Overexpression analysis suggests that *FON2-LIKE CLE PROTEIN1* is involved in rice leaf development

Yoshihiro Ohmori[†], Yukiko Yasui and Hiro-Yuki Hirano*

Department of Biological Sciences, Graduate School of Science, The University of Tokyo,
Bunkyo-ku, Tokyo 113-8654, Japan

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Peptide signaling plays important roles in various developmental processes of plants. Genes encoding CLE proteins, which are processed into CLE signaling peptides, are required for maintenance of the shoot apical meristem and for vascular differentiation. *FON2-LIKE CLE PROTEIN1* (*FCP1*), a member of the CLE gene family, negatively regulates meristem maintenance in both shoot and root apical meristems of rice (*Oryza sativa*). Here, we examined the role of *FCP1* in leaf development. We found that overexpression of *FCP1* affects various aspects of leaf development in shoots regenerated from calli, making it difficult to distinguish between the leaf blade and leaf sheath. Differentiation of tissues such as vascular bundle and sclerenchyma was strongly inhibited by *FCP1* overexpression. Spatial expression patterns of developmental genes *DROOPING LEAF* (*DL*) and *OsPINHEAD1* (*OsPNH1*) were severely affected in the *FCP1*-overexpressing shoots. Whereas *DL* was expressed in the central region of leaf primordia in control shoots, *DL* expression was expanded throughout the leaf primordia of the *FCP1*-overexpressing shoots in early developmental stages. By contrast, *OsPNH1*, which is expressed in provascular and developing vascular tissues in normal seedlings, was strongly repressed by *FCP1* overexpression. Taken together, our results suggest that *FCP1* is involved in the regulation of cell fate determination during leaf development.

Key words: cell differentiation, CLE peptide, *FCP1*, leaf development, rice

Intercellular communication is particularly important for development in plants, because plant cells are fixed by rigid cell walls and do not migrate. Many types of small peptides are involved in communication between cells and between functional domains within tissues and organs to regulate developmental processes such as stem cell maintenance, vascular differentiation, and stomatal development. In *Arabidopsis thaliana*, stem cell maintenance in the shoot apical meristem is negatively regulated by a CLAVATA3 (CLV3) CLE peptide, which acts through receptor complexes such as CLV1, CLV2/CRN, and RPK2 (reviewed in Aichinger et al., 2012; Ha et al., 2010; Miyawaki et al., 2013). Mutation in *CLV3* leads to an enlarged meristem consisting of over-proliferated stem cells, due to a failure in the repression of *WUSCHEL* (*WUS*) activity, which promotes stem cell identity (Fletcher et al., 1999). Conversely, overexpression of *CLV3* results in premature termination of the meristem

(Brand et al., 2000; Fletcher et al., 1999).

In rice, mutations in the genes *FLORAL ORGAN NUMBER1* (*FON1*) and *FON2* give rise to enlargement of the floral meristem, resulting in increases in the numbers of floral organs such as carpels and stamens (Suzaki et al., 2004, 2006). *FON1* encodes a CLV1-like LRR receptor kinase, whereas the protein encoded by *FON2* contains a CLE domain highly similar to that of *Arabidopsis* CLV3. Unlike *Arabidopsis clv* mutants, however, rice *fon* mutants do not display abnormal phenotypes in the vegetative phase. In addition, overexpression of *FON2* does not affect vegetative development, whereas it disturbs inflorescence and flower development probably due to defects in maintenance of the reproductive meristem. Thus, CLV signaling appears to be partially conserved in rice (reviewed in Miyawaki et al., 2013; Pautler et al., 2013).

FON2-LIKE CLE PROTEIN1 (*FCP1*) encodes a CLE protein closely related to *FON2* and plays an important role in the vegetative development of rice (Suzaki et al., 2008; Ohmori et al., 2013). Constitutive overexpression of *FCP1* causes premature termination of the meristem in shoots regenerating from calli (Suzaki et al., 2008). Like-

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* Corresponding author. E-mail: hyhirano@bs.s.u-tokyo.ac.jp

[†] Present address: Graduate School of Agriculture and Life Sciences, The University of Tokyo, Yayoi, Bunkyo, Tokyo 113-8657, Japan

wise, inducible overexpression of *FCP1* results in a failure in meristem maintenance (Ohmori et al., 2013). When *FCP1* expression is strongly induced, the undifferentiated cell marker *OSH1* and the stem cell marker *FON2* are downregulated and leaf initiation is compromised. Conversely, inducible knockdown of *FCP1* and *FCP2* leads to increased expression of *OSH1* and *FON2* and expansion of the expression domains of these genes, suggesting that meristem activity is upregulated. Although it is not known whether a *WUS* ortholog functions similarly in rice, a member of the *WUSCHEL-RELATED HOMEODOMAIN* (*WOX*) gene family, *OsWOX4*, is involved in meristem maintenance (Ohmori et al., 2013). Inducible knockdown of *OsWOX4* results in downregulation of *OSH1* and *FON2*. Consistent with this observation, the expression of *OsWOX4* is negatively regulated by *FCP1*. Thus, the role of *FCP1* in meristem maintenance is relatively well understood. In the current work, we have analyzed the function of *FCP1* in other developmental processes such as leaf development and shoot regeneration.

We previously found that overexpression of *FCP1* (*FCP1-OX*) results in the regeneration of abnormal shoots from calli (Suzaki et al., 2008). To examine the role of *FCP1* in regeneration, we characterized the regeneration process in detail. Calli were induced from the scutellum and used for *Agrobacterium*-mediated transformation (Hiei et al., 1994). After selection for hygromycin resistance, calli were transferred to regeneration medium. In calli carrying the empty vector, tiny green spots were observed about 10 d after transfer, and green leaf-like

organs were subsequently observed (Fig. 1, A and B). Shoot-like structures and roots were formed after 25 and 30 d on the regeneration medium, respectively (Fig. 1, C and D). Normal shoots, which were very similar in appearance to those of seedlings, were obtained within 40 d (Fig. 1E). By contrast, regeneration was delayed in the *FCP1-OX* calli. Even after 18 d on the regeneration medium, we observed only green calli that lacked leaf-like structures (Fig. 1, F and G). Many *FCP1-OX* calli failed to form a shoot-like structure after 30 d (Fig. 1, H and I). After 40 d, shoot-like structures with malformed morphology were apparent, but no root regeneration was observed in the *FCP1-OX* calli (Fig. 1J). At this stage, we often observed abnormal calli that produced several small, leaf-like organs (Fig. 1K). The shoots that did form on *FCP1-OX* calli did not grow further and eventually died. Thus, *FCP1* overexpression disturbed shoot regeneration from callus and resulted in the production of malformed shoots.

To elucidate the effects of *FCP1* overexpression, we first made cross-sections of regenerating shoots and observed the inner tissues. Normal leaves were formed in the shoots carrying empty vector, and leaf blades and leaf sheaths were easily distinguished from one another, as in seedlings germinated from seeds (Fig. 2, A and G). The leaf blade contained small cytoplasm-dense cells, whereas the leaf sheath had larger cells that were less stained with toluidine blue. Vascular bundles were differentiated in both the leaf blade and leaf sheath, with large and small vascular bundles distinguishable in the leaf blade (Fig. 2, A and H). In the leaf sheath, sclerenchyma tis-

Fig. 1. Shoot regeneration from callus. (A–E) Calli carrying empty vector. (F–K) *FCP1-OX* calli. The number of days after transfer to the regeneration medium is indicated. Arrowheads indicate the following: red, green spot (A, F, G); blue, leaf-like structure (B, C, H, I); green, shoot (D); white, root (D). Scale bars = 1 mm in (A–D, F–K), 1 cm in (E).

Fig. 2. Effect of *FCP1* overexpression on cell differentiation and expression of marker genes. (A–C, H) Regenerating shoots carrying empty vector. (D–F, I) *FCP1*-OX regenerating shoots. (G) A shoot germinated from a seed. (A, D) Cross-section of the apex of the regenerating shoots. (B, E) *In situ* hybridization showing spatial distribution of *DL* transcripts. (C, F) *In situ* hybridization showing spatial distribution of *OsPNH* transcripts. (H, I) Close-up views of the regions indicated with squares in (A) and (D), respectively. (G) Cross-section of the shoot apex of a seedling germinated from a seed. Arrowheads and asterisks indicate vascular bundles and air spaces, respectively. Arrows indicate abnormal margins. Brackets indicate sclerenchyma tissues. LB, leaf blade; LS, leaf sheath; LV, large vascular bundle; SV, small vascular bundle. Scale bars = 100 μ m in (A–G), 50 μ m in (H, I).

sues adaxial to the vascular bundles were differentiated and air spaces were formed (Fig. 2H). By contrast, it was hard to distinguish between leaf blade and leaf sheath in the *FCP1*-OX shoots (Fig. 2D). Although vascular-like structures were sometimes observed in inner leaves, their number and tissue patterns were abnormal. In outer leaves, no vascular-like tissues were formed (Fig. 2, D and I). Sclerenchyma and air spaces were also absent (Fig. 2, D and I). In addition, the morphology of the marginal region was abnormal (Fig. 2D). Taken together, these observations showed that cell differentiation was highly compromised in the *FCP1*-OX shoots.

Next, we examined the expression patterns of two genes, *DROOPING LEAF* (*DL*) and *OsPINHEAD1* (*OsPNH1*), that are expressed in leaf primordia (Nishimura et al., 2002; Ohmori et al., 2011; Yamaguchi et al., 2004). *DL* is required for midrib formation in the leaf and is expressed in the central region of the leaf primordium

(Ohmori et al., 2011; Yamaguchi et al., 2004). In shoots carrying empty vector, *DL* transcripts were localized to the central region (Fig. 2B), as in the wild type seedlings. By contrast, *DL* transcripts were observed in the *FCP1*-OX shoots throughout the leaf primordia at earlier developmental stages (P2 and P3), but were absent in the leaf primordia at subsequent stages (Fig. 2E). Thus, the spatial and temporal expression pattern of *DL* was deregulated in the *FCP1*-OX shoots.

OsPNH1 is responsible for leaf development and meristem maintenance (Nishimura et al., 2002). In the empty-vector control shoots, *OsPNH1* was expressed in the presumptive region of vascular differentiation and the adaxial region of the leaf primordia (Fig. 2C), as previously described (Nishimura et al., 2002). By contrast, *OsPNH1* expression in the *FCP1*-OX shoots was not detected in the leaf primordia except for weak expression in a narrow domain of the leaf center (Fig. 2F). These

data suggest that overexpression of *FCP1* strongly represses *OsPNH1* expression in the regenerating shoot.

We previously showed that *FCP1* is expressed in the leaf primordia in addition to the shoot apical meristem in wild-type shoot, suggesting that *FCP1* functions in leaf development (Suzaki et al., 2008). Our current results from overexpression analysis suggest that *FCP1* plays a role in determining cell fate during leaf development. In control regenerating shoots, as in seedlings germinated from seeds, cells differentiate to produce the vascular bundle, sclerenchyma, and marginal tissues. By contrast, cellular differentiation was strongly inhibited in the *FCP1-OX* shoots. The cells in the *FCP1-OX* leaf primordia appeared largely homogeneous at a stage when the control had formed leaf sheath cells (Fig. 2, D and I). In addition, air space formation, a process involving programmed cell death in wild-type leaf sheaths (Inada et al., 2002), was absent in the *FCP1-OX* shoots.

Expression of developmental genes was also altered by *FCP1* overexpression, which caused *DL* and *OsPNH1* to be up- and downregulated throughout young leaf primordia, respectively (Fig. 2, E and F). *DL* promotes cell proliferation in the central region of the leaf primordia in normal seedlings (Ohmori et al., 2011; Yamaguchi et al., 2004). The leaf primordia were thicker in the *FCP1-OX* shoots than in the control (Fig. 2, A and D), which may result from enhanced cell proliferation caused by ectopic *DL* expression in the *FCP1-OX* shoots. In the wild-type shoot, *FCP1* is expressed throughout the leaf primordia from the P1 to P3 stage, whereas both *DL* and *OsPNH1* are expressed in specific and distinct regions from P1 to P4 (Nishimura et al., 2002; Ohmori et al., 2011; Yamaguchi et al., 2004). This spatiotemporally overlapping expression pattern between *FCP1* and *DL/OsPNH1* is consistent with the idea that *FCP1* is likely to be involved in the regulation of both *DL* and *OsPNH1* in wild type. We conclude that an appropriate level of *FCP1* expression appears to be required for normal leaf development through the function of key developmental factors regulating cell differentiation.

Differentiation of vascular tissues was compromised in *FCP1-OX* shoots. *OsPNH1* was expressed in the presumptive regions of vascular differentiation and developing vascular bundles in the control, whereas this *OsPNH1* expression was strongly repressed by *FCP1* overexpression (Fig. 2, C and F). It has been reported that antisense suppression of *OsPNH1* results in partial failure of vascular differentiation (Nishimura et al., 2002). Therefore, defects in vascular development in the *FCP1*-overexpressing shoots may be caused by the downregulation of *OsPNH1*. Alternatively, downregulation of *OsWOX4* may be associated with a failure of vascular development in the *FCP1-OX* shoots. In *Arabidopsis*, *WOX4* plays a crucial role in the maintenance of vascular stem cells (Hirakawa et al., 2010). In rice, we have

shown that *OsWOX4* is expressed in the provascular and developing vascular tissues, suggesting that *OsWOX4* is involved in vascular development as well as meristem maintenance (Ohmori et al., 2013). In addition, *OsWOX4* is negatively regulated by *FCP1* (Ohmori et al., 2013). It is therefore also plausible that the failure in vascular development in the *FCP1-OX* shoots is due to downregulation of *OsWOX4*. These two hypotheses are not mutually exclusive.

Progress in shoot regeneration was delayed in *FCP1-OX* shoots. This suggests that cell cycle progression or cell proliferation is partially compromised by *FCP1* overexpression. This delay may also be related to the disturbance of cell differentiation.

Together, our results suggest that *FCP1* is involved in the regulation of cell differentiation in leaf development, likely affecting the expression of many genes. This highlights the importance of understanding *FCP1* signaling and its downstream genetic networks in the leaf.

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