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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Flower meristem maintenance by *TILLERS ABSENT 1* is essential for ovule development in rice

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ABSTRACT

Plant development depends on the activity of pluripotent stem cells in meristems, such as the shoot apical meristem and the flower meristem. In *Arabidopsis thaliana*, *WUSCHEL* (*WUS*) is essential for stem cell homeostasis in meristems and integument differentiation in ovule development. In rice (*Oryza sativa*), the *WUS* ortholog *TILLERS ABSENT 1* (*TAB1*) promotes stem cell fate in axillary meristem development, but its function is unrelated to shoot apical meristem maintenance in vegetative development. In this study, we examined the role of *TAB1* in flower development. The ovule, which originates directly from the flower meristem, failed to differentiate in *tab1* mutants, suggesting that *TAB1* is required for ovule formation. Expression of a stem cell marker was completely absent in the flower meristem at the ovule initiation stage, indicating that *TAB1* is essential for stem cell maintenance in the 'final' flower meristem. The ovule defect in *tab1* was partially rescued by *floral organ number 2* mutation, which causes overproliferation of stem cells. Collectively, it is likely that *TAB1* promotes ovule formation by maintaining stem cells at a later stage of flower development.

KEY WORDS: Flower development, Meristem, Ovule, Rice (*Oryza sativa*), Stem cell maintenance, *TILLERS ABSENT 1* (*TAB1*)

INTRODUCTION

Plants have a unique ability to produce lateral organs, such as leaves and floral organs, continuously throughout their life cycle. This ability depends on the activity of pluripotent stem cells, which are located at the tip of above-ground meristems, such as the shoot apical meristem (SAM) and flower meristem. In these meristems, stem cells self-renew to maintain a constant number, while supplying cells for organ differentiation.

In *Arabidopsis* (*Arabidopsis thaliana*), the CLAVATA–WUSCHEL (CLV–WUS) negative feedback loop plays a central role in coordinating stem cell self-renewal with organ differentiation in the meristem. WUS, a homeodomain transcription factor, positively regulates stem cell proliferation (Laux et al., 1996; Mayer et al., 1998). Loss of function of *WUS* causes a failure in stem cell maintenance, leading to premature termination of the meristem (Mayer et al., 1998). In the vegetative phase, the *wus* mutant terminates shoot development after forming a few leaves, and *wus* flowers lack carpels and have reduced numbers of stamens in the reproductive phase (Laux et al., 1996). In contrast to *WUS*, the CLV signaling pathway plays a major role in repressing the proliferation of stem cells (Clark et al., 1993, 1995; Fletcher et al., 1999; Schoof et al., 2000). The protein encoded by *CLV3* is processed into a CLE peptide, which acts as a ligand of CLV1 – a leucine-rich repeat (LRR)-receptor kinase (Clark et al., 1997; Fletcher et al., 1999). The CLV signaling pathway negatively regulates *WUS* expression, whereas WUS promotes *CLV3* transcription by directly binding to its promoter (Brand et al., 2000; Schoof et al., 2000; Yadav et al., 2011).

The genetic mechanisms underlying stem cell maintenance have been also well-studied in grasses such as maize (*Zea mays*) and rice (*Oryza sativa*). In particular, several signaling pathways that negatively regulate stem cell proliferation are well documented in both maize and rice (Taguchi-Shiobara et al., 2001; Suzaki et al., 2004, 2006, 2008, 2009; Bommert et al., 2005, 2013; Pautler et al., 2015; Je et al., 2016, 2018; Wu et al., 2018; Suzuki et al., 2019a). In rice, for example, a signaling pathway containing *FLORAL ORGAN NUMBER 1* (*FON1*) and *FON2* plays an important role in negative regulation of the flower meristem (Suzaki et al., 2004, 2006). *FON1* encodes a CLV1-like LRR-receptor kinase, whereas *FON2* encodes a CLE protein closely related to CLV3. Loss of function of either *FON1* or *FON2* induces stem cell overproliferation, leading to an increase in the number of stamens and carpels. Other *CLE* genes, such as *FON2-LIKE CLE PROTEIN 1* (*FCP1*) and *FON2 SPARE 1* (*FOS1*), are also involved in the negative regulation of the stem cell maintenance in the SAM and/or flower meristem (Suzaki et al., 2008, 2009). By contrast, our knowledge of positive regulators of meristem maintenance remains limited in rice.

Unlike *WUS* in *Arabidopsis*, its rice ortholog, *TILLERS ABSENT 1* (*TAB1*; *OsWUS*), is not involved in positive regulation of the SAM in the vegetative phase (Tanaka et al., 2015; Suzuki et al., 2019b). *TAB1* is not expressed in the SAM and the *tab1* mutant produces the same number of leaves as wild type (Tanaka et al., 2015). Instead, *TAB1* promotes stem cells during axillary meristem development. In the *tab1* mutant, no axillary shoot (tiller) is formed due to a failure in stem cell maintenance at the early stage of axillary meristem development (Tanaka et al., 2015; Hirano and Tanaka, 2020; Tanaka and Hirano, 2020). *TAB1* function is partially repressed by *FON2* because the *TAB1* expression domain is expanded in an enlarged meristem at the early stage of axillary meristem development in *tab1* (Tanaka and Hirano, 2020). Therefore, the genetic relationship between *TAB1* and *FON2* in axillary meristem development in rice is similar to that between *WUS* and *CLV3* in SAM maintenance in *Arabidopsis*. In the vegetative SAM, *WOX4*, a close paralog of *TAB1*, maintains stem cells (Ohmori et al., 2013). In axillary meristem development,

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WOX4 starts to be expressed at a later stage and is responsible for maintaining the established axillary meristem (Tanaka et al., 2015). In summary, the positive regulator differs depending on the type of meristem in rice, and, as yet, a positive regulator in the flower meristem is not known.

In *Arabidopsis*, the ovule is initiated from the placenta, which is differentiated from the carpel margin meristem – a meristematic tissue present along the margins of two fused carpel primordia (Skinner et al., 2004; Roeder and Yanofsky, 2006; Reyes-Olalde et al., 2013). Specification of ovule identity is regulated by *SEEDSTICK* (*STK*), which acts redundantly with three other *AGAMOUS* (*AG*) clade MADS-box genes: *AG*, *SHATTERPROOF 1* (*SHP1*) and *SHP2* (Pinyopich et al., 2003). After specification, *WUS* plays an important role in ovule development. In transgenic plants in which *WUS* is knocked out in an ovule-specific manner, integument differentiation is completely inhibited (Groß-Hardt et al., 2002). *WUS* is specifically expressed in the nucellus but not in the integument, indicating that *WUS* promotes integument development in a non-cell-autonomous manner. The nucellus-specific expression of *WUS* is regulated by class III homeodomain leucine zipper (HD-ZIPIII) genes in collaboration with *BELL1* (Yamada et al., 2016).

Unlike in *Arabidopsis*, ovule formation depends on the activity of the flower meristem in rice (Yamaki et al., 2005, 2011). The ovule is differentiated directly from the flower meristem, which remains even after carpel specification (Yamaki et al., 2005). Loss of function of *LONELY GUY* (*LOG*), which encodes a cytokinin-activating enzyme, results in a severe defect in the flower meristem, leading to a reduced number of floral organs, such as stamens and carpels (Kurakawa et al., 2007). A weak allele of *log* displays an ovule-less phenotype, consistent with the observation that the ovule initiates from the flower meristem (Yamaki et al., 2011). Loss of function of rice *OsMADS13*, an ortholog of *STK*, results in the conversion of an ovule into a carpelloid structure (Dreni et al., 2007). Thus, the genetic regulators that specify ovule identity seem to be conserved between *Arabidopsis* and rice.

In our previous study, we showed that the *tab1* mutant produces abnormal floral organs (Tanaka et al., 2015); however, the function of *TAB1* in flower development remains to be elucidated. In this study, we conducted developmental analysis focusing on maintenance of the flower meristem and ovule formation in *tab1*. As a result, we found that ovule development is strongly compromised in *tab1*. Furthermore, expression of meristem markers was found to disappear precociously from the flower meristem in *tab1*. Collectively, our results suggest that, by maintaining stem cells at a later stage of flower development, *TAB1* plays an essential role in ovule development in rice.

RESULTS

Pistil structure is partially compromised in the *tab1-1* flower

We previously reported that *tab1-1*, a complete loss-of-function mutant, produces the same number of leaves in the vegetative phase as wild type (Tanaka et al., 2015). To examine the function of *TAB1* in the reproductive phase, we first examined the number of floral organs in *tab1-1*. Analysis of 180 flowers indicated that there was no significant difference in the number of lodicules, stamens or pistils between wild type and *tab1-1* (Fig. 1A). However, about half of the *tab1-1* flowers were abnormal such that the palea/lemma was reduced in size or absent (Fig. S1).

We found that the *tab1-1* mutant was completely sterile, as reported previously (Lu et al., 2015). Therefore, to check whether the pistil is functional in *tab1-1*, we crossed wild-type pollen with *tab1-1* pistils. As a result, no seeds were obtained even after performing more than 70 crosses. We carefully observed the morphology of the *tab1-1* pistil; however, only minor abnormalities, such as a slightly thinner ovary and an increase in stigma number, were found (Fig. 1B,C,E,F).

To examine the inner features of the pistil, we cleared the *tab1-1* pistils and observed them under differential interference contrast optics. In wild type, two long vascular bundles ran from the base of the ovary to the tip of each stigma, and a short vascular bundle reached almost to the central region of the ovary (Fig. 1D). In *tab1-1*, long vascular bundles similarly stretched towards the stigmas (Fig. 1G,H); however, the short vascular bundle ended in an abnormal position in some cases (Fig. 1G), or branched abnormally and had a mass of proliferated cells in others (Fig. 1H). The internal structure of the ovary also appeared to be affected by *tab1* mutation: an elliptical structure, presumed to be an ovule, was clearly observed inside the ovary in wild type (Fig. 1D), but no such structure was detected in *tab1-1* (Fig. 1G,H).

Collectively, these results suggest that pistil formation is likely to be partially compromised in the *tab1-1* flower.

Ovule formation is severely affected in the *tab1-1* flower

We prepared longitudinal sections to observe the internal structure of the ovary in more detail. In wild type, an ovule, surrounded by inner and outer integuments, adhered to the inner wall of the ovary and an embryo sac was clearly observed inside the ovule (Fig. 2A). In *tab1-1*, by contrast, no ovule-like structure was observed. In severe cases, the ovary had a hollow structure, implying that it lacked an ovule (Fig. 2B,D); in others, an unidentified structure with neither embryo sac nor integuments was present inside the ovary (Fig. 2C,D). These observations suggest that ovule formation is severely defective in the *tab1-1* mutant.

tab1 shows serious defects in ovule initiation and development

To examine further the defect in ovule formation in *tab1-1*, we carried out developmental analysis using marker genes for flower development. First, we analyzed the expression pattern of *DROOPING LEAF* (*DL*), which is required for carpel specification (Yamaguchi et al., 2004). We confirmed that *DL* is expressed in the carpel primordia in wild type (Fig. 3A,C), as reported previously (Yamaguchi et al., 2004). The carpel primordia in *tab1-1* had morphology similar to that in wild type and showed a similar expression pattern of *DL* (Fig. 3B,D), implying that carpel specification is not affected by the *tab1* mutation.

Next, we focused on ovule development. At the early stage of development in wild type, a small bulge considered to be an ovule primordium was observed between the carpels (Fig. 3A). By contrast, a flattened structure instead of a bulge was observed between the carpel primordia in *tab1-1* (Fig. 3B), suggesting that normal ovule initiation is inhibited by *tab1* mutation. At a later stage in wild type, an ovule primordium with initiated integuments was observed inside the ovary (Fig. 3C). In *tab1-1*, by contrast, the putative ovule primordium lacked the integument-like structure (Fig. 3D).

We also examined the expression of *OsMADS13*, which has an important role in ovule development in rice (Dreni et al., 2007). As previously reported (Dreni et al., 2007), *OsMADS13* was expressed in the ovule primordium and the adaxial side of developing carpels in wild type (Fig. 3E,G). Although a similar expression pattern of *OsMADS13* was detected in *tab1-1*, the morphology of the ovule was aberrant during its development (Fig. 3F,H): in some cases, an abnormal tissue formed instead of an ovule primordium (Fig. 3F); in others, the ovule primordium was completely absent inside the ovary (Fig. 3H).

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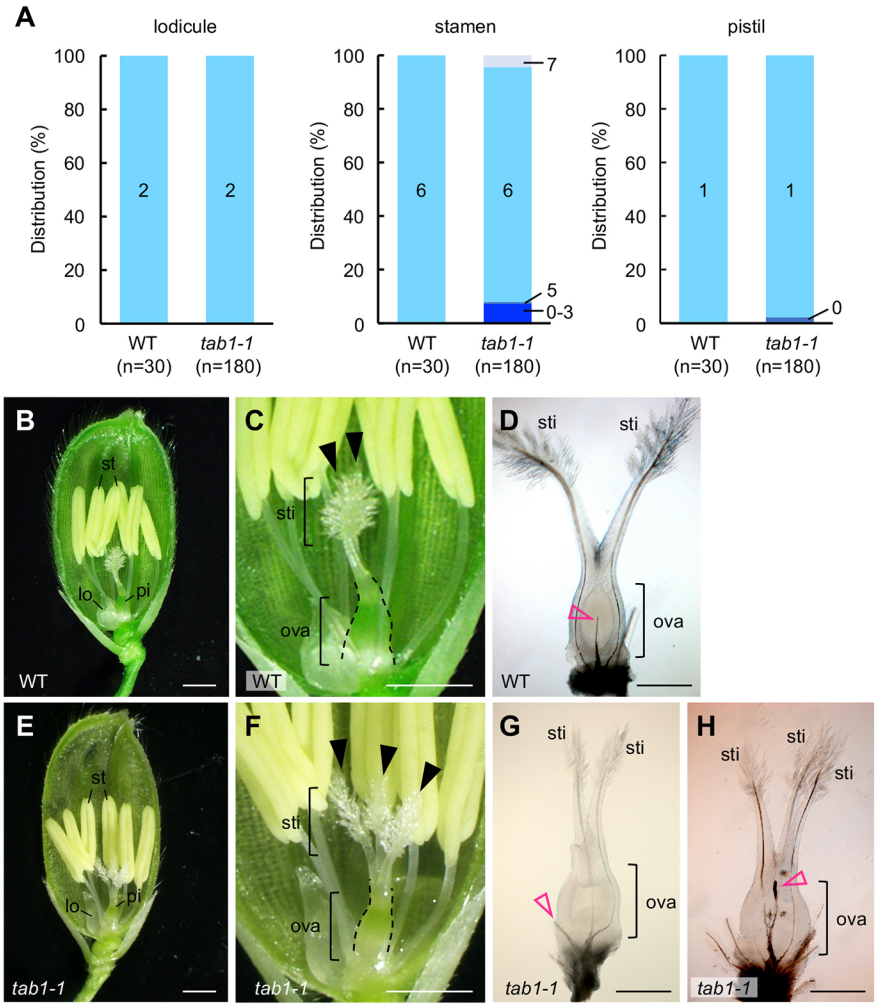

Fig. 1. Flower phenotype of the *tab1* mutant. (A) Distribution of the number of lodicules, stamens and pistils (*n*=30 for wild type, *n*=180 for *tab1-1*). (B,C, E,F) Flower phenotypes of wild type (B,C) and the *tab1-1* mutant (E,F). Arrowheads indicate the stigmas. (D,G,H) Phenotypes of pistils in wild type (D) and *tab1-1* (G,H). Arrowheads indicate the vascular bundles. lo, lodicule; ova, ovary; pi, pistil; st, stamen; sti, stigma; WT, wild type. Scale bars: 1 mm (B,C,E,F); 0.5 mm (D, G,H).

These morphological observations indicated that ovule development is seriously impaired in *tab1-1*; however, the expression pattern of *OsMADS13* was similar between wild type and *tab1-1*. Therefore, the defects in ovule initiation and development observed in *tab1-1* do not seem to be associated with the function of *OsMADS13*.

Maintenance of the final flower meristem is compromised in *tab1* plants

Because the ovule is formed from the flower meristem, we examined the expression pattern of a meristem marker, *ORYZA SATIVA HOMEOBOX 1* (*OSH1*) (Sato et al., 1996; Tsuda et al., 2011), during flower development. In wild type, *OSH1* was expressed throughout the flower meristem at an early stage of flower development (Fig. 4A), and a similar pattern of *OSH1* expression was detected in most *tab1-1* plants (Fig. 4B). At a middle stage, when stamen primordia emerged, *OSH1* expression was observed in the flower meristem between stamen primordia in wild type (Fig. 4C). Again, a similar expression pattern of *OSH1* was observed in the flower meristem in *tab1-1* (Fig. 4D).

At a later stage, after carpel primordia initiation, *OSH1* was expressed in a small region adjacent to the carpel primordia in wild

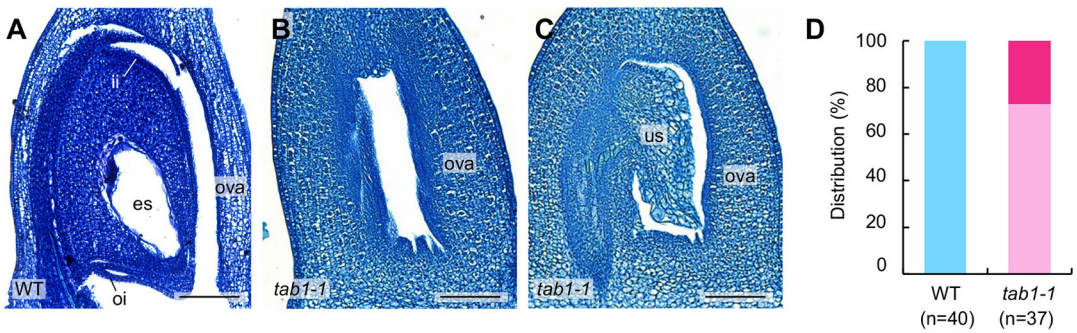

Fig. 2. Ovule phenotype of the *tab1* mutant. (A-C) Longitudinal sections of the ovary in wild type (A) and *tab1-1* (B,C). (D) Distribution of ovule phenotypes in wild type and *tab1-1* (*n*=40 for wild type, *n*=37 for *tab1-1*). Blue, wild type-like ovule; pink, unidentified structure without embryo sac or integuments; magenta, hollow ovary. es, embryo sac; ii, inner integument; oi, outer integument; ova, ovary; us, unidentified structure; WT, wild type. Scale bars: 100 µm.

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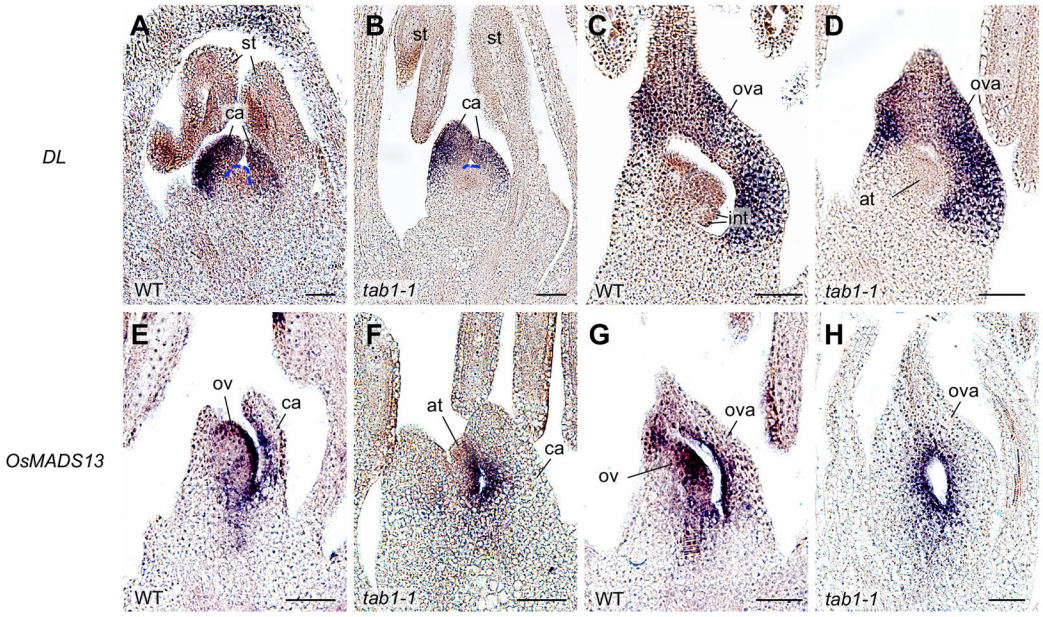

Fig. 3. Spatial expression patterns of marker genes during ovule development. (A,B) Expression pattern of *DL* at the early stage of ovule development in wild type (A) and *tab1-1* (B). Dashed blue lines indicate ovule primordia, which initiate between the carpels. (C,D) Expression pattern of *DL* at the late stage of ovule development in wild type (C) and *tab1-1* (D). (E-H) Spatiotemporal expression pattern of *OsMADS13* during ovule development in wild type (E,G) and *tab1-1* (F, H). The reproducibility of *in situ* hybridization was confirmed in more than six samples. at, abnormal tissue; ca, carpel; int, integument; ov, ovule; ova, ovary; st, stamen; WT, wild type. Scale bars: 50 µm.

type (Fig. 4E); however, such *OSH1* expression was not detected in any *tab1-1* plant (Fig. 4F). This result suggests that meristematic cells are lost precociously at a late developmental stage in the *tab1* flower. Hereafter, we refer to the meristem at this late stage as the 'final flower meristem'.

TAB1 is likely to act as a stem cell-promoting factor in the final flower meristem

Our recent study showed that *TAB1* promotes stem cell fate during axillary meristem development (Tanaka and Hirano, 2020). To clarify whether *TAB1* is associated with stem cell maintenance in flower development, we analyzed the expression pattern of the stem cell marker *FON2* (Suzaki et al., 2006). In wild type, *FON2* was expressed in a small central region of the flower meristem at the early stage of flower development (Fig. 5A), as reported previously (Suzaki et al., 2006). In *tab1-1*, *FON2* expression was similarly observed in the early flower meristem (Fig. 5B). At a later stage, when carpel primordia emerged, *FON2* expression was restricted to a few outer layers of the final flower meristem in wild type (Fig. 5C). In *tab1-1*, by contrast, *FON2* expression was completely absent (Fig. 5D), suggesting that stem cells had disappeared. This result suggests that *TAB1* is required for maintaining stem cells in the final flower meristem.

TAB1 is expressed in the final flower meristem

Next, we analyzed the spatiotemporal expression patterns of *TAB1* during flower development. *TAB1* transcript was first detected in the apical central region of the flower meristem at the early stage of

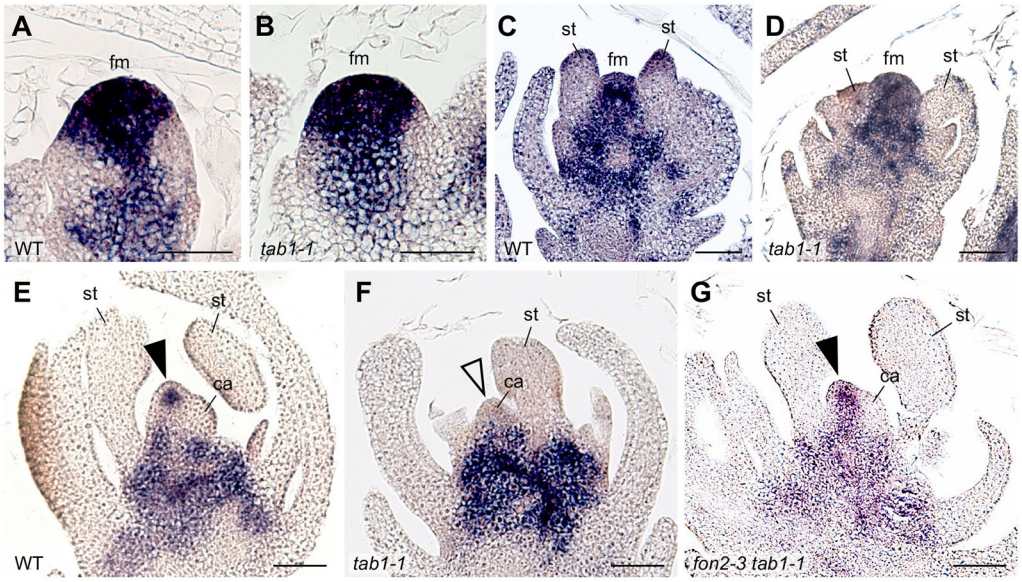

Fig. 4. Spatial expression pattern of a meristem marker during flower development. (A,B) Expression pattern of *OSH1* at the early stage of flower development in wild type (A) and *tab1-1* (B). (C,D) Expression pattern of *OSH1* at the middle stage of flower development in wild type (C) and *tab1-1* (D). (E-G) Expression pattern of *OSH1* at the late stage of flower development in wild type (E), *tab1-1* (F) and *fon2-3 tab1-1* (G). Filled arrowheads indicate strong expression in the final flower meristem; unfilled arrowhead indicates absence of expression. The reproducibility of *in situ* hybridization was confirmed in at least six samples and in more than ten samples when no signal was detected. ca, carpel; fm, flower meristem; st, stamen; WT, wild type. Scale bars: 50 µm.

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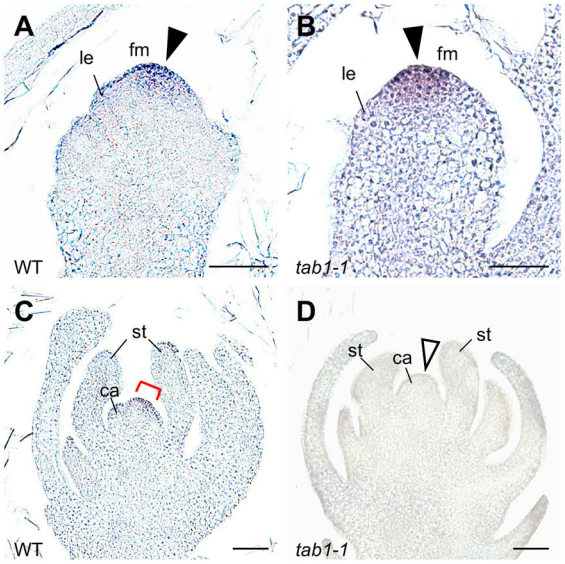

Fig. 5. Expression pattern of a stem cell marker during flower development. (A,B) Expression pattern of *FON2* at the early stage of flower development in wild type (A) and *tab1-1* (B). (C,D) Expression pattern of *FON2* at the late stage of flower development in wild type (C) and *tab1-1* (D). Filled arrowheads and bracket indicate *FON2* signals in the early flower meristem and final flower meristem, respectively; unfilled arrowhead indicates absence of *FON2* expression. The reproducibility of *in situ* hybridization was confirmed in at least six samples and in more than ten samples when no signal was detected. ca, carpel; fm, flower meristem; le, lemma; st, stamen; WT, wild type. Scale bars: 50 µm.

flower development (Fig. 6A). At the later stage, when carpel primordia emerged, *TAB1* expression was observed in the final flower meristem (Fig. 6B). This result supports the idea that *TAB1* functions in the flower meristem.

TAB1 expression was also detected during ovule development. Soon after an ovule primordium emerged, *TAB1* was expressed in the margin of the ovule primordium (Fig. 6C). Following differentiation of the ovule tissues, *TAB1* transcript was observed in the embryo sac and the boundary region between the nucellus and integuments (Fig. 6D).

To examine the genetic relationship between *TAB1* and *FON2*, we analyzed the expression pattern of *TAB1* in the *fon2-3* mutant, in which the flower meristem is enlarged as a result of overproliferation of stem cells (Suzaki et al., 2006, 2019a). At the early stage, *TAB1* was expressed in a wider region in the enlarged flower meristem of *fon2-3* compared with wild type (Fig. 6A,E). At a later stage, the expression region of *TAB1* was similarly expanded in the final flower meristem in *fon2-3* (Fig. 6B,F).

Next, we analyzed the expression pattern of *WOX4*, which is responsible for maintaining the vegetative SAM in rice (Ohmori et al., 2013) during flower development. The results showed that *WOX4* was expressed in the flower meristem at both the early and late stages of flower development (Fig. 6G,H).

Ovule defects in *tab1* are partially rescued by the *fon2* mutation

To examine the effect of *fon2* mutation on the *tab1* phenotype, we generated a *fon2-3 tab1-1* double mutant and analyzed its phenotype. Consistent with a previous report (Suzaki et al., 2006), the number of pistils was significantly increased in *fon2-3* (Fig. S2). There was no significant difference in pistil number among wild type, *tab1-1* and *fon2-3 tab1-1*.

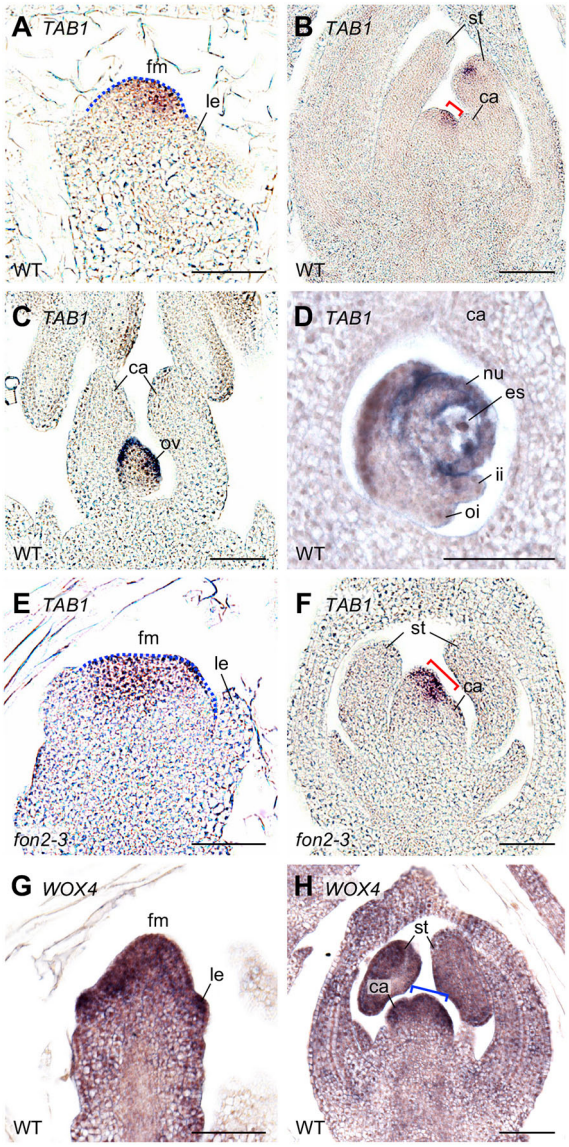

Fig. 6. Spatial expression patterns of *TAB1* and *WOX4*. (A-D) Expression pattern of *TAB1* in the early flower meristem (A), in the final flower meristem (B) and in ovule development (C,D) in wild type. (E,F) Expression pattern of *TAB1* in the early flower meristem (E) and in the final flower meristem (F) in *fon2-3*. Brackets indicate *TAB1* signals in the final flower meristem. Dotted blue lines delineate the flower meristem. (G,H) Expression pattern of *WOX4* in the early flower meristem (G) and in the final flower meristem (H) in wild type. The bracket indicates *WOX4* signal in the final flower meristem. The reproducibility of *in situ* hybridization was confirmed in more than six samples. ca, carpel; es, embryo sac; fm, flower meristem; ii, inner integument; le, lemma; nu, nucellus; oi, outer integument; ov, ovule; st, stamen; WT, wild type. Scale bars: 50 µm.

We then focused on ovule morphology. Ovule structure in *fon2-3* was similar to that in wild type (Fig. 7A,B,G). As described above, an ovule-like structure was not observed in any *tab1-1* plants, and a hollow ovary was detected in the most severe cases (Fig. 7C,G). In *fon2-3 tab1-1*, by contrast, about 40% of the flowers formed an ovule with an embryo sac and integuments (Fig. 7D,G), although the morphology differed slightly from that of wild type. In addition, about 30% of the *fon2-3 tab1-1* flowers formed an ovule-like structure with integuments but without an embryo sac (Fig. 7E,G). Furthermore, the proportion of severe morphological abnormalities, such as the hollow ovary observed in the *tab1-1* single mutant, was

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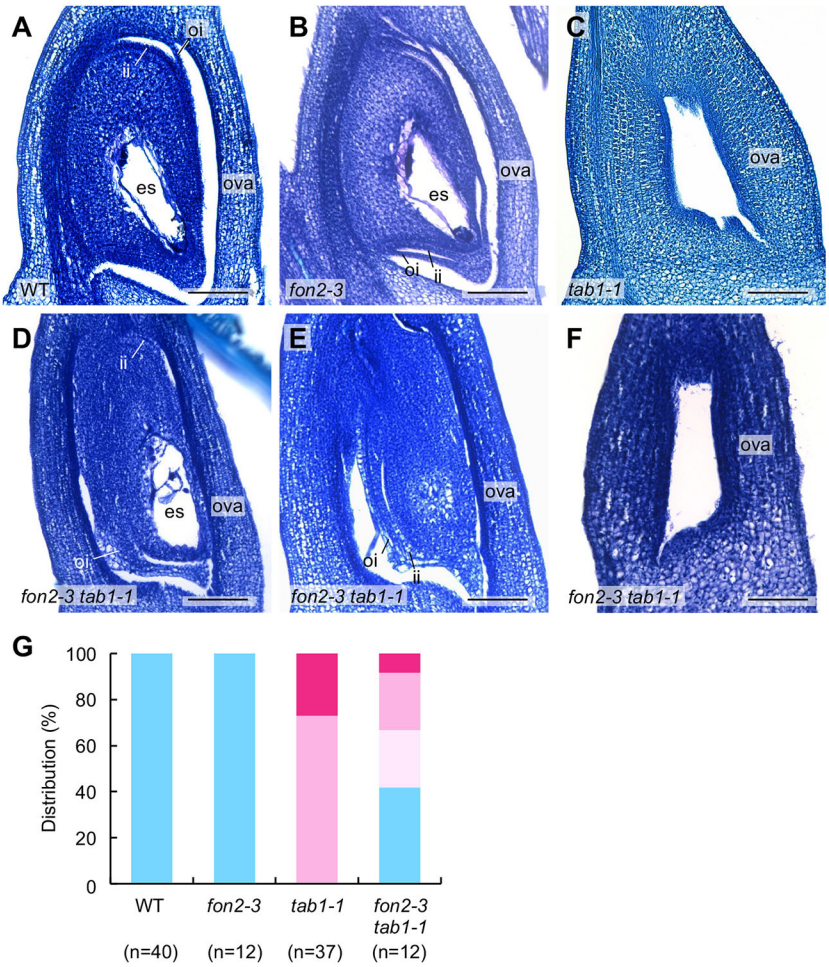

Fig. 7. Ovule phenotype of the *fon2 tab1* double mutant. (A-F) Longitudinal sections of the ovary in wild type (A), *fon2-3* (B), *tab1-1* (C) and *fon2-3 tab1-1* (D-F). (G) Distribution of ovule phenotypes (*n*=40 for wild type, *n*=12 for *fon2-3*, *n*=37 for *tab1-1*, *n*=12 for *fon2-3 tab1-1*). Blue, wild type-like ovule; pale pink, ovule-like structure without embryo sac; pink, unidentified structure without embryo sac or integuments; magenta, hollow ovary. es, embryo sac; ii, inner integument; oi, outer integument; ova, ovary; WT, wild type. Scale bars: 100 µm.

markedly reduced in the double mutant (Fig. 7F,G). These results suggest that the *fon2* mutation partially rescued the ovule defects in the *tab1* mutant.

To test whether this rescue was associated with meristem function, we analyzed the expression pattern of *OSH1*. *In situ* hybridization analysis showed that *OSH1* was clearly expressed in the final flower meristem in *fon2-3 tab1-1* (Fig. 4G), similar to its expression in wild type (Fig. 4E). This result indicates that the activity of the final flower meristem that was lost in *tab1-1* was restored by the *fon2* mutation.

Taken together, these observations suggest that the partial rescue of ovule formation in *fon2-3 tab1-1* seems to be associated with restoration of the activity of the final flower meristem.

DISCUSSION

TAB1 is required for stem cell maintenance in the final flower meristem

In rice, *TAB1* acts as a stem cell-promoting factor during axillary meristem development (Tanaka and Hirano, 2020). However, *TAB1* is not involved in stem cell maintenance in the SAM in the vegetative phase (Tanaka et al., 2015; Suzuki et al., 2019b); instead, *WOX4* is required for SAM maintenance (Ohmori et al., 2013). In *Arabidopsis*, by contrast, *WUS* promotes stem cell maintenance in both the developing axillary meristem and the established SAM (Laux et al., 1996; Mayer et al., 1998; Wang et al., 2017). Thus, in rice, *TAB1* and *WOX4* are likely to share, in part, the function of *WUS* in *Arabidopsis*.

In the present study, we identified a function of *TAB1* in flower development: namely, *TAB1* is predominantly required for maintaining stem cells in the final flower meristem at the late stage of flower development (Fig. 8). At this stage, expression of the meristem marker *OSH1* and the stem cell marker *FON2* disappeared

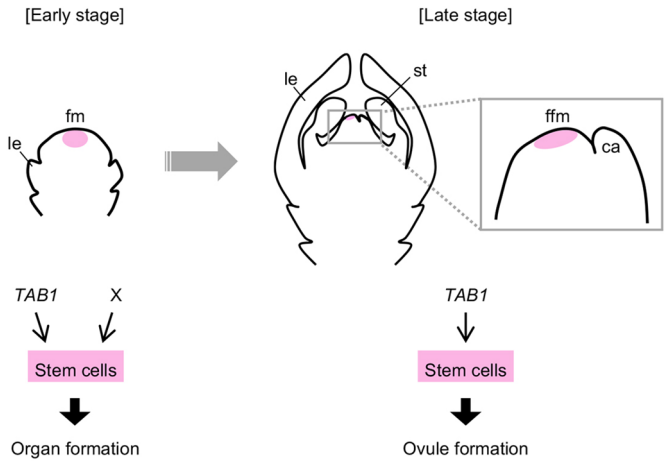

Fig. 8. Representation of the regulation of stem cell maintenance in the flower meristem in rice. Pink regions indicate stem cells. X indicates an unknown gene, which acts redundantly with *TAB1* to promote stem cell fate in the early flower meristem. ca, carpel; ffm, final flower meristem; fm, flower meristem; le, lemma; st, stamen.

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completely in the *tab1* mutant. As a result, *tab1* plants failed to form the ovule, which should be differentiated from the final flower meristem. Thus, the importance of *TAB1* function is evident in the final flower meristem.

In *Arabidopsis*, loss of function of the stem cell regulator *WUS* leads to a reduction of the number of floral organs (Laux et al., 1996). However, we observed no significant change in the number of floral organs in *tab1* mutants. *OSH1* and *FON2* were expressed normally in the flower meristem at the early-to-middle stages of flower development in *tab1*, suggesting that stem cell activity was not affected by the *tab1* mutation at these stages. Therefore, there seems to be a putative gene (X) that promotes stem cell fate at the early-to-middle stages (Fig. 8). As deduced from the *tab1* phenotype, the action of gene X is expected to be reduced at the late stage. We first considered that *WOX4* is a possible candidate for gene X owing to its function in the SAM (Ohmori et al., 2013). Unexpectedly, however, *in situ* hybridization analysis showed that *WOX4* was expressed in the flower meristem at the late stage in addition to the early-to-middle stages. Therefore, it is likely that *WOX4* alone is insufficient to maintain the flower meristem at the late stage, and probably also at the early-to-middle stages. Thus, *WOX4* does not seem to be a candidate for gene X. Identification of gene X is an important issue to be resolved in a future study in order to understand stem cell regulation in the flower meristem.

Our assumption of the existence of gene X indicates that several positive regulators are involved in stem cell maintenance in rice. Our group have revealed that rice has multiple CLV3-like negative regulators of stem cell maintenance such as FON2, FCP1 and FOS1 (Suzaki et al., 2006, 2008, 2009). The diversification of both positive and negative regulators implies that the genetic network of stem cell regulation is highly complex and varies depending on the type of meristem in rice. In *Arabidopsis*, a CLV3-related CLE peptide, CLE27, has been reported to act as a negative regulator of meristem maintenance (Je et al., 2016). In addition, the *wus* mutant forms axillary shoots from adventitious meristems and flowers on the shoots (Laux et al., 1996), suggesting that an unknown positive regulator promotes stem cells in adventitious meristems and flower meristems. Therefore, the regulatory mechanisms underlying stem cell maintenance in *Arabidopsis* may also not be simple.

TAB1 promotes ovule formation by maintaining stem cells in the final flower meristem

In *Arabidopsis*, the flower meristem is consumed after carpel initiation, and ovules are initiated from the placenta, which develops from the carpel margin meristem (Skinner et al., 2004; Roeder and Yanofsky, 2006; Reyes-Olalde et al., 2013). In rice, by contrast, the flower meristem remains even after carpel initiation, and the ovule is initiated directly from the meristem (Yamaki et al., 2005, 2011).

Various defects regarding ovule formation were observed in *tab1*. The loss-of-ovule phenotype seems to result from a failure in ovule initiation. The unidentified structure detected inside the ovary instead of the ovule probably results from inhibition of ovule differentiation. Despite these defects, *OsMADS13* was expressed in *tab1* as it is in wild type; thus, the potential for ovule specification does not seem to be affected by loss of *TAB1* activity. So why is ovule development defective in *tab1*? Considering the role of *TAB1* in the final flower meristem discussed above, we suggest that the reduced stem cell numbers and subsequent shortage of ovule initial cells might lead to the ovule defects seen in *tab1* mutants. In support of this suggestion, a weak allele of *LOG*, which is required for maintaining the flower meristem, also results in ovule loss (Yamaki et al., 2011).

When combined with *fon2* mutation, *tab1* showed partial rescue of the ovule defects. Furthermore, expression analysis of *OSH1* revealed that the activity of the final flower meristem in *tab1* was recovered by the *fon2* mutation. These observations imply that the excess of stem cells in *fon2* mutants is probably associated with the recovered meristem activity, eventually leading to the partial rescue of ovule formation in *fon2 tab1* plants. Taken together with our suggested role of *TAB1*, it is likely that *TAB1* promotes ovule formation by maintaining stem cells in the final flower meristem (Fig. 8).

In *Arabidopsis*, the *wus* mutation is epistatic to *clv3*; thus, the *wus clv3* double mutant has a *wus* phenotype (Schoof et al., 2000). The reason why the *fon2* mutation partially rescues the *tab1* phenotype in rice may be related to the unknown gene X that promotes stem cell at the early-to-middle stages of flower development (Fig. 8), as discussed above. The activity of gene X might be elevated as a result of the *fon2* mutation, generating an excess amount of stem cells. Previously, we showed that the *tab1* defects in axillary meristem development were partially rescued by *fon2* mutation (Tanaka and Hirano, 2020). This rescue was found to be due to precocious expression of *WOX4*. The difference in the phenotypes between *tab1 fon2* in rice and *wus clv3* in *Arabidopsis* seems to result from the existence of an additional gene(s) promoting stem cells in rice.

Although the activity of the final flower meristem in *tab1* mutants was recovered by *fon2* mutation, ovule formation was not fully rescued. In addition, fertility was not restored in *fon2 tab1* plants. *In situ* hybridization analysis indicated that *TAB1* is expressed in the developing ovule, in addition to the flower meristem, suggesting that *TAB1* plays some role in ovule differentiation. Therefore, the incomplete rescue of the *tab1* ovule by *fon2* mutation seems to be related to a failure in ovule differentiation, in addition to a deficiency of stem cells. In *Arabidopsis*, *WUS* is reported to be involved in ovule development, especially in integument development (Groß-Hardt et al., 2002; Yamada et al., 2016). Indeed, the *CLV1::WUS; wus* plant, in which loss of *WUS* activity is specific to ovules, fails to initiate integuments (Groß-Hardt et al., 2002). We observed that integument differentiation was similarly inhibited in the *tab1* mutant in rice. Therefore, a role in integument development may be conserved between rice *TAB1* and *Arabidopsis WUS*. However, the integument defect in *tab1* was partially rescued by *fon2* mutation to some extent, which also suggests that this defect is a secondary effect of insufficient cells for ovule development. The role of *TAB1* in ovule differentiation, including integument formation, will be an interesting issue to explore in future studies.

In this study, we have shown that *TAB1* is essential for ovule development in rice. *TAB1* seems to promote ovule formation by maintaining stem cells in the 'final flower meristem' at a late stage of flower development. In *Arabidopsis*, ovule formation is not associated with the flower meristem, because the ovule is initiated from the placenta. Therefore, the direct necessity of stem cell activity in ovule formation seems to be unique to rice.

MATERIALS AND METHODS

Plant materials and growth conditions

Oryza sativa L. ssp. *japonica* cultivar Taichung 65 (T65) was used as the wild type in phenotype comparisons and *in situ* hybridization analysis. The *tab1-1* and *fon2-3* mutants have been reported previously (Suzaki et al., 2006; Tanaka et al., 2015). *tab1-1* has a nucleotide substitution in the splicing site of intron 1 that causes premature termination of TAB1 protein translation in the intron (Tanaka et al., 2015). *fon2-3* has a point mutation at a conserved amino acid in the CLE domain and shows phenotypes very similar to those of a complete loss-of-function allele (Suzaki et al., 2006). Plants were grown outdoors in pots containing soil.

Clearing experiment

Pistils were dissected and immersed in a fixing solution (acetic acid and ethanol, in a ratio of 1:3). After 5 days, the samples were transferred to sterile water for 1 h, and then to a clearing solution (chloral hydrate, water, and glycerol in a ratio of 8:3:1) for more than 24 h. Samples were observed under differential interference contrast optics using a BX50 optical microscope (Olympus) coupled with an Axiocam 506 color camera (Carl Zeiss).

Histological analysis

Plant tissues, such as pistils and young panicles, were dissected, fixed and dehydrated according to the methods of Toriba and Hirano (2018). The tissues were replaced with xylene, and then embedded in Paraplast Plus (McCormick Scientific LLC). Microtome sections (8 µm) were prepared for histological analysis, mounted on glass slides, and stained with 0.05% Toluidine Blue-O for observation using a BX50 optical microscope (Olympus) coupled with an Axiocam 506 color camera (Carl Zeiss).

In situ hybridization

To prepare the *FON2* probe, RNA was transcribed with T7 RNA polymerase using digoxigenin-labeled UTP (Roche Applied Science), and treated by alkaline hydrolysis, as described previously (Suzaki et al., 2006). The *TAB1*, *OSH1*, *WOX4*, *DL* and *OsMADS13* probes were prepared as described previously (Yamaguchi et al., 2004; Dreni et al., 2007; Ohmori et al., 2013; Tanaka et al., 2015). Microtome sections (8-10 µm) were made from tissues embedded in Paraplast Plus as described above and mounted on glass slides. *In situ* hybridization and immunological detection were performed according to the methods of Toriba and Hirano (2018). Observation was performed using a BX50 optical microscope (Olympus) coupled with an Axiocam 506 color camera (Carl Zeiss).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: W.T., H.-Y.H.; Methodology: W.T., H.-Y.H.; Investigation: W.T., S.O., H.-Y.H.; Resources: W.T., H.-Y.H.; Writing - original draft: W.T., H.-Y.H.; Writing - review & editing: W.T., H.-Y.H.; Supervision: W.T., N.K., H.-Y.H.; Funding acquisition: W.T., H.-Y.H.

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References

Brand, U., Fletcher, J. C., Hobe, M., Meyerowitz, E. M. and Simon, R. (2000). Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by *CLV3* activity. *Science* **289**, 617-619. doi:10.1126/science.289.5479.617

Bommert, P., Lunde, C., Nardmann, J., Vollbrecht, E., Running, M., Jackson, D., Hake, S. and Werr, W. (2005). *thick tassel dwarf1* encodes a putative maize ortholog of the *Arabidopsis CLAVATA1* leucine-rich repeat receptor-like kinase. *Development* **132**, 1235-1245. doi:10.1242/dev.01671

Bommert, P., Je, B. I., Goldshmidt, A. and Jackson, D. (2013). The maize Gα gene *COMPACT PLANT2* functions in CLAVATA signalling to control shoot meristem size. *Nature* **502**, 555-558. doi:10.1038/nature12583

Clark, S. E., Running, M. P. and Meyerowitz, E. M. (1993). *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. *Development* **119**, 397-418. doi:10.1242/dev.119.2.397

Clark, S. E., Running, M. P. and Meyerowitz, E. M. (1995). *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. *Development* **121**, 2057-2067. doi:10.1242/dev.121.7.2057

Clark, S. E., Williams, R. W. and Meyerowitz, E. M. (1997). The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* **89**, 575-585. doi:10.1016/s0092-8674(00)80239-1

Dreni, L., Jacchia, S., Fornara, F., Fornari, M., Ouwerkerk, P. B. F., An, G., Colombo, L. and Kater, M. M. (2007). The D-lineage MADS-box gene *OsMADS13* controls ovule identity in rice. *Plant J.* **52**, 690-699. doi:10.1111/j.1365-313X.2007.03272.x

Fletcher, J. C., Brand, U., Running, M. P., Simon, R. and Meyerowitz, E. M. (1999). Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems. *Science* **283**, 1911-1914. doi:10.1126/science.283.5409.1911

Groß-Hardt, R., Lenhard, M. and Laux, T. (2002). *WUSCHEL* signaling functions in interregional communication during *Arabidopsis* ovule development. *Genes Dev.* **16**, 1129-1138. doi:10.1101/gad.225202

Hirano, H.-Y. and Tanaka, W. (2020). Stem cell maintenance in the shoot apical meristems and during axillary meristem development. *Cytologia* **85**, 3-8. doi:10.1508/cytologia.85.3

Je, B. I., Gruel, J., Lee, Y. K., Bommert, B., Arevalo, E. D., Eveland, A. L., Wu, Q., Goldshmidt, A., Meeley, R., Bartlett, M. et al. (2016). Signaling from maize organ primordia via FASCIATED EAR3 regulates stem cell proliferation and yield traits. *Nat. Genet.* **48**, 785-791. doi:10.1038/ng.3567

Je, B. I., Xu, F., Wu, Q., Liu, L., Meeley, R., Gallagher, J. P., Corcilius, L., Payne, R. J., Bartlett, M. E. and Jackson, D. (2018). The CLAVATA receptor FASCIATED EAR2 responds to distinct CLE peptides by signaling through two downstream effectors. *Elife* **7**, e35673. doi:10.7554/eLife.35673

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., Sakakibara, H. and Kyozuka, J. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* **445**, 652-655. doi:10.1038/nature05504

Laux, T., Mayer, K. F. X., Berger, J. and Jürgens, G. (1996). The *WUSCHEL* gene is required for shoot and floral meristem integrity in *Arabidopsis*. *Development* **122**,, 87-96. doi:10.1242/dev.122.1.87

Lu, Z., Shao, G., Xiong, J., Jiao, Y., Wang, J., Liu, G., Meng, X., Liang, Y., Xiong, G., Wang, Y. et al. (2015). *MONOCULM 3*, an ortholog of *WUSCHEL* in rice, is required for tiller bud formation. *J. Genet. Genomics* **42**, 71-78. doi:10.1016/j.jgg.2014.12.005

Mayer, K. F. X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G. and Laux, T. (1998). Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem. *Cell* **95**, 805-815. doi:10.1016/S0092-8674(00)81703-1

Ohmori, Y., Tanaka, W., Kojima, M., Sakakibara, H. and Hirano, H.-Y. (2013). *WUSCHEL-RELATED HOMEOBOX4* is involved in meristem maintenance and is negatively regulated by the CLE gene *FCP1* in rice. *Plant Cell* **25**, 229-241. doi:10.1105/tpc.112.103432

Pautler, M., Eveland, A. L., LaRue, T., Yang, F., Weeks, R., Lunde, C., Je, B. I., Meeley, R., Komatsu, M., Vollbrecht, E. et al. (2015). *FASCIATED EAR4* encodes a bZIP transcription factor that regulates shoot meristem size in maize. *Plant Cell* **27**, 104-120. doi:10.1105/tpc.114.132506

Pinyopich, A., Ditta, G. S., Savidge, B., Liljegren, S. J., Baumann, E., Wisman, E. and Yanofsky, M. F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature* **424**, 85-88. doi:10.1038/nature01741

Reyes-Olalde, J. I., Zuñiga-Mayo, V. M., Chávez Montes, R. A., Marsch-Martínez, N. and de Folter, S. (2013). Inside the gynoecium: at the carpel margin. *Trends Plant Sci.* **18**, 644-655. doi:10.1016/j.tplants.2013.08.002

Roeder, A. H. K. and Yanofsky, M. F. (2006). Fruit development in Arabidopsis. *Arabidopsis Book* **4**, e0075.

Sato, Y., Hong, S. K., Tagiri, A., Kitano, H., Yamamoto, N., Nagato, Y. and Matsuoka, M. (1996). A rice homeobox gene, *OSH1*, is expressed before organ differentiation in a specific region during early embryogenesis. *Proc. Natl. Acad. Sci. USA* **93**, 8117-8122. doi:10.1073/pnas.93.15.8117

Schoof, H., Lenhard, M., Haecker, A., Mayer, K. F. X., Jürgens, G. and Laux, T. (2000). The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. *Cell* **100**, 635-644. doi:10.1016/S0092-8674(00)80700-X

Skinner, D. J., Hill, T. A. and Gasser, C. S. (2004). Regulation of ovule development. *Plant Cell* **16**, S32-S45. doi:10.1105/tpc.015933

Suzaki, T., Sato, M., Ashikari, M., Miyoshi, M., Nagato, Y. and Hirano, H.-Y. (2004). The gene *FLORAL ORGAN NUMBER1* regulates floral meristem size in rice and encodes a leucine-rich repeat receptor kinase orthologous to *Arabidopsis* CLAVATA1. *Development* **131**, 5649-5657. doi:10.1242/dev.01441

Suzaki, T., Toriba, T., Fujimoto, M., Tsutsumi, N., Kitano, H. and Hirano, H.-Y. (2006). Conservation and diversification of meristem maintenance mechanism in *Oryza sativa*: Function of the *FLORAL ORGAN NUMBER2* gene. *Plant Cell Physiol.* **47**, 1591-1602. doi:10.1093/pcp/pcl025

Suzaki, T., Yoshida, A. and Hirano, H.-Y. (2008). Functional diversification of CLAVATA3-related CLE proteins in meristem maintenance in rice. *Plant Cell* **20**, 2049-2058. doi:10.1105/tpc.107.057257

Suzaki, T., Ohneda, M., Toriba, T., Yoshida, A. and Hirano, H.-Y. (2009). *FON2 SPARE1* redundantly regulates floral meristem maintenance with *FLORAL ORGAN NUMBER2* in rice. *PLoS Genet.* **5**, e1000693. doi:10.1371/journal.pgen.1000693

Suzuki, C., Tanaka, W. and Hirano, H.-Y. (2019a). Transcriptional corepressor ASP1 and CLV-like signaling regulate meristem maintenance in rice. *Plant Physiol.* **180**, 1520-1534. doi:10.1104/pp.19.00432

Suzuki, C., Tanaka, W., Tsuji, H. and Hirano, H.-Y. (2019b). *TILLERS ABSENT1*, the *WUSCHEL* ortholog, is not involved in stem cell maintenance in the shoot apical meristem in rice. *Plant Signal Behav.* **14**, 1640565.

Taguchi-Shiobara, F., Yuan, Z., Hake, S. and Jackson, D. (2001). The *fasciated ear2* gene encodes a leucine-rich repeat receptor-like protein that regulates shoot meristem proliferation in maize. *Genes Dev.* **15**, 2755-2766. doi:10.1101/gad.208501

Tanaka, W. and Hirano, H.-Y. (2020). Antagonistic action of *TILLERS ABSENT1* and *FLORAL ORGAN NUMBER2* regulates stem cell maintenance during axillary meristem development in rice. *New Phytol.* **225**, 974-984. doi:10.1111/nph.16163

Tanaka, W., Ohmori, Y., Ushijima, T., Matsusaka, H., Matsushita, T., Kumamaru, T., Kawano, S. and Hirano, H.-Y. (2015). Axillary meristem formation in rice requires the *WUSCHEL* ortholog *TILLERS ABSENT1*. *Plant Cell* **27**, 1173-1184. doi:10.1105/tpc.15.00074

Toriba, T. and Hirano, H.-Y. (2018). Two-color in situ hybridization: a technique for simultaneous detection of transcripts from different loci. In *Plant Transcription Factors - Methods And Protocols* (ed. N. Yamaguchi), pp. 269-287. New York: Springer.

Tsuda, K., Ito, Y., Sato, Y. and Kurata, N. (2011). Positive autoregulation of a *KNOX* gene is essential for shoot apical meristem maintenance in rice. *Plant Cell* **23**, 4368-4381. doi:10.1105/tpc.111.090050

Wang, J., Tian, C., Zhang, C., Shi, B., Cao, X., Zhang, T.-Q., Zhao, Z., Wang, J. W. and Jiao, Y. (2017). Cytokinin signaling activates *WUSCHEL* expression during axillary meristem initiation. *Plant Cell* **29**, 1373-1387. doi:10.1105/tpc.16.00579

Wu, Q., Regan, M., Furukawa, H. and Jackson, D. (2018). Role of heterotrimeric Gα proteins in maize development and enhancement of agronomic traits. *PLoS Genet.* **14**, e1007374. doi:10.1371/journal.pgen.1007374

Yadav, R. K., Perales, M., Gruel, J., Girke, T., Jönsson, H. and Reddy, G. V. (2011). WUSCHEL protein movement mediates stem cell homeostasis in the *Arabidopsis* shoot apex. *Genes Dev.* **25**, 2025-2030. doi:10.1101/gad.17258511

Yamada, T., Sasaki, Y., Hashimoto, K., Nakajima, K. and Gasser, C. S. (2016). *CORONA*, *PHABULOSA* and *PHAVOLUTA* collaborate with *BELL1* to confine *WUSCHEL* expression to the nucellus in *Arabidopsis* ovules. *Development* **143**, 422-426. doi:10.1242/dev.129833

Yamaguchi, T., Nagasawa, N., Kawasaki, S., Matsuoka, M., Nagato, Y. and Hirano, H.-Y. (2004). The *YABBY* Gene *DROOPING LEAF* regulates carpel specification and midrib development in *Oryza sativa*. *Plant Cell* **16**, 500-509. doi:10.1105/tpc.018044

Yamaki, S., Satoh, H. and Nagato, Y. (2005). Gypsy embryo specifies ovule curvature by regulating ovule/integument development in rice. *Planta* **222**, 408-417. doi:10.1007/s00425-005-1547-z

Yamaki, S., Nagato, S., Kurata, N. and Nonomura, K. (2011). Ovule is a lateral organ finally differentiated from the terminating floral meristem in rice. *Dev. Biol.* **351**, 208-216. doi:10.1016/j.ydbio.2010.12.006