The Roles of Two *FLORAL ORGAN NUMBER* Genes, *FON1* and *FON2*, Differ in Axillary Meristem Development

Wakana Tanaka^{1,2*} and Hiro-Yuki Hirano²

¹ Graduate School of Integrated Sciences for Life, Hiroshima University, 1–4–4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739–8528, Japan

² Department of Biological Sciences, School of Science, The University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–8654, Japan

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Summary Plant development depends on the activity of stem cells. The shoot branch, which is called a tiller in grasses, is derived from stem cells in the axillary meristem, which continuously emerges in the leaf axil during plant growth. *TILLERS ABSENT1* (*TAB1*) and *FLORAL ORGAN NUMBER2* (*FON2*) play important roles in axillary meristem development in rice. In this process, *TAB1* is required for stem cell maintenance, whereas *FON2* negatively regulates stem cell fate by restricting *TAB1* transcription. *FON2* encodes a signaling molecule, which was originally identified as a negative regulator of stem cell maintenance in the floral meristem, and is known to act in the same genetic pathway as *FON1* encoding a putative receptor of *FON2*. Here, we investigated whether *FON1* is involved in axillary meristem development. The *fon1* mutation did not affect axillary meristem development or tiller formation. Consistent with this result, *FON1* expression was not detected throughout axillary meristem development. In addition, the *fon1* mutation, unlike *fon2*, did not rescue the failure in tiller formation in *tab1*. Therefore, the roles of *FON1* and *FON2* likely differ from each other in axillary meristem development, and *FON1* seems not to be involved in stem cell maintenance. These results also suggest that *FON2* might regulate axillary meristem development *via* a receptor other than *FON1*.

Keywords Axillary meristem, *CLV*, *FON1*, Rice, *Oryza sativa*, Stem cell maintenance, Tiller.

In a plant, the activity of stem cells in the shoot apical meristem (SAM) is vital for its development. The stem cells not only provide cells for organogenesis but also divide for self-renewal. Thus, the balance of these two processes, stem cell maintenance, ensure continuous development of the plant. In Arabidopsis (*Arabidopsis thaliana*), the maintenance of stem cell homeostasis in the SAM is regulated by a negative feedback loop between WUSCHEL (*WUS*) and CLAVATA (*CLV*) signaling (reviewed in Ha *et al.* 2010, Somssich *et al.* 2016). *WUS* is a member of the WUSCHEL-related homeobox (*WOX*) transcription factors and promotes stem cell fate (Laux *et al.* 1996, Mayer *et al.* 1998). By contrast, *CLV* proteins such as *CLV1* and *CLV3*, encoding a LRR-receptor kinase and a CLE peptide, respectively, negatively regulate stem cell fate by repressing *WUS* expression (Clark *et al.* 1993, 1997, Fletcher *et al.* 1999, Brand *et al.* 2000). *WUS* in turn promotes *CLV3* expression by directly binding its promoter (Schoof *et al.* 2000, Yadav *et al.* 2011).

The *CLV* signaling that negatively regulates stem cell maintenance is likely to be conserved in rice (*Oryza sativa*) (reviewed in Tanaka *et al.* 2014, Fletcher 2018, Hirano and Tanaka 2020). *FLORAL ORGAN NUMBER1*

(*FON1*) and *FON2*, the respective orthologs of Arabidopsis *CLV1* and *CLV3*, are involved in the maintenance of the floral meristem (Suzaki *et al.* 2004, 2006). In both *fon1* and *fon2* mutants, the floral meristem is enlarged and the expression domain of the stem cell marker is expanded, suggesting that the two genes negatively regulate stem cell fate in the floral meristem. A double mutant of *fon1* and *fon2* shows a similar phenotype to that of the single mutant, suggesting that the two genes act in the same genetic pathway (Suzaki *et al.* 2006).

Unlike *FON* signaling, the function of the rice *WUS* ortholog, named *TILLERS ABSENT1* (*TAB1*), differs from that of Arabidopsis *WUS*. Our previous study revealed that *TAB1* is unrelated to SAM maintenance, but is required for stem cell maintenance during axillary meristem formation (Tanaka *et al.* 2015, 2019, Suzuki *et al.* 2019, Tanaka and Hirano 2020). In the *tab1* mutant, axillary meristem formation is arrested at an early developmental stage because of a failure in stem cell maintenance, resulting in no-tiller formation (Tanaka *et al.* 2015, 2019, Tanaka and Hirano 2020). *TAB1* is transiently expressed in an early stage of axillary meristem development, and its expression disappears in the established axillary meristem (Tanaka *et al.* 2015). Thus, *TAB1* is required for axillary meristem formation by promoting stem cell fate only at an early stage, during its formation. Recently, we revealed that *FON2* is

* Corresponding author, e-mail: wakanat@hiroshima-u.ac.jp
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also involved in axillary meristem development (Tanaka and Hirano 2020). In *fon2*, a bulge named the premeristem zone, which is first detected in the leaf axil at an early stage of axillary meristem development was significantly enlarged in comparison to a wild type. *FON2* is expressed during axillary meristem development and in the established axillary meristem. Furthermore, *TAB1* was expressed in a wider region of the premeristem zone in *fon2*, compared with the wild type. Therefore, *FON2* negatively regulates stem cell fate by restricting *TAB1* expression during axillary meristem development. We also found that the defect in axillary meristem formation in *tab1* was partially suppressed by the *fon2* mutation. This suppression is likely to be associated with the precocious expression of *WOX4*, a *WOX* gene closely related to *TAB1* (Tanaka and Hirano 2020).

In this study, we carried out a molecular genetic analysis to reveal the function of *FON1* in axillary meristem development in rice. The result indicated that the *fon1* mutation did not affect axillary meristem development and that *FON1* is not expressed throughout axillary meristem formation. In addition, the *fon1* mutation did not suppress the *tab1* phenotype. All these results are in contrast to those obtained in a *FON2* study (Tanaka and Hirano 2020). Therefore, *FON1*, unlike *FON2*, seems not to be involved in axillary meristem development, suggesting that *FON1* is unrelated to the *FON2* signaling pathway during this process.

Materials and methods

Plant materials and growth conditions

The rice strains used in this study were *O. sativa japonica*; Taichung 65 was used as a wild-type strain. The *fon1-5* and *tab1-1* mutants have been reported previously (Suzaki *et al.* 2008, Tanaka *et al.* 2015). Plants were usually grown in an NK System Biotron (model LH-220S; Nippon Medical & Chemical Instruments).

Histological analysis and in situ hybridization experiment

Basal shoot tissues containing premeristem zones and axillary meristems were dissected, fixed, and dehydrated in accordance with the methods of Toriba and Hirano (2018). The tissues were then replaced with xylene and embedded in Paraplast Plus (McCormick Scientific). For histological analysis, microtome sections (8–10 μ m) were mounted on glass slides and stained with 0.05% toluidine blue O for observation by a BX50 optical microscope (Olympus) and an Axiocam 506 color camera (Carl Zeiss).

The *FON1* probes were transcribed as described previously (Suzaki *et al.* 2004). Microtome sections (10 μ m) were mounted on glass slides, in a similar manner to that described above. *In situ* hybridization and immunological detection of *FON1* signals were conducted in accor-

dance with the methods of Toriba and Hirano (2018).

Measurement of the premeristem zone area

To measure the area of the premeristem zone, basal shoot tissues containing the premeristem zone were dissected and immersed in a fixing solution (acetic acid and ethanol, in a ratio of 1:3). After fixation for three days, the samples were transferred to sterile water for one hour, and then to a clearing solution (of chloral hydrate, water, and glycerol, ratio 8:3:1) for 24 h. Samples were observed under DIC optics by a BX50 optical microscope (Olympus) coupled with an Axiocam 506 color camera (Carl Zeiss). The area of the premeristem zone of each plant was then measured, using Fiji software (<https://imagej.net/Fiji>).

Results

The *fon1* mutation does not suppress the *tab1* phenotype

To examine the function of the *FON1* gene during axillary meristem development, we first performed genetic analysis. One month after germination, *fon1-5* formed the same number of tillers as the wild type (Fig. 1A, C), similar to *fon2-3* (Fig. 1B). Consistent with the previous report, *tab1-1* did not form tillers (Fig. 1D) (Tanaka *et al.* 2015). Next, we generated a *fon1-5 tab1-1* double mutant and analyzed the effect of *fon1* mutation on the *tab1* mutant. The result indicated that the *fon1-5 tab1-1* double mutant did not form tillers (Fig. 1F). Statistical analysis indicated that there was no significant difference in tiller numbers between *tab1-1* and *fon1-5 tab1-1* (Fig. 1G). This observation contrasted with that of *fon2-3 tab1-1*, in which the number of tillers was significantly higher than in *tab1-1* (Fig. 1E, G) (Tanaka and Hirano 2020). This result suggests that, unlike *fon2* mutation, the *fon1* mutation did not suppress the defect in tiller formation of *tab1*.

We then examined the morphology of axillary buds formed on the main stem. In the wild type, an outermost specialized leaf of the axillary bud, called a prophyll, was detected at the axil of the leaf (Fig. 2A). A prophyll with a similar appearance to that of the wild type was observed in *fon2-3*, *fon1-5*, and *fon2-3 tab1-1* (Fig. 2B, C, E). In *fon1-5 tab1-1*, by contrast, an abnormally elongated prophyll was formed at the axil of the leaf (Fig. 2F), and this phenotype was indistinguishable from that of the *tab1-1* single mutant (Fig. 2D). To gain further insight, we observed the inner structure of those prophylls by preparing longitudinal sections. In *fon2-3* and *fon1-5*, a dome-shaped axillary meristem was observed, similar to the wild type (Fig. 2G–I). As previously reported (Tanaka *et al.* 2015, Tanaka and Hirano 2020), no such dome-like meristem was observed in *tab1-1* (Fig. 2J), whereas a dome-shaped axillary meristem similar to that of the wild type was often observed in the *fon2-3 tab1-1* double mutant (Fig. 2K). By contrast, however, no meri-

Fig. 1. Shoot phenotype of *fon1-5 tab1-1*. (A–F) Shoot phenotypes of one-month-old plants of the wild type (A), *fon2-3* (B), *fon1-5* (C), *tab1-1* (D), *fon2-3 tab1-1* (E), and *fon1-5 tab1-1* (F). Arrowheads indicate tillers. (G) Comparison of tiller numbers. Error bars indicate SE. Asterisks indicate significant differences (Student's *t*-test, $p < 0.01$); ns, not significant (Student's *t*-test, $p > 0.05$). Scale bars = 2 cm.

Fig. 2. The phenotype of axillary bud. (A–F) Basal region of the main stem after the removal of leaves in the wild type (A), *fon2-3* (B), *fon1-5* (C), *tab1-1* (D), *fon2-3 tab1-1* (E), and *fon1-5 tab1-1* (F). White arrowheads indicate normal prophylls, whereas pink arrowheads indicate abnormally elongated prophylls. (G–L) Longitudinal sections of the prophyll in the wild type (G), *fon2-3* (H), *fon1-5* (I), *tab1-1* (J), *fon2-3 tab1-1* (K), and *fon1-5 tab1-1* (L). The arrowheads in (J) and (L) indicate the absence of dome-like meristem. am, axillary meristem; pp, prophyll; P1, plastochron one-leaf primordium. Scale bars in (A) to (F) = 1 mm; scale bars in (G) to (L) = 50 μ m.

stem was detected in any *fon1-5 tab1-1* double mutants examined (Fig. 2L). This observation suggests that the failure of axillary meristem formation in *tab1* was not suppressed by the *fon1* mutation, and that the effects of

the *fon2* and *fon1* mutations on *tab1* differ.

Fig. 3. The phenotype of the premeristem zone in *fon1-5*. (A, B) Longitudinal sections of the premeristem zones in the wild type (A) and *fon1-5* (B). The region inside the dotted line indicates the area of the premeristem zone. (C) Comparison of the areas of the premeristem zones, between the wild type and *fon1-5*. Error bars indicate $\pm$ SE; ns, not significant (Student's *t*-test, $p > 0.05$). Scale bars = 25 μ m.

Fig. 4. Absence of *FON1* expression during axillary meristem development. (A–C) Expression patterns of *FON1* in the SAM (A) and the premeristem zone (B) at an early stage during axillary meristem development, and the axillary meristem (C), respectively. am, axillary meristem; pp, prophyll; P1, plastochron one-leaf primordium; sam, shoot apical meristem. Scale bars = 50 μ m.

Axillary meristem development is not affected by the fon1 mutation

We previously characterized the developmental process of axillary meristem formation in rice. We found that a small bulge named the premeristem zone, where a stem cell marker starts to be expressed is first detected in the leaf axil at an early stage of this process (Tanaka *et al.* 2015, Tanaka and Hirano 2020). We examined whether the *fon1* mutation affects the morphology of the premeristem zone. To observe the premeristem zone, we prepared longitudinal sections of the basal tissue of the shoot. The premeristem zone of *fon1-5* appears to be similar to that of the wild type in both size and morphology (Fig. 3A, B). To analyze quantitatively, we next prepared the cleared tissues and measured the area of the premeristem zone, using Fiji software. We found no significant difference in the area of the premeristem zone between the wild type and *fon1-5* (Fig. 3C), suggesting that the activity of the premeristem zone is not affected by the *fon1* mutation. As described previously (Tanaka and Hirano 2020), the area of the premeristem zone was significantly enlarged in the *fon2* mutant due to an expansion of the stem cell region. Therefore, the roles of

FON1 and *FON2* are likely to differ from each other in axillary meristem development.

Lastly, we analyzed the expression of *FON1* during axillary meristem development. Clear *FON1* expression was detected in the SAM (Fig. 4A), as described previously (Suzaki *et al.* 2004). By contrast, no *FON1* signal was detected either in the premeristem zone (Fig. 4B) or in the established axillary meristem (Fig. 4C). Thus, *FON1* is likely not expressed during axillary meristem development.

Discussion

In the floral meristem, both *FON1* and *FON2* act as negative regulators of stem cell maintenance (Suzaki *et al.* 2004, 2006). A mutation in either *FON1* or *FON2* causes an enlargement of the floral meristem because of an overproliferation of stem cells. Our recent study showed that *FON2* negatively regulates stem cell maintenance in axillary meristem development as well (Tanaka and Hirano 2020). In *fon2*, the size of the premeristem zone is enlarged due to an expanded stem cell region. In the present study, *fon1*, unlike *fon2*, exhibited no en-

largement of the premeristem zone. Consistent with this observation, *FON1* was not expressed throughout axillary meristem development. In addition, the *fon1* mutation, unlike *fon2*, did not rescue the *tab1* mutant. Therefore, it seems likely that the function of *FON1* is not involved in axillary meristem development, and that the role of *FON1* differs from that of *FON2* in this process.

FON1 and *FON2* are likely acting in the same genetic pathway in the floral meristem, since the double mutant of *fon1* and *fon2* shows a similar phenotype to that of the single mutant (Suzaki *et al.* 2006). *FON1* is considered to be a putative receptor of *FON2* in the floral meristem, based on the fact that overexpression of *FON2* has no effect on the *fon1* mutant, unlike the wild type (Suzaki *et al.* 2006). By contrast, *FON1* seems to be unrelated to *FON2* action in axillary meristem development, as discussed above. Therefore, *FON2* probably acts through a receptor other than *FON1* to maintain stem cells during axillary meristem development. In the rice genome, there are five *FON1*-like genes. Future studies will therefore need to elucidate whether the *FON1*-like genes perceive a *FON2* signal during axillary meristem development.

Unlike *fon2*, the *fon1* mutation did not suppress the phenotype of *tab1*, which is defective in tiller formation. We previously showed that this suppression resulted from precocious expression of *WOX4* during axillary meristem formation (Tanaka and Hirano 2020). This result suggests that a signal from *FON2* action suppresses the early expression of *WOX4* during axillary meristem development in the wild type. Thus, the failure in suppression of the *tab1* phenotype by the *fon1* mutation is probably associated with the fact that *FON1* is not involved in the *FON2* signaling to repress *WOX4*. As discussed above, *FON1* seems not to mediate *FON2* signaling for the negative regulation of stem cell proliferation. Therefore, our results presented in this study agree with each other.

In this study, we clearly showed that the *fon1* mutation did not affect tiller formation. This result is consistent with previous studies using other *fon1* alleles (Nagasawa *et al.* 1996, Suzaki *et al.* 2004), although one exceptional report claimed that a *fon1* allele exhibited reduced tiller number (Shao *et al.* 2019). Although the mutant used in the latter study was named *fon1-5*, its mutation was different from our original *fon1-5* allele (Suzaki *et al.* 2008). Furthermore, the authors arrived at an odd conclusion, that is, *FON1* was required for axillary bud outgrowth that is unrelated to its original function, stem cell maintenance.

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