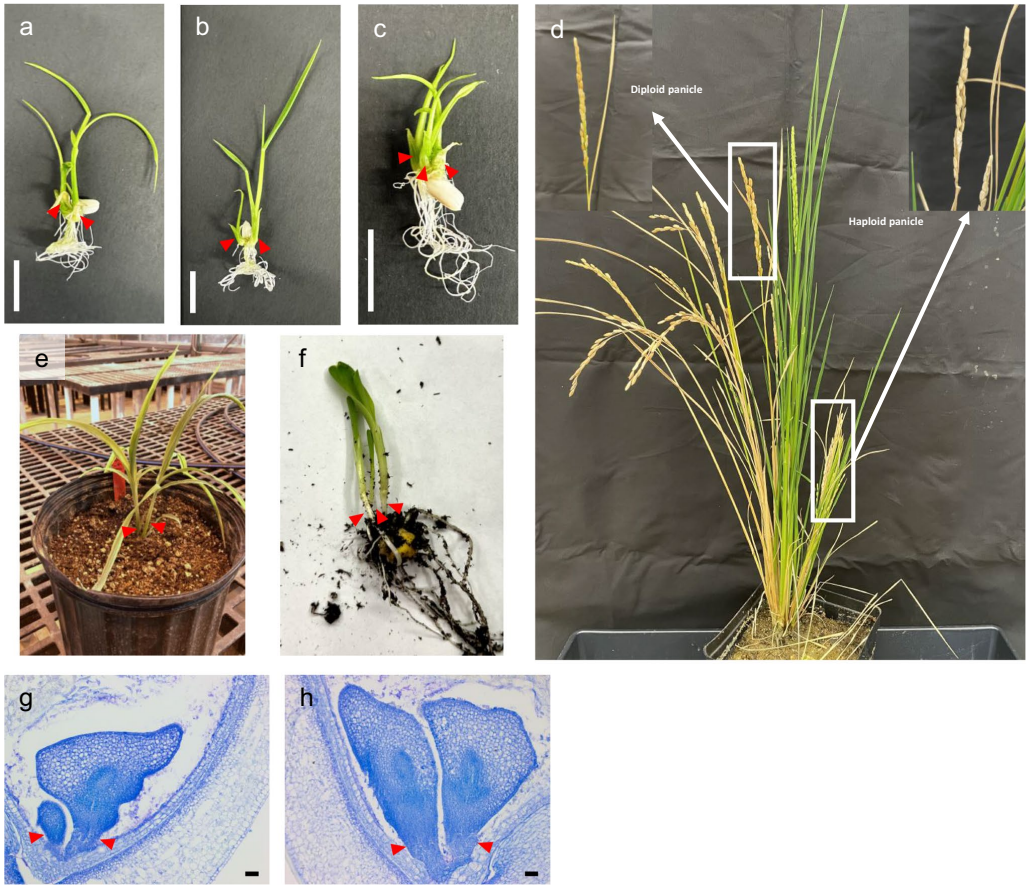


Fig. 1 Examples of twins and triplets in parthenogenetic lines of rice and maize with egg cell expression of *OsBBM1* and *ZmBBM1* respectively. **a** *Oryza sativa* haploid-haploid twins **b** unknown-haploid twins and **c** haploid-haploid-haploid triplets. Scalebars, 1 cm. **d** *Oryza sativa* diploid-haploid twins. **e** *Zea mays* haploid-haploid twins and **f** triplets. **g** and **h** Cross sections of *Oryza sativa* twin embryos 5 days after pollination of different sizes (**g**) and of similar sizes (**h**). Scalebars, 100 um. Arrowheads point to the individual seedlings or embryos that constitute twins or triplets

maize-Tripsacum hybrids (Grimanelli et al. 2003), and in polyembryonic ApIII rice (Mu et al. 2010). The association of high frequencies of twins with parthenogenesis was described in the classic studies of the Salmon haploid induction system in wheat (Tsunewaki et al. 1968; Kobayashi and Tsunewaki 1978).

Despite the ubiquity of twin formation during induced parthenogenesis using BBM1 and related transcription factors, the underlying mechanisms are as yet unexplored. In addition to the intrinsic biological interest of this curious phenomenon, there are also implications for the field application of synthetic apomixis technology. For crops such as maize which has been bred for non-tillering to preserve uniformity of plant structure and grain production for more intense management (Lusvardi 2022), twins are undesirable. Insights into this phenomenon may enhance our understanding of basic mechanisms of seed initiation, as well as provide important information for the adoptability of synthetic apomixis to agriculture.

Genetic mechanisms for twin embryos in sexual plants

Arabidopsis mutants in which twin embryos occur at high frequencies (up to 10%) have been previously described, where the second embryo arises from conversion of

suspensor cells to embryonic fate. However, these mutants are associated with severe developmental defects in the embryo proper and in the seedlings (Vernon and Meinke 1994; Zhang and Somerville 1997). In contrast, twins associated with parthenogenesis undergo normal embryo development and give rise to normal adult plants, and are therefore unlikely to arise from such a mechanism. Direct evidence that the parthenogenetic twins are not suspensor derived is provided by microscopy observations of embryos in parthenogenetic lines, showing that twin embryos possess separate suspensors (Fig. 1g, h).

Other studies in *Arabidopsis* have found that a mechanism that can lead to the development of two embryos from a single embryo sac is the formation of a supernumerary egg cell, giving rise to two genetically identical dizygotic twins after pollination. These supernumerary egg cells are generally derived from the accessory synergid cells in the embryo sac. The plasticity of synergid cell fate has been extensively demonstrated using genetic cell ablation (Lawit et al. 2013), as well as mutants affecting female gametophyte cell differentiation, such as *lachesis*, *eostre,* and *amp1* (Groß-Hardt et al. 2007; Pagnussat et al. 2007; Kong et al. 2015). The current model is that a wild-type egg cell suppresses the differentiation of adjacent synergids into egg cells, and that egg cell ablation or gametophyte mutations lead to loss of this suppression, and consequently the switch of a synergid to egg cell (Hater et al. 2020). In some instances, the supernumerary eggs could be fertilized and developed into twin embryos, but the seeds subsequently abort due to the absence of endosperm from an unfertilized central cell, because both sperm cells from pollen have fused with the two egg cells. Another possible twinning mechanism could be the division of the fertilized egg to form two zygotes, as sometimes observed in mammals (Scott 2002). Evidence that this mechanism is possible in flowering plants comes from a study in which levels of ascorbate were manipulated in tobacco to produce monozygotic twins at a low frequencies (~2%), by the longitudinal splitting of the zygote after fertilization (Chen and Gallie 2012).

Models for twinning and polyembryony from induced parthenogenesis

Based on our observations of twins in parthenogenetic lines and understanding of the potential mechanisms that underlie twinning, two models can be proposed for the developmental origin of twin progeny in plants ectopically expressing *BBM1* in the egg cell (Fig. 2). In all cases, to produce viable seeds the central cell must be fertilized to form the endosperm, which requires the availability of a sperm cell and at least one synergid cell. In the first model we hypothesize that after the unfertilized egg cell transitions to a zygote under the action of BBM1, one of the synergids can switch its cell fate identity to an egg (Fig. 2a). In this dizygotic model, the synergid-derived egg can then give rise to one of two different types of twins: i) It may also undergo parthenogenesis, so that the resulting embryos will be genetically identical haploid-haploid parthenogenetic dizygotic twins; or ii) The supernumerary egg may be fertilized resulting in genetically non-identical parthenogenetic haploid-sexual diploid dizygotic twins (Fig. 2a). In the second model, we hypothesize that a parthenogenetic zygote derived from the egg cell divides to produce two zygotes, due to maintenance of pluripotency after division, resulting in monozygotic haploid-haploid twins (Fig. 2b).

We have recently obtained high frequencies of parthenogenesis using a combination of two transcription factors, *BBM1* and *WOX9A* (Ren et al. 2024). In this study, 364 twin pairs were analyzed, and ratios of haploid-haploid to haploid-diploid twins were found to range from 0.3 to 3.4 with an average ratio of 2.1 in the T2 through T4 generation of two transformant lines. Because we used inbred rice lines, we could not distinguish between sexual diploids arising from fertilization and the possibility of diploids that arose from asexual haploids through spontaneous genome doubling. However, in our study of *BBM1*-induced parthenogenesis in maize, cross pollination with a different maize inbred allowed identification of sexual diploids (Skinner et al. 2023). In this study, molecular analysis revealed that 10 of 13 genotyped pairs of twins were presumptive haploid-haploid while the remaining three consisted of haploid-sexual diploid twins, i.e., the haploid twins had only the maternal marker allele and therefore parthenogenetic, whereas the diploid twins were heterozygous for the maternal and paternal alleles, and therefore sexual in origin. The occurrence of these discordant twins supports the dizygotic model, in which a synergid switches fate to become a supernumerary egg cell (Fig. 2a). Interestingly, an early study of apomictic *Tripsacum* had first suggested that synergid derived embryos might be source of twin formation (Farquharson 1955), and a model in which haploid-diploid twins arise from the egg cell and synergid had been proposed for the complex Salmon haploid induction system in wheat, in which a unknown parthenogenetic factor results in haploid-diploid twins, with the difference that the synergid was assumed to be capable of being fertilized, since the plasticity of the synergid to egg cell fate switch in the absence of a functional egg cell was not known at that time (Tsunewaki et al. 1968). Because haploid-haploid twins can also be explained by the possibility (i) of the synergid to egg switch model (see above), a separate mechanism involving zygote splitting is not necessary to account for the occurrence of twins.

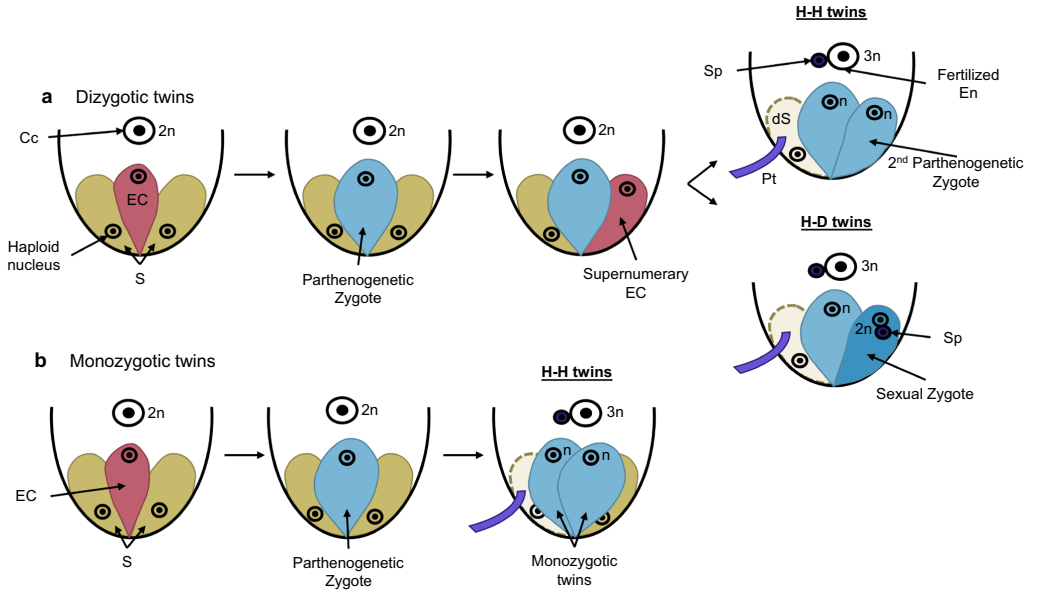


Fig. 2 Schematic representation of twin zygotes formation. Gametophytes shown are from lines with ectopic *BBM1* expression under an egg cell (EC) specific promoter. **a** Dizygotic twins. A parthenogenetic zygote arises from the egg cell allowing a synergid (S) to convert to a supernumerary egg cell. The supernumerary egg cell gives rise to haploid-haploid twins by parthenogenesis or haploid-sexual diploid twins by fertilization. **b** Monozygotic twins. A parthenogenetic zygote arises from the egg cell. Haploid-haploid twins form when the zygote divides to form two zygotes due to maintenance of pluripotency. The endosperm (En) forms in all pathways by fertilization of the central cell (Cc) by a sperm cell (Sp). A degenerated synergid (dS) is a byproduct of fusion with the pollen tube (Pt)

However, a more complex picture emerges when considering the results from the analysis of triplets, which are also observed in the parthenogenetic lines, although much less frequently. From the study we recently conducted of 2 independent parthenogenesis induction lines in rice (Ren et al. 2024), out of 511 progeny of which 373 were polyembryonic, only 9 were triplets; of these 7 were haploid-haploid-haploid triplets and 2 were haploid-haploid-diploid triplets (unpublished results). Both types of triplets develop into viable plants, as they are derived from seeds with mature endosperm. At least one synergid is required to attract the pollen tube for fertilization of the endosperm, and that synergid will be degenerated in the process. Thus, the haploid-haploid-haploid triplets could not have been generated by both synergids switching to egg cells; endosperm formation requires that only one synergid can switch to an egg cell. Therefore, the existence of these triplets is best explained by a mechanism involving monozygotic twin formation by splitting of a parthenogenetic zygote, as illustrated in the second model (Figs. 2b, 3). Interestingly, viable tetraploid-diploid-diploid triplets have been reported in parthenogenetic lines with unreduced gametes in rice (Dan et al. 2024). The tetraploid individual likely arose from fertilization of a supernumerary egg as proposed in the synergid to egg cell fate switch model. Therefore, it is likely that both of the mechanisms invoked by the two different models are at play, to account for the occurrence of the different types of polyembryony observed during induction of parthenogenesis.

Conclusion

In summary, the formation of twins and triplets in parthenogenetic induction likely arises from the action of two different mechanisms discussed above, both operating independently but capable of acting in combination. The formation of monozygotic twins is of particular interest, because there is very limited information on this process in flowering plants. Further studies are needed to understand how the pluripotency of the parthenogenetic zygote is maintained in the daughter cells after the initial division, and whether additional divisions can occur to form triplets or higher order polyembryony. In addition, there are practical implications for the application of synthetic apomixis to farmers' fields, when polyembryony is not desirable. Tuning the dosage of the egg cell expressed *BBM1* transgene will likely be

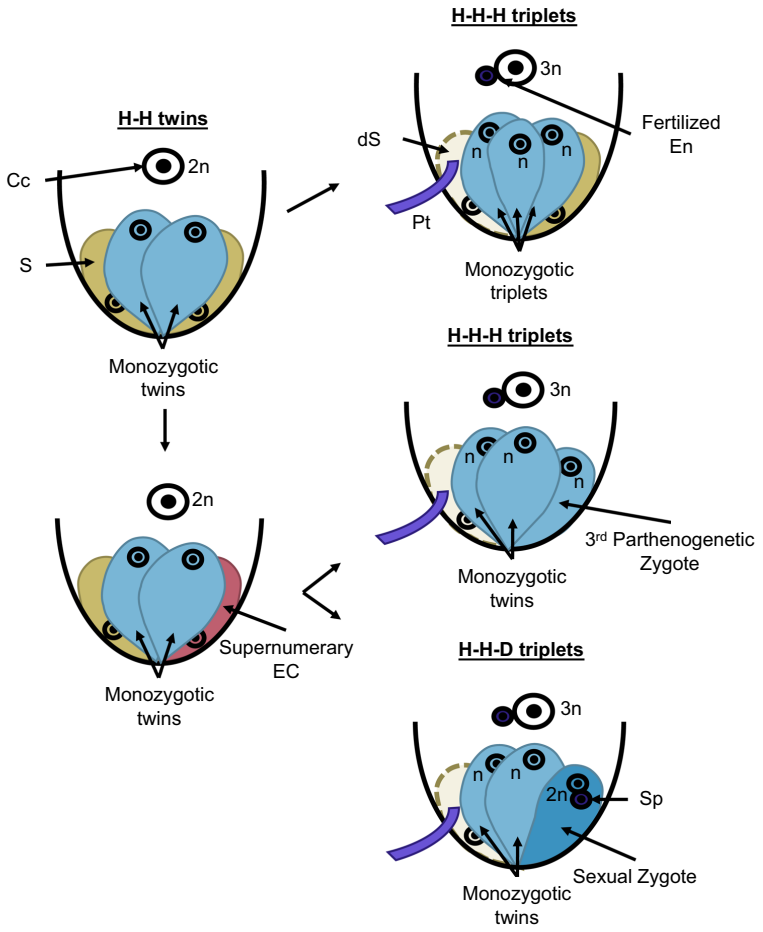


Fig. 3 Monozygotic haploid-haploid twins (Fig. 2b) can explain the formation of triplets. Haploid-haploid-haploid triplets form when a zygote splits again to form monozygotic triplets (top) or a syngergid derived supernumerary egg cell (EC) enables a second parthenogenetic event (middle). Haploid-haploid-diploid triplets form when a syngergid derived supernumerary egg cell is fertilized (bottom). The endosperm (En) forms in all pathways by fertilization of the central cell (Cc) by a sperm cell (Sp). A degenerated synergid (dS) is a byproduct of fusion with the pollen tube (Pt)

required to prevent twin formation. In the synergid-to-egg cell switch mechanism, delaying parthenogenesis, by egg cell expression of *BBM1* under a delayed onset promoter would reduce the time frame when a supernumerary egg could form. In the monozygotic twin mechanism, retention of the pluripotent state even after the division of the parthenogenetic zygote might be the result of an excess of *BBM1* gene product that suppresses differentiation of cell fates in the two celled proembryo. Altering the timing and expression levels of *BBM1* in parthenogenesis induction may reveal the role it plays in twin formation and in conferring zygotic fate, as well as provide an improved methodology for synthetic apomixis without the complications of polyembryony.

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Author contribution statement HP and HR contributed equally as co-first authors. Conceptualization, HP, VS; Data, HR, DS; Images, HR, DS; Writing, HP, VS; editing, VS, DS, HR. All authors have read and agreed to the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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Chapter 4 Synthetic apomixis in triploid rice

4.1 Introduction

Heterosis, or hybrid vigor, is a phenomenon in which hybrid offspring exhibit superior traits when compared to their inbred parents, particularly in terms of crop yield[1,2]. There are two primary genetic models proposed to explain heterosis. The dominance model proposes that each parent carries different, slightly deleterious recessive alleles, which are masked in the hybrid offspring. The complementation of these alleles in the hybrid enhances genetic plasticity, which allows the hybrid to better adapt to variable environmental conditions, as well as to exhibit increased biomass production and fertility. The overdominance model proposes that heterosis arises from the superior performance of heterozygous alleles, whereby certain heterozygous loci confer advantages that do not present in either homozygous state[1–3]. Neither of these models fully accounts for the complexity of heterotic effects. While the dominance model can explain certain aspects of heterosis, emerging evidence suggests that genomic dosage effects also play a crucial role in the mechanism of heterosis[4].

In maize (*Zea mays*), heterosis has been a cornerstone of modern breeding programs. Traits such as seedling biomass, plant height, tolerance to biotic and abiotic stresses, and grain yield exhibit strong heterotic responses[5]. Grain yield, in particular, is the most intensively studied heterotic trait due to its pronounced heterotic effect and significant agricultural and economic relevance. The situation is similar to other important crops like rice (*Oryza sativa*) and tomato (*Solanum lycopersicum*), in which most heterotic phenotypes are associated with yield[6–8]. Despite its benefits, a key limitation of heterosis lies in its instability across generations. Due to genetic segregation, high-yield hybrids cannot be maintained in the F_2 population and have to be generated

afresh for each growing season through controlled crosses, which demands substantial time, labor, and financial resources[9].

This limitation has sparked interest in developing synthetic apomixis for hybrid crop plants, which self-propagate through seeds while maintaining the hybrid condition. Achieving this goal would represent a transformative advance in crop science, offering a more sustainable and cost-effective approach to hybrid agriculture.

Apomixis is a form of asexual reproduction in plants where seeds are produced without fertilization, resulting in offspring that are genetically identical to the mother plant[10,11]. In natural apomixis, the process bypasses the typical sexual reproduction pathway, eliminating the need for meiosis and the fusion of male and female gametes. Apomixis occurs naturally in various wild plant species, such as *Pennisetum*, *Poa,* and *Hieracium*[11,12], but has not been identified in major crop plants. Harnessing naturally occurring apomictic mechanisms for crops has been unsuccessful so far. Recently, it was reported that emasculated sunflowers (*Helianthus annuus*) spontaneously form parthenogenic haploid seeds, with a haploid yield reaching up to 199 haploids per head under preferred conditions[13]. Although the underlying mechanism is not yet understood, this opens new possibilities for exploring apomixis or apomixis-like pathways in crop breeding, particularly for the propagation of seeds with desirable hybrid traits without the need for repeated crossing.

With the lack of natural apomixis in major crops, the possibility of engineering synthetic apomixis in plants, which recapitulates a natural mode of apomixis, has been explored[14,15]. In many natural apomicts exhibiting diplospory, a form of gametophytic apomixis, the megaspore mother cell undergoes aberrant or suppressed meiosis, leading to the formation of an unreduced embryo

sac[10,11]. The unreduced egg cell inside the embryo sac then develops via parthenogenesis, resulting in clonal progeny.

To produce the unrecombined and unreduced diploid egg cell in crop plants, meiosis can be converted to mitosis through the combination of mutations in three genes - *REC8*, *SPO11-1*/*PAIR1* and *OSD1* (MiMe - Mitosis instead of Meiosis)[16,17]. Each mutation targets one of three main, different features between meiosis and mitosis. First, *spo11-1* abolishes meiotic recombination. Second, *rec8* causes the separation of sister chromatids at the first meiotic division, instead of the distribution of homologous chromosomes. Last, *osd1* causes the skipping of the second meiotic division. As a result, meiosis in MiMe occurs without recombination and distributes sister chromatids in a single round of division, mimicking a mitotic division and producing the diploid unrecombined and unreduced gametes. These diploid gametes can undergo sexual reproduction to form tetraploid plants, which carry all the parental chromosomes, or undergo *BBM*-mediated parthenogenesis to form diploid clonal plants[14].

Therefore, to mimic the natural apomixis model, synthetic apomixis has two key components: the formation of an unrecombined and unreduced diploid egg cell and the parthenogenetic development of this egg cell, leading to a clonal diploid zygote[10,11]. Our previous demonstration of synthetic apomixis in rice, which combined a system of MiMe-produced unrecombined and unreduced (2n) gametes with *OsBBM1*-induced parthenogenesis, mimicked this type of natural apomixis and proved that synthetic apomixis in crop plants is feasible[14].

Triploid hybrids, which contain three sets of chromosomes (3n), often display enhanced hybrid vigor compared to diploid hybrid plants[18]. In many plants, for example, willow and maize, the triploid hybrids tend to be larger in terms of plant size and produce higher yields[4,18,19]. These

attributes can make triploidy attractive for certain agricultural applications[19]. Triploid plants are usually sterile because three sets of chromosomes cannot segregate properly during meiosis. In fruits, such as watermelons and grapes, the sterility of triploids is a desirable trait, as it enables the production of seedless fruits, which are favored by consumers and have higher market value.

From a breeding perspective, triploid hybrids can be bred to have further yield advantages conferred by both heterosis and increased genomic content[18,20]. This can result in superior phenotypic performance of triploid hybrids compared to both diploid hybrids and triploid inbred lines. However, the sterility of triploids also presents a major limitation in that they cannot reproduce sexually due to unequal segregation of homologous chromosomes at meiosis, and therefore, they cannot self-propagate. This restricts their use in crops like rice and maize, where seed-based propagation is essential for large-scale cultivation. The possibility of synthetic apomixis arising recently also provides a transformative solution for seed-propagated crops. Its integration into breeding programs could address long-standing challenges in hybrid agriculture and contribute significantly to the sustainability and resilience of global food systems. In this study, I constructed apomictic triploids in rice and demonstrated that they could be stably propagated as triploids through seeds.

4.2 Materials and methods

Plant materials and growth conditions.

Rice cultivar Kitaake (*Oryza sativa subsp. japonica*) was used in this study. Diploid apomixis line Os-Apo-Fr#27d has the all-in-one construct with both *pOsECA1::OsBBM1* and CRISPR-MiMe components (Fig. 4.1a)[15]. F_1 Triploid plants are generated from the crosses

between Os-Apo-Fr#27d and wild-type. Three independent lines were analyzed for synthetic apomixis.

Wild-type and Os-Apo-Fr#27d seeds were germinated on half-strength Murashige and Skoog's (MS) medium[21] containing 1% sucrose and 0.3% phytagel in a growth chamber for 12 days, under a 14h light/10 h dark cycle with 28/25 °C day/night temperatures and relative humidity > 65%. Seedlings were then transferred to the greenhouse and grown under natural light conditions at 80% relative humidity and 28/20 °C day/night temperatures in Davis, California. Supplemental light and heat were used during the winter months.

Crosses and embryo rescue.

Flowers from Os-Apo-Fr#27d T_1 transgenic rice plants were emasculated around the anthesis stage as the female parent, bagged, and then crossed to wild-type plants the next day. Flowers from wild-type plants were hand-pollinated on the emasculated flowers during the anthesis stage. Embryos were collected at 7 days after pollination, and embryo rescue was performed on the same day. Then the embryos were grown on embryo rescue media 1 containing 1.8g MS, 20g sucrose, and 2.4g phytagel per liter in a growth chamber in the dark for 1-2 weeks. After the shoots emerging from the rescued embryos, they were moved to embryo rescue media 2 containing 1.8g MS, 12g sucrose, and 1.1g phytagel per liter and kept in a growth chamber under a 14h light/10h dark cycle until transferring to soil.

Genotyping

Genotyping of MiMe genes was carried out by PCR-amplifying DNA at the mutation site with primers *OSD1* Seq F 5'- TTA CTT GGA AGA GGC AGG AGC C -3' and *OSD1* Seq R 5'-

ACC TTG ACG ACT GAC GTG ATG TC -3', *PAIR1* Seq F 5'- GTG GTG TGG TGT GTT CAG GAG -3' and *PAIR1* Seq R 5'- TGG AAT CCC CAA TCA GTA AGG CAC -3', *REC8* Seq F 5'- GCA CTA AGG CTC TCC GGA ATT CTC -3' and *REC8* Seq R 5'- AAT GGA TCA AGG AGG AGG CAC C -3'. Sanger sequencing of the three PCR amplicons of 364 bp, 344 bp and 328 bp, respectively, were performed.

Flow cytometry.

Nuclei for fluorescence-activated cell sorting (FACS) analysis were isolated by a leaf-chopping method[22]. The isolated nuclei were stained with propidium iodide at 40 μg/ml in Galbraith's buffer. FACS analysis and DNA-content estimation were carried out by using the Cytoflex flow cytometer and CytExpert 2.6 software.

4.3 Results

To create triploid apomixis rice, I used a synthetic apomixis diploid line Os-Apo-Fr#27d generated by introducing an all-in-one construct with both *pOsECA1::OsBBM1* and CRISPR-MiMe components[15] (Fig. 4.1a). These Os-Apo-Fr#27d plants provided the diploid (2n) egg cell as the female parent, and were crossed to wild-type plants that served as the male parent to provide the haploid (1n) sperm cell (Fig. 4.1b). For the endosperm development in these crosses, the maternal-to-paternal genome ratio is 4:1, which deviates significantly from the normal 2:1 ratio. The unbalanced endosperm genome ratio could lead to a post-zygotic reproductive barrier, which is also called the triploid block, and cause the seeds to abort early in development[23]. To prevent seed abortion in these crosses, embryo rescue was performed at 7 days after pollination. The expected progeny resulting from the crossing would be the triploid plants with the all-in-one

construct. Since the apomixis line Os-Apo-Fr#27d is the maternal parent, which could also produce diploid progeny resulting from parthenogenesis events that occurred before fertilization, I used flow cytometry to determine the ploidy of the F_1 progeny to identify the triploid progeny resulting from the designed crossing (Fig. 4.1c, 4.1d).

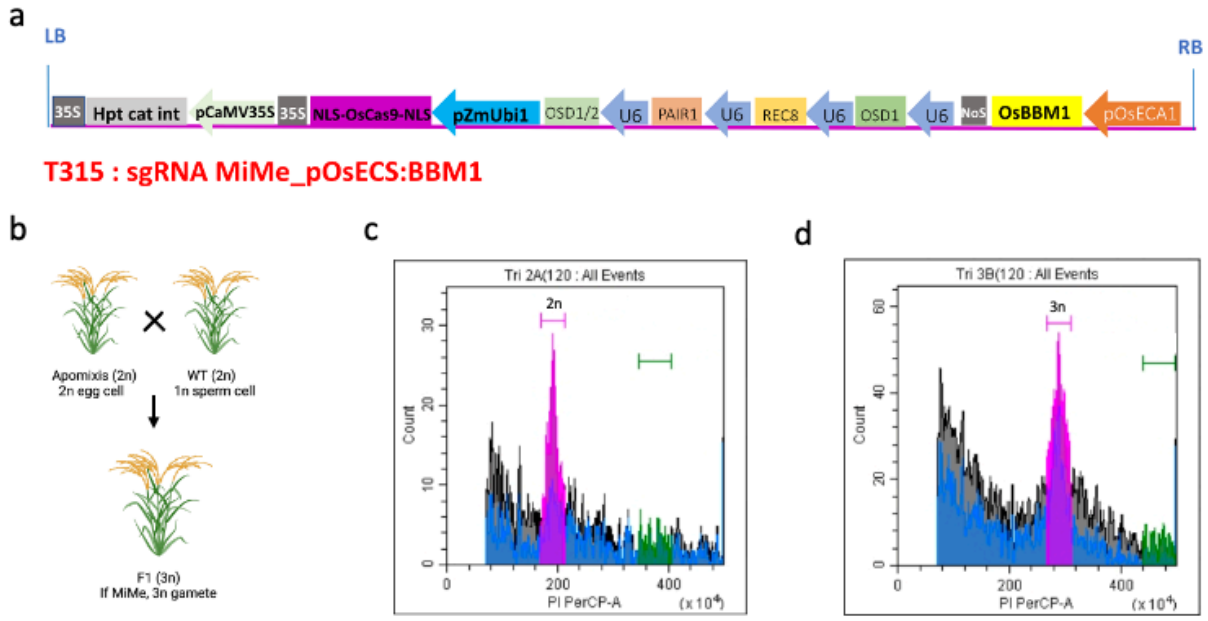


Fig. 4.1. Triploid rice plants were obtained by crossing Os-Apo-Fr#27d and wild-type. a, The schematic of the all-in-one construct that Os-Apo-Fr#27d has, containing both *pOsECA1:OsBBM1* and MiMe components. **b**, The scheme for creating apomixis triploid rice. Os-Apo-Fr#27d (Apomixis) plant served as the female parent, which provided the diploid (2n) egg cell, and was crossed to the wild-type plant that served as the male parent, which provided the haploid (1n) sperm cell, to form the triploid (3n) embryo. **c-d**, Flow cytometry analysis for ploidy determination. **c**, A diploid plant sample showing a diploid (2n) peak. **d**, A triploid plant sample showing a triploid (3n) peak. PI PerCP-A, propidium iodide and Peridinin Chlorophyll Protein - A refer to the fluorescence channel.

There were 13 F_1 progeny recovered from embryo rescue, and 7 of them were triploid plants (Table 4.1), confirmed by flow cytometry. The phenomenon of twin plants discussed in Chapter 3 was also observed in these F_1 progeny, and the two twin seedlings emerging from the same seed were named as 7A and 7B. Triploid plants generally have similar plant size and panicle length to diploid plants (Fig. 4.2a, 4.2b), but with larger grains (Fig. 4.2c). Triploid rice plants have awns on the florets, while diploid plants usually do not (Fig. 4.2c).

Plant #	1	2	3	4	5	6	7A	7B	8	9	10	11	12	13
Ploidy	2n	2n	2n	3n	3n	3n	3n	2n	2n	2n	2n	3n	3n	3n

Table 4.1. Ploidy of F_1 progeny of Os-Apo-Fr#27d by wild-type crosses. Ploidy was confirmed by flow cytometry. 2n, diploid. 3n, triploid.

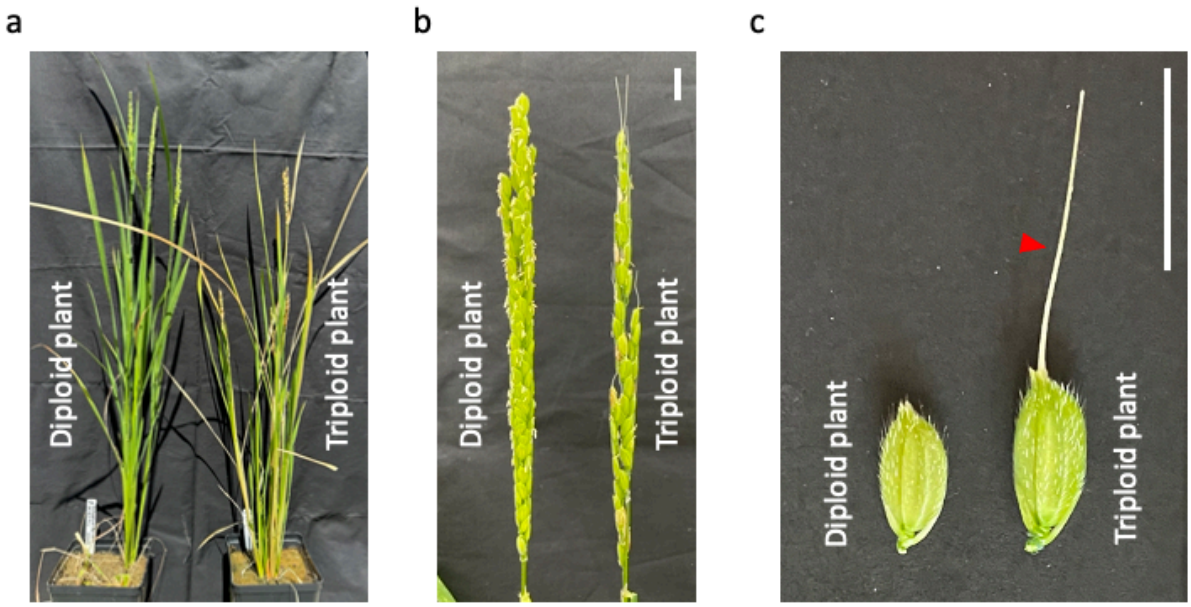


Fig. 4.2. Phenotypic differences between triploid rice and diploid rice. **a,** The diploid (left) and triploid (right) plants have a similar size. **b,** The panicle lengths of the diploid plant (left) and triploid plant (right) are similar. Scale bar, 1cm. **c,** The floret of triploid plants (right) is larger than the floret of diploid plants (left). Triploid plants have awns (red arrowhead) on the floret while diploid plants do not.

Although the *pOsECA1:: OsBBM1*, contained in the all-in-one construct, confers parthenogenesis in these triploid plants, the cross to wild-type introduced wild-type copies of the MiMe genes, which need to be knocked out to achieve synthetic apomixis. Specifically for each MiMe gene, the two alleles coming from Os-Apo-Fr#27d are non-functional, but there is still one functional allele coming from the wild-type paternal parent, which needs to be edited by CRISPR-MiMe (provided by the all-in-one construct) to make the triploid plant MiMe. Three out of seven F_1 triploid progeny were confirmed MiMe by genotyping three MiMe genes (Table 4.2). These three plants were expected to be synthetic apomicts.

	REC8			*PAIR1*			*OSD1*		
	Allele 1	Allele 2	Allele 3	Allele 1	Allele 2	Allele 3	Allele 1	Allele 2	Allele 3
Os-Apo-Fr #27d	-3 bp	-6 bp	/	-1 bp	+1 bp	/	+1 bp	+1 bp	/
Triploid #4	-3 bp	-6 bp	+1 bp	-1 bp	+1 bp	+1 bp	+1 bp	+1 bp	+1 bp
Triploid #7A	-3 bp	-6 bp	+1 bp	-1 bp	+1 bp	+1 bp	+1 bp	+1 bp	+2 bp
Triploid #12	-3 bp	-6 bp	+1 bp	-1 bp	+1 bp	+1 bp	+1 bp	+1 bp	+1 bp

Table 4.2. Genotype of three selected triploid F_1 progeny for MiMe genes. Os-Apo-Fr #27d is an apomixis line confirmed by phenotype and flow cytometry, so the two in-frame-mutation alleles of *REC8* in this line must also be non-functional.

To analyze whether these F_1 triploid plants can self-propagate through apomixis, I collected seeds from them and germinated F_2 seeds. For Triploid #12, there were 6 F_2 plants recovered from seeds, and only 1 of them is triploid, named as Triploid #12-1, confirmed by flow cytometry (Table 4.3). I again collected seeds from the F_2 Triploid #12-1 and germinated these F_3 seeds. Among the F_3 progeny of Triploid #12-1, there were 4 triploid plants resulting from parthenogenesis and 11 hexaploid plants (Table 4.4), which resulted from fertilization of two 3n gametes, giving a parthenogenesis efficiency of about 26.7%. Hexaploid plants are much smaller than triploid plants in terms of plant size (Fig. 4.3), and they are sterile, possibly due to excessively high genome dosage.

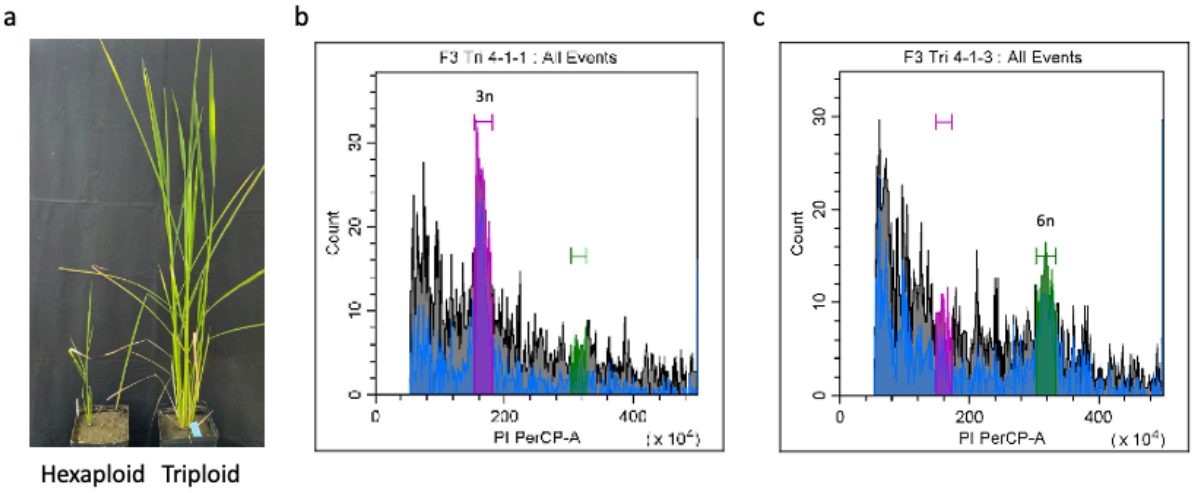


Fig. 4.3. Phenotypic differences between hexaploid rice and triploid rice. **a,** The plant sizes differ between hexaploid (left) and triploid (right) plants. The hexaploid plants are much smaller compared to the triploid plants. **b-c,** Flow cytometry analysis for ploidy determination. **b**, A triploid plant sample showing a triploid (3n) peak. **c**, A hexaploid plant sample showing a hexaploid (6n) peak. PI PerCP-A, propidium iodide and Peridinin Chlorophyll Protein - A refer to the fluorescence channel.

F_1 parent plant #	F_2 progeny		
	3n	6n	Parthenogenesis efficiency
Triploid #4	3	2	60%
Triploid #7A	1	1	50%
Triploid #12	1	5	16.7%

Table 4.3. Ploidy analysis of F_2 progeny of synthetic apomixis triploid rice. 3n, triploid plants result from the parthenogenesis events on 3n egg cells. **6n,** hexaploid plants which come from the fusion of two 3n gametes.

A similar analysis was performed for the F_1 Triploid #7A. Although there were only two seeds recovered from F_1 Triploid #7A, one of them was triploid, named #7A-1, confirmed by flow cytometry (Table 4.3). I collected F_3 seeds from F_2 Triploid #7A-1 and germinated them to analyze their ploidy. Seven out of 28 were triploid plants, giving a parthenogenesis efficiency of about 25.0% (Table 4.4). Therefore, synthetic apomixis can be achieved by combining *BBM*-induced parthenogenesis and MiMe in triploid rice plants, successfully overcoming the limitation that triploid plants cannot self-propagate.

F_2 parent plant #	F_3 progeny		
	3n	6n	Parthenogenesis efficiency
Triploid #7A-1	7	21	25.0%
Triploid #12-1	4	11	26.7%

Table 4.4. Ploidy analysis of F_3 progeny of synthetic apomixis triploid rice. 3n, triploid plants result from the parthenogenesis events on 3n egg cells. **6n,** hexaploid plants which come from the fertilization of two 3n gametes.

4.4 Discussion

Synthetic apomixis in triploid plants offers a solution to the sterility of seed-propagated triploid crop plants by bypassing meiosis and enabling clonal seed reproduction, thereby allowing triploids to be more broadly utilized in agronomy. Moreover, it enables the combination of triploid with heterosis to further enhance agronomic performance.

However, there are still some technical issues that need to be addressed. The fertility of the triploid apomixis plants is lower than diploid apomixis plants growing at the same time. The maternal-to-paternal ratio in endosperm is the same 2:1 ratio as normal diploid plants. But there is an overall high genomic dosage in the endosperm (9n), which may result in seed abortion, as the tetraploid rice is sterile. This problem needs to be investigated and solved to achieve high fertility to make triploid apomixis agriculturally applicable in rice. The parthenogenesis efficiency of these triploid lines also went lower compared to their diploid apomixis parent, whose parthenogenesis efficiency is 84%[15] (Table 4.3, 4.4). All of the F_1 progeny from crosses to generate triploids went through an embryo rescue procedure. The tissue culture step could lead to changes in the expression level of the transgenic *OsBBM1* in the egg cell[24], and affect the parthenogenesis efficiency.

While challenges remain in achieving consistent parthenogenesis efficiency and agronomically applicable fertility, I have shown that synthetic apomixis can be achieved in triploid rice. Interestingly, many apomictic species are triploid, including the common dandelion, which is a vigorous plant that propagates asexually[25]. Our results demonstrate the feasibility of triploid and triploid hybrid propagation in crop plants. More generally, it outlines how genome combinations that would not normally be tolerated in meiosis can be constructed and propagated in crop plants, holding great promise for applications to agriculture in the future.

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Chapter 5 Conclusion and future directions

5.1 Synergistic actions of paternal genome-encoded transcription factors to promote early embryogenesis in rice

The zygotic genome activation occurs shortly after fertilization in plants; however, the molecular mechanisms underlying this process have not been well understood yet. In early rice zygotes, transcription is largely maternally driven, though certain transcription factors are expressed from the paternal genome[1], suggesting a coordination between maternal and paternal genomes during embryo development. *OsBBM1* is a key paternal-expressed transcription factor whose ectopic expression in the egg cell (*OsBBM1*-ee) is sufficient to induce parthenogenesis in rice, thereby bypassing the need of fertilization for embryogenesis[2]. These results indicate that the fertilization requirement for initiating embryogenesis in rice is primarily mediated by the paternal expression of *OsBBM1*.

There are other transcription factors evolved to be paternally expressed to enhance the action of *OsBBM1*. In Chapter 2, we demonstrated that *OsWOX9A*, another transcription factor expressed from the paternal genome, is not capable of independently inducing parthenogenesis by its egg cell ectopic expression. However, when it was co-expressed with *OsBBM1* in the egg cell (*OsBBM1*-ee + *OsWOX9A*-ee), it significantly enhanced the parthenogenesis efficiency, with 4- to 15- fold increases when compared to *OsBBM1*-ee alone. To investigate the function of *OsWOX9A* in rice early embryogenesis, we used CRISPR to knock out *OsWOX9A* and its paralog *OsWOX9C*. The genotyped *Oswox9a/Oswox9a Oswox9c/Oswox9c* double mutant embryos showed delayed growth compared to wild-type embryos from 3 days after pollination (DAP), then underwent cell

proliferation without corresponding developmental patterning at 5 DAP, and were developmentally arrested when observed at later stages. Thus, *OsWOX9A* and *OsWOX9C* together are required for normal embryo patterning and development as early as the 3 DAP stage.

Together, we proposed a model in which early zygote development is mediated synergistically by the paternal factors (Fig. 5.1). *OsBBM1* is expressed in rice sperm cells but not egg cells, while *OsWOX9A* is not expressed in sperm cells or egg cells. After fertilization, both *OsBBM1* and *OsWOX9A* are expressed *de novo* from the male genome and turn on the expression of downstream genes. They may operate in distinct pathways to promote embryo initiation and establishment as they are not direct targets of each other. *OsBBM1* likely serves as the initial trigger, while *OsWOX9A* acts in subsequent steps, together promoting the stable establishment of the zygotic state and progression of embryogenesis.

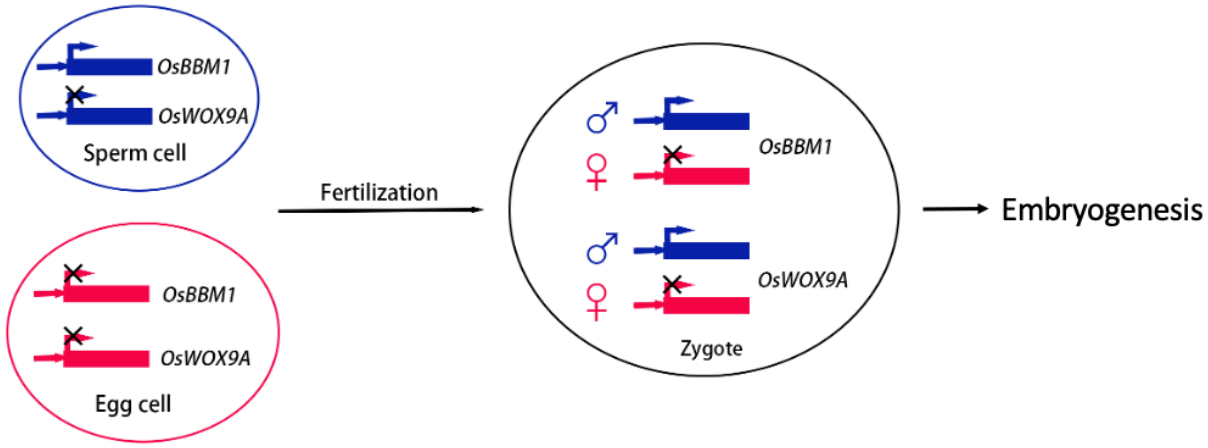


Fig. 5.1. Model for the functional role of *OsBBM1* and *OsWOX9A* in rice early embryogenesis. Red, maternal genome; Blue, paternal genome.

5.2 Polyembryony progeny in parthenogenetic plants

One intriguing phenomenon associated with the combination of *OsWOX9A*-ee and *OsBBM1*-ee is that the high frequency of polyembryony progeny occurred, especially twinning events. We have provided a brief description of the phenomenon and discussed the potential mechanisms for twin formation in flowering plants, and proposed two possible models in Chapter 3. While twin progeny have previously been observed in *OsBBM1*-ee lines[3,4], their occurrence was substantially higher (50%-100%) in plants expressing both *OsBBM1*-ee and *OsWOX9A*-ee than in *OsBBM1*-ee alone. This raises an important question: why does *OsWOX9A*-ee, despite its inability to induce parthenogenesis alone, so dramatically enhance both the frequency and complexity of polyembryony when expressed with *OsBBM1*-ee?

A plausible explanation lies in the distinct functional roles of the two genes. It was known that overexpression of *OsBBM1* can induce somatic embryo-like structures, indicating its strong capacity to de-differentiate cells and confer pluripotency[2]. When we ectopically express *OsBBM1* in rice egg cells, the egg cells become zygotes without fertilization, and embryogenesis starts[2]. In parallel, *WOX9* has been shown to be required for shoot apical maintenance[5] and maintains the undifferentiated status of the shoot apical meristem in addition to its role in embryonic patterning. Therefore, when *OsWOX9A*-ee is co-expressed with *OsBBM1*-ee, it may stabilize the undifferentiated status of the developing parthenogenetic embryo for a longer window, allowing time for symmetrical cell divisions that give rise to monozygotic twins prior to asymmetrical cell divisions that give rise to embryos.

Alternatively, the coordination between *OsBBM1*-ee and *OsWOX9A*-ee may affect the developmental outcome of the parthenogenesis events. *OsBBM1*-ee may trigger embryo initiation

at an overly early time point before fertilization, so the parthenogenetic embryo develops before the fertilization of the central cell, which is essential for proper endosperm formation and seed viability. In this case, the absence of central cell fertilization at this early stage may lead to embryo abortion. Adding *OsWOX9A*-ee may mitigate this issue by prolonging the undifferentiated status of the parthenogenetic embryo, thereby providing a broader temporal window for central cell fertilization. The extended window allows the central cell to be fertilized by a sperm cell and initiate the development of endosperm, which is an indispensable part of the viable seed. In parallel, one of the synergid cells may undergo cell fate transition, acquiring egg cell identity and subsequently forming a second embryo either through fertilization by the other sperm cell or via another independent parthenogenetic event, thereby facilitating the formation of the dizygotic twins.

Despite the scientific interest in polyembryony, it presents agronomic challenges for major crops like maize and sorghum. To address this issue, two strategies are proposed for future investigation:

Promoter optimization is proposed as the first approach. The current promoter that was used is an Arabidopsis egg cell-specific promoter (*pEC1.2*), which functions in rice but may lack spatial-temporal precision. Identifying rice-specific egg cell promoters that are active strictly in mature egg cells could minimize the early activation of *OsBBM1*, reducing twin formation. In this case, only the mature egg cell would be reprogrammed into an embryo, while the central cell is also mature and can be fertilized by the sperm cell, ensuring viable seed formation and reducing twinning events.

Fine-tuning expression levels is the second. High expression levels of *OsBBM1*-ee may prematurely trigger parthenogenesis. Using a weaker promoter to drive *OsBBM1* expression could slow down the accumulation of *OsBBM1*, thereby delaying the onset of parthenogenesis. This temporal shift may narrow the window during which a synergid cell could convert into a second egg cell before the central cell is fertilized. As a result, the likelihood of twin embryo formation could be reduced.

5.3 Implications of synthetic apomixis in rice

In Chapter 4, I demonstrated that synthetic apomixis can be achieved in triploid rice using MiMe and *OsBBM1*-ee, which overcomes the typical meiotic problem of triploids and makes fertile triploid rice. While MiMe penetrance was robust, the parthenogenesis efficiency in these lines was notably lower than in their parent diploid apomixis line. To optimize this system, incorporating *OsWOX9*-ee alongside *OsBBM1*-ee is a promising avenue. As proved in Chapter 2, *OsWOX9A*-ee can significantly enhance the parthenogenesis efficiency by 4- to 15-fold over *OsBBM1*-ee alone, potentially solving the problem.

Moreover, as discussed in Chapter 4, a critical limitation observed in these triploid apomictic plants is the low seed set. This may be due to overly early initiation of embryogenesis triggered by *OsBBM1*-ee. In this case, embryo development proceeds without concurrent endosperm formation, ultimately resulting in embryo abortion due to a lack of endosperm. To address this, refining the promoter driving *OsBBM1* expression, ideally one that is active specifically in mature egg cells, could finetune the onset of parthenogenesis to allow normal fertilization of the central cell. This adjustment would restore proper endosperm development, thereby improving both seed viability and overall reproductive success in triploid apomictic plants.

Together, these findings not only deepen our understanding of paternal regulatory factors in early embryogenesis but also open practical pathways for improving synthetic apomixis systems in crop breeding. By fine-tuning the expression of *OsBBM1*-ee and *OsWOX9A*-ee, we may achieve both high parthenogenesis efficiency with normal seed set and desirable single-embryo seed development, which are important for apomictic crop production.

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Appendix I: Induction of parthenogenesis in dicot plants

Abstract

Egg cell expression of *OsBBM1* induces parthenogenesis in rice[1], and its efficiency is significantly enhanced by co-expression of *OsWOX9A* (Chapter 2), indicating that *OsBBM1* and *OsWOX9A* function as key regulators of embryo initiation. To investigate whether this regulatory mechanism is conserved in dicotyledonous species, I examined the effects of egg cell expression of candidate genes in Arabidopsis and tomato. In Arabidopsis, egg cell expression of *AtBBM*, either alone or in combination with *AtWOX9* or *AtSSP*, failed to produce haploid progeny. However, egg cell expression of *AtDAZ3*, the Arabidopsis ortholog of *PARTHENOGENESIS* (*PAR*), which was identified as a natural parthenogenesis allele from apomictic dandelion[2], was sufficient to induce parthenogenesis at low frequency in Arabidopsis. In tomato, egg cell expression of *SlBBM* and *SlWOX9* is not sufficient to induce parthenogenesis, suggesting that additional regulatory factors may be required to initiate embryogenesis. Together, these findings indicate that, unlike in monocots, egg cell expression of *BBM* alone or in combination with *WOX9* is insufficient to induce parthenogenesis in Arabidopsis and tomato, and other regulatory factors may constrain haploid induction in dicots.

Main Text

The egg cell expression of *OsBBM1* (*OsBBM1*-ee) is sufficient to initiate embryo development in rice without fertilization[1], suggesting that the requirement for fertilization in embryogenesis initiation in rice is primarily mediated by the paternal expression of *OsBBM1*. Orthologous *BBM* genes are present in most angiosperms, and their capacity to induce somatic

embryogenesis has been demonstrated in multiple dicot lineages, including *Brassicaceae*[3], *Solanaceae*[4], and *Theobroma cacao*[5], which indicates their potential in inducing parthenogenesis in dicots through ectopic expression of *BBM* in the egg cell.

WOX9 genes may have evolved to be expressed from the male genome to enhance or coordinate with the action of *BBM*, thereby improving the parthenogenesis efficiency. In rice, *OsBBM1*-ee alone is sufficient to induce parthenogenesis, while the egg cell expression of *OsWOX9A* (*OsWOX9A*-ee) is not required for parthenogenesis induced by *OsBBM1*-ee. However, co-expression of *OsWOX9A* with *OsBBM1* in the egg cell significantly increased the parthenogenesis efficiency by 4- to 15-fold when compared to *OsBBM1*-ee alone[1,6]. In Arabidopsis, *AtWOX9* is expressed *de novo* from the paternal genome after fertilization[7], similar to *BBM* genes, suggesting that *AtWOX9* may also have the potential to induce parthenogenesis.

To test this parthenogenesis potential in dicot plants, *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) were selected as model species, as Arabidopsis is a well-studied genetic model, and tomato is an agriculturally important crop with significant heterosis. Successful induction of haploid progeny in either species would identify the key regulatory genes capable of initiating the embryogenesis program without fertilization in dicot plants and provide a framework for engineering clonal propagation in economically important dicot crops.

Egg cell expression of paternal factors and parthenogenesis induction in Arabidopsis:

To test whether *AtBBM* can induce parthenogenesis in Arabidopsis, I used the Arabidopsis egg cell-specific promoter *pEC1.2* (promoter of At2g21740)[8] to drive *AtBBM* (At5g17430) (*AtBBM*-ee) expression in the egg cell (Fig. A.1a). The *AtBBM* coding sequence was used, which

was cloned from a plasmid obtained from the Arabidopsis Biological Resource Center (ABRC) (Stock number: TOPO-U15-C07)[9]. The *pEC1.2::AtBBM* construct was introduced into *Agrobacterium tumefaciens* strain GV3101, and then transformed into *A. thaliana* Col-0 wild-type via the floral dip method[10]. The primary transformants (T1 generation) were selected on antibiotic-containing media, and the derived T2 seeds were used for the examination of ploidy levels. A total of three lines, 200 plants for each line, were screened for haploid progeny, but no haploid was identified. Similar analysis was done by using *pEC1.2* to drive the *AtWOX9* (At2g33880) coding sequence, which was cloned from a plasmid obtained from the ABRC (Stock number: TOPO-U15-F01)[9] (*AtWOX9*-ee), for the expression of *AtWOX9* in the egg cell (Fig. A.1a), with no haploid plant identified.

To investigate if there is coordination between *AtWOX9* and *AtBBM* in embryogenesis in Arabidopsis, I co-expressed *AtWOX9*-ee with *AtBBM*-ee for haploid induction by crossing *AtWOX9*-ee plants with *AtBBM*-ee plants. However, three lines, 250 plants derived from each line, were screened for the combined egg cell expression of *AtBBM* and *AtWOX9*, but no haploid plant was identified either.

As discussed in Chapter 1, in the Arabidopsis *YODA* signaling pathway, *SHORT SUSPENSOR* (*AtSSP*) transcripts are produced in mature pollen and translated post-fertilization in the zygote[11,12], marking *AtSSP* as another paternal-expressed gene that may act as a signal for fertilization. Since in Arabidopsis, *AtSSP* activates the YODA MAP kinase cascade and acts upstream of both *AtWOX8* and *AtWOX9*[13,14], expressing *AtSSP* in egg cells could simplify the complexity of redundantly acting *AtWOX8* and *AtWOX9*. Therefore, I ectopically expressed the *AtSSP* (At2g17090) coding sequence in Arabidopsis egg cells (*AtSSP*-ee) for parthenogenesis

induction. *AtSSP* was amplified from the Arabidopsis flower complementary DNA (cDNA) library, and then expressed under the *pEC1.2* promoter (Fig. A.1a). This construct was transformed into Arabidopsis Col-0 wild-type via *Agrobacterium*-mediated transformation as described above, and primary transformants were selected by antibiotic-containing media. *AtBBM*-ee plants were crossed with *AtSSP*-ee plants to obtain the transgenic plants with both *AtBBM* and *AtSSP* expressed in the egg cell. Four lines (250 plants for each line) were screened, but no haploid plant was found. Since the *pEC1.2* promoter has been demonstrated to be an egg cell-specific promoter in Arabidopsis, and *pEC1.2*::GFP showed green fluorescence in the Arabidopsis egg cells[8], the egg cell expression of *AtBBM*, *AtWOX9*, and *AtSSP* was not directly confirmed in this study.

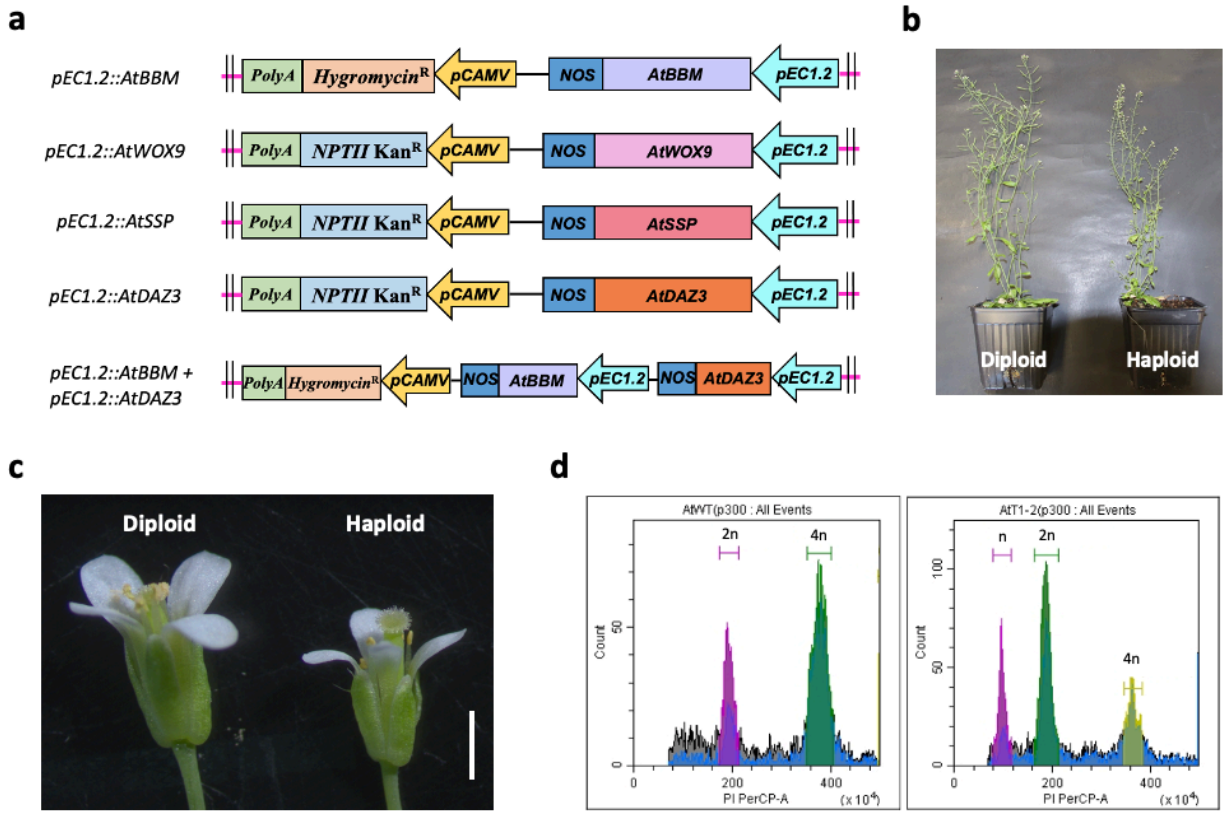


Fig. A.1. Parthenogenesis induction in Arabidopsis. a, Schematic of the *pEC1.2::AtBBM*, *pEC1.2::AtWOX9*, *pEC1.2::AtSSP*, *pEC1.2::AtDAZ3*, and *pEC1.2::AtBBM+pEC1.2::AtDAZ3* binary vectors for egg cell expression of these genes. **b,** T2 diploid and haploid *AtDAZ3*-ee progenies. The plant sizes differ between diploid (left) and haploid (right) siblings. **c,** Differences in flower size and morphology between diploids and haploids. Left: *AtDAZ3*-ee diploid. Right: *AtDAZ3*-ee haploid. Scale bar, 1 mm. **d,** Flow cytometry analysis for ploidy determination. Left: A Col-o wild-type plant leaf sample showing a diploid (2n) peak. The tetraploid (4n) peak is due to endoreduplication. Right: A haploid plant leaf sample from *AtDAZ3*-ee showing a haploid (1n) peak. The diploid (2n) and tetraploid (4n) peaks are due to endoreduplication. PI PerCP-A, propidium iodide and Peridinin Chlorophyll Protein-A refer to the fluorescence channel.

There is a natural parthenogenesis allele, *PARTHENOGENESIS (PAR)* has recently been identified from apomictic dandelion (*Taraxacum officinale*)[2], as discussed in Chapter 1. There are two orthologs in Arabidopsis: *DAZ3* (*DUO1 activated zinc finger 3 (ZF3)*; At4g35700) and *DAZ3like/TREE1* (*transcriptional repressor of EIN3-dependent ethylene-response 1*; At4g35610). To test if egg cell expression of *AtDAZ3* can induce parthenogenesis in Arabidopsis, I used *pEC1.2* to drive the *AtDAZ3* coding sequence to express it in the egg cell (*AtDAZ3*-ee) (Fig. A.1a). The *AtDAZ3* coding sequence was cloned from a plasmid (ABRC Stock number: TOPO-U06-D05)[9]. The resulting expression construct was transformed into Arabidopsis Col-0 wild-type via *Agrobacterium*-mediated transformation as described above. Eight independent lines, 3469 plants in total, were screened (Table A.1), with 2 haploid plants identified. Both haploid plants are from line #104-1, of which 2111 plants were screened, giving a parthenogenesis efficiency of 0.1%. Haploid Arabidopsis is smaller in size than the diploid plants, and it is sterile (Fig. A.1b, A.1c). Flow cytometry was performed as described[15] to confirm the ploidy (Fig. A.1d).

T1 plants	Diploid (n=)	Haploid (n=)	Parthenogenesis efficiency (%)
#104-1	2109	2	0.1
#104-2	404	0	0
#104-3	189	0	0
#104-4	109	0	0
#106-1	89	0	0
#106-2	281	0	0
#106-4	80	0	0
#106-5	206	0	0

Table A.1. Parthenogenesis efficiency in *AtDAZ3*-ee plants. T1 plants are the primary transformants that are germinated and selected from the antibiotic-containing media.

To investigate if there is a coordination between *AtDAZ3* and *AtBBM*, I also transformed *pEC1.2::AtDAZ3* into the *AtBBM*-ee line #66-1, which is homozygous for *pEC1.2::AtBBM*, via *Agrobacterium*-mediated transformation as described above. Five independent lines, 1082 plants in total, were screened for haploids, but no haploid plant was identified. In addition, I made a single construct containing both *pEC1.2::AtBBM* and *pEC1.2::AtDAZ3* (Fig. A.1a), and transformed it into Arabidopsis Col-0 wild-type to minimize the negative effect from double selection of the antibiotic-containing media. Six independent lines, 1008 plants, were screened for haploid plants, but no haploid plant was identified. The reason why I identified haploid plants in *AtDAZ3*-ee lines but not in *AtDAZ3-ee* + *AtBBM-ee* lines might be that the number of plants that were screened for the *AtDAZ3-ee* + *AtBBM-ee* combination was too small in terms of the low parthenogenesis efficiency (0.1%) observed in the *AtDAZ3*-ee line.

Egg cell expression of paternal factors and parthenogenesis induction in Tomato:

Based on the published phylogeny of *BBM* genes[16], as well as BLAST searches that I performed, the tomato *BBM* ortholog (*SlBBM*) was identified as Solyc11g008560. Its coding sequence was amplified from the tomato flower cDNA library, and then expressed under the *pEC1.2* promoter (*SlBBM*-ee) (Fig. A.2a). Although the earliest steps of the zygotic transition are as yet uncharacterized in tomato, tomato has only one *WOX9* ortholog (*SlWOX9*: Solyc02g077390) and lacks a *WOX8* ortholog. Moreover, *SSP* is not found outside of the *Brassicaceae*[17], suggesting a simpler or different regulatory pathway in tomato. To bypass all hypothetical upstream regulators of *SlWOX9*, I amplified *SlWOX9* from the tomato flower cDNA library and directly ectopically expressed it in the egg cell with the *pEC1.2* promoter (*SlWOX9*-ee). To investigate if *SlWOX9*-ee can enhance *BBM*-mediated parthenogenesis, I co-expressed *SlBBM*-ee and *SlWOX9*-ee using the

pEC1.2 promoter fused to both *SlBBM* and *SlWOX9* (Fig. A.2b). The T-DNA binary vectors carrying these fusions were transformed into tomato (*Solanum lycopersicum*) cv. Moneymaker, a variety preferred for its high efficiency of transformation and regeneration, via *Agrobacterium*-mediated transformation by the UC Davis transformation facility.

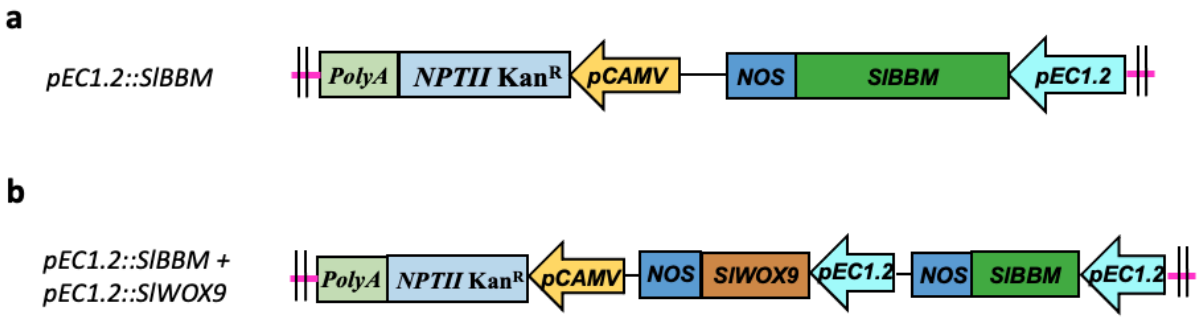


Fig. A.2. Schematic diagram of constructs used to induce parthenogenesis in tomato. a, *pEC1.2::SlBBM* binary vector for the egg cell expression of *SlBBM*. **b,** *pEC1.2::SlBBM* + *pEC1.2::SlWOX9* binary vector for the combined egg cell expression of *SlBBM* and *SlWOX9*.

Transgenic tomato plants (T0) were self-fertilized, and the T1 progeny were screened for haploid progeny that are produced by parthenogenesis. Pollen staining and flow cytometry were performed as described for the screening of haploid plants[15,18].

For *SlBBM*-ee: Ten T0 primary transformants were obtained. Seeds from six different T0 lines with good fertility were germinated. From the progeny of these lines, 140 plants were screened for haploids using the pollen staining method, and 66 plants were screened using flow cytometry. No haploid plant was found.

For *SlBBM*-ee + *SlWOX9*-ee: Nine T0 primary transformants were obtained. Seeds from seven different T0 lines with good fertility were germinated. From the progeny of these lines, 43 plants were screened using the pollen staining method, and 387 plants were screened using the flow cytometry method. No haploid plant was found either.

The egg cell expression of *SlBBM*, either alone or in combination with *SlWOX9*, was not directly confirmed in this study. However, since the *pEC1.2* promoter has been demonstrated to drive egg cell expression in both Arabidopsis[8] and rice[1], it is highly likely that *pEC1.2* functions similarly in tomato. Under this assumption, the egg cell expression of *SlBBM* alone or together with *SlWOX9* is insufficient to induce parthenogenesis in tomato, indicating that additional regulatory factors may be required to initiate embryogenesis. It remains possible that *pEC1.2* is not strictly egg cell-specific in tomato, and further experimental validation will be necessary to clarify its expression pattern.

Since *PAR* was identified from apomictic dandelion (*Taraxacum officinale*), which is a dicot plant, the underlying mechanism initiating embryogenesis might be more relevant to dicots such as tomato. Egg cell expression of *PAR* and its Arabidopsis ortholog *AtDAZ3* has been shown to trigger embryo development in an unfertilized egg cell in *Lactuca sativa*[2] and *Arabidopsis thaliana* (in this work), respectively. These findings suggest that the tomato ortholog of *PAR* may have the potential to induce parthenogenesis in tomato and could represent a promising candidate for future validation and engineering for clonal reproduction in dicot crops.

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