Journal of Experimental Botany, Vol. 64, No. 17, pp. 5359–5369, 2013
doi:10.1093/jxb/ert301 Advance Access publication 16 September, 2013

Journal of
Experimental
Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

A CLE–WOX signalling module regulates root meristem maintenance and vascular tissue development in rice

Huangwei Chu[1,2,*], Wanqi Liang[1,*], Juan Li[3], Fan Hong[1], Yunfei Wu[1], Likai Wang[3], Juan Wang[2,3], Ping Wu[4], Chunming Liu[5], Qifa Zhang[3], Jian Xu[2,3,†] and Dabing Zhang[1,†]

[1] School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China
[2] Department of Biological Sciences and NUS Centre for BioImaging Sciences, Faculty of Science, National University of Singapore, Singapore 117543
[3] National Key Laboratory of Crop Genetic Improvement, National Center of Plant Gene Research (Wuhan), Huazhong Agricultural University, Wuhan 430070, China
[4] School of Life Science, Zhejiang University, Hongzhou 310058, China
[5] Institute of Botany, the Chinese Academy of Sciences, Beijing 100093, China

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: zhangdb@sjtu.edu.cn or dbsxj@nus.edu.sg

Received 12 May 2013; Revised 22 July 2013; Accepted 8 August 2013

Abstract

CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR)-related (CLE) proteins belong to a small peptide family conserved in plants. Recent studies in *Arabidopsis* and rice have revealed a key role for CLEs in mediating cell–cell communication and stem cell maintenance during plant development, but how CLE signalling controls root development in the rice remains largely unknown. Here it is shown that exogenous application of a synthetic dodeca-amino acid peptide corresponding to the CLE motif of the rice FON2-LIKE CLE PROTEIN2 (FCP2p) protein or overexpression of *FCP2* terminates root apical meristem (RAM) activity and impairs late metaxylem formation. FCP2p treatment suppresses the expression of the rice *QUIESCENT-CENTER-SPECIFIC HOMEOBOX* (*QHB*) gene, a putative orthologue of *Arabidopsis WUSCHEL* (*WUS*)*-RELATED HOMEOBOX 5* (*WOX5*) gene, in both quiescent centre and late metaxylem cells; whereas inducible overexpression of *QHB* reduces the sensitivity of rice to FCP2p treatment. These results together suggest that in rice RAM maintenance and late metaxylem development are probably controlled by the mutual regulation between *FCP2* and *QHB*. Moreover, a cross-species peptide treatment experiment in *Arabidopsis* implies that FCP2 has both evolutionarily conserved and species-specific roles in root development.

Key words: *FCP2*, *QHB*, signalling, rice, root meristem, vascular tissue identity.

Introduction

In higher plants, post-embryonic organogenesis depends on the activity of meristems, which contain a pool of non-differentiated stem cells that are destined to differentiate into a diverse range of specialized cell types (Weigel and Jurgens, 2002; Laux, 2003). Stem cells in plants are maintained as a pluripotent population which are preserved in the shoot apical meristem (SAM), root apical meristem (RAM), and vascular meristem (procambium or cambium) (Simon and Stahl, 2006).

In the model dicotyledonous plant *Arabidopsis thaliana*, the maintenance of stem cell activity in the SAM is regulated by a feedback loop mechanism containing WUSCHEL (WUS) homeodomain transcription factor and CLAVATA (CLV) proteins including CLV1, CLV2, CLV3, and SOL2/CORYNE (CRN) (Clark *et al.*, 1997; Mayer *et al.*, 1998; Fletcher *et al.*, 1999; Jeong *et al.*, 1999; Casamitjana-Martinez *et al.*, 2003; Muller *et al.*, 2008). CLV3, a member of the CLV3/ENDOSPERM SURROUNDING REGION

(ESR)-related (CLE) family, is produced in and secreted from the central zone (CZ) of the SAM, and processed to a mature CLE peptide consisting of 12 amino acids most probably by a serine protease (Ni *et al.*, 2011). The secreted mature CLE peptide of CLV3 negatively regulates *WUS* expression in the organizer centre where *WUS* maintains stem cells in an undifferentiated state (Brand *et al.*, 2000; Schoof *et al.*, 2000). Likewise, *WUS-RELATED HOMEOBOX 5* (*WOX5*), a homologue of the *WUS* gene, is expressed in the quiescent centre (QC) and acts as a key regulator of the root stem cell population, indicating a similar regulatory mechanism in the root (Sarkar *et al.*, 2007; Stahl *et al.*, 2009). Consistent with this indication, CLV3 and some other CLE peptides are able to alter the root meristem activity (Casamitjana-Martinez *et al.*, 2003; Fiers *et al.*, 2005). The expression of *WOX5* in the QC and stem cell proliferation in the root, however, were found to be controlled by the CLE40 signal originated from differentiated root cap cells (Stahl *et al.*, 2009), while in the shoot, CLE signalling is derived within the stem cell area.

The identification and functional analyses of orthologues of key components of the *Arabidopsis* CLV signalling pathway in monocotyledonous crops, such as *FLORAL ORGAN NUMBER1* (*FON1*; orthologue of *CLV1*) (Suzaki *et al.*, 2004) and *FON4*/*FON2* (Chu *et al.*, 2006; Suzaki *et al.*, 2006) in rice, and *fascinated ear2* (*fea2*; orthologue of *CLV2*) (Taguchi-Shiobara *et al.*, 2001) and *thick tassel dwarf1* (*td1*; orthologue of *CLV1*) (Bommert *et al.*, 2005) in maize, have shown that a conserved regulatory mechanism might be involved in regulating the stem cell population in the SAM of monocots. A similar regulatory mechanism may also exist in the RAM of monocots, as rice seedlings treated with CLE peptides of OsCLE202 and FON2-LIKE CLE PROTEIN1 (FCP1, also named OsCLE402) showed a severe defect in the RAM (Kinoshita *et al.*, 2007; Suzaki *et al.*, 2008). The putative orthologue of *WOX5* in rice, *QUIESCENT-CENTER-SPECIFIC HOMEOBOX* (*QHB*), was found to be specifically expressed in the QC cells of the RAM (Kamiya *et al.*, 2003) and suppressed by exogenous application of an FCP1 peptide (Suzaki *et al.*, 2008). The roles of *QHB* and other *CLE* genes in the rice root, however, remain unknown.

The vasculature system in plants has dynamic and diverse cellular patterns. It consists of xylem and phloem, two differentiated conductive tissues, as well as undifferentiated cambial or procambial stem cells (Dettmer *et al.*, 2009; Lucas *et al.*, 2013). Within young tissues of shoot and root, the primary xylem and phloem are generated by the procambium, acting as the apical vascular meristem (Shininger, 1979). In the older parts of plants, the secondary phloem and xylem are formed from the cambium as a lateral vascular meristem (Sachs, 1981). Compared with the SAM and RAM, little is known about the roles of CLEs in the regulation of vascular meristem activity. Only TDIF (tracheary element differentiation inhibitory factor), a CLE-related peptide isolated from mesophyll cell culture of *Zinnia elegans*, was shown to inhibit the differentiation of cultured cells into tracheary elements (Ito *et al.*, 2006). In *Arabidopsis*, TDIF, derived from the conserved C-terminal domain of CLE41 and CLE44, is capable of promoting the proliferation of procambial cells and suppressing the differentiation of vascular stem cells into xylem through a CLV1-related leucine-rich repeat receptor kinase TDR/PXY (putative TDIF receptor) (Fisher and Turner, 2007; Hirakawa *et al.*, 2008). A *WUSCHEL-related HOMEOBOX* gene, *WOX4*, has been shown to be a key component of the TDIF signalling pathway. The expression of *WOX4*, preferentially in procambium and cambium, could be up-regulated by TDIF signalling. Genetic analysis reveals that *WOX4* is essential for promoting the proliferation of procambial cells, but not for suppressing the differentiation of vascular stem cells into xylem, suggesting that the TDIF–TDR–WOX4 signalling pathway is crucial for the maintenance of the organization of the vascular meristem during secondary growth in *Arabidopsis* (Hirakawa *et al.*, 2010). This raises the question of whether *CLE* and *WOX* genes regulate vascular tissue patterning in rice.

Here it is shown that both overexpression of *FON2-LIKE CLE PROTEIN2* (*FCP2*; also known as *CLE50*) and exogenous application of synthetic FCP2 peptide (FCP2p) suppress root elongation and reduce the RAM size in rice, indicating that *FCP2* may have a redundant function with *FCP1* in regulating RAM activities (Suzaki *et al.*, 2008). In addition, it is found that FCP2p perturbs late metaxylem formation in the procambium, suggesting that the vascular tissue and the RAM may be regulated by the same set of CLE peptides in rice. FCP2p could repress the expression of the *QHB* gene in the QC and late metaxylem in procambium; and overexpression of the *QHB* gene reduced the sensitivity of rice roots to FCP2p treatment, suggesting that CLE peptide may negatively regulate RAM activity and late metaxylem formation in rice root by suppressing the expression of *QHB*. Together, the results provide a new insight into the role of CLE–WOX signalling in RAM maintenance and vascular tissue development.

Materials and methods

Plant materials and growth conditions

Sterilized seeds of the 9522 background (*Oryza sativa* L. ssp. *Japonica*) were germinated in liquid medium containing half-strength Murashige and Skooge (MS) microelements and macroelements, and 6% sucrose at 28 °C under continuous light. *Arabidopsis* Columbia-0 (Col-0), *WOX5-GUS* (Sarkar *et al.*, 2007), *QC46*, and *ACR4-H2B-YFP* (Gifford *et al.*, 2003) were used for peptide treatment under the conditions described previously (Fiers *et al.*, 2005).

DNA construction of transgenic rice plants and rice transformation

To generate the *FCP2-overexpressing* (*FCP2-OE*) construct, an *FCP2* cDNA fragment was amplified by using the *FCP2* forward and reverse primers (Supplementary Table S1 available at *JXB* online). The PCR product was inserted in pMD18-T (TaKaRa), then this fragment, digested with *Hin*dIII and *Sac*I, was subcloned into the pHB vector at the MCS (multiple cloning site) in sense orientation downstream of the double *Cauliflower mosaic virus* (CaMV) 35S promoter (Mao *et al.*, 2005).

To obtain the *QHB-GUS* reporter construct, the 1940 bp promoter sequence of *QHB* containing a 1928 bp DNA fragment upstream of the start codon and a 12 bp encoding region was fused to a *GUS* (β-glucuronidase) reporter gene in the binary vector pBI101.3 (Clontech Laboratories, Inc., Mountain View, CA, USA).

Inducible vector pER12 was used to generate the *QHB-ind* construct. The QHB-coding region was amplified by using the QHB forward and reverse primers (Supplementary Table S1 at *JXB* online). The PCR product was digested with *Sal*I and *Spe*I, and then cloned into pER12 between the *Xho*I and *Spe*I sites (Zuo *et al.*, 2000).

These constructs were introduced into *Agrobacterium tumefaciens* EHA105 by electroporation. *Agrobacterium*-mediated transformation was performed as previously reported by Hiei *et al.* (1994).

Histochemical localization of GUS activity

Staining for GUS activity was performed according to the protocol of Schoof *et al.* (2000). The amount of time used for GUS staining is 2h for *QHB-GUS*, 8h for H729, and 4h for *WOX5-GUS* and QC46, respectively. GUS-stained samples were cleared following the method of Sabatini *et al.* (1999) and analysed using a Leica microscope equipped with Nomarski optics. For transverse observation of the localization of GUS activity, samples were embedded in 5% LMPAgarose (Promega V2111), and cut into 100 μm sections by a vibrating microtome (Leica VT1200), then analysed by using a Leica microscope equipped with Nomarski optics.

In situ *hybridization*

The *QHB*-specific probe was prepared from the full-length cDNA clone J033087F22 kindly provided by the Rice Genome Resource Center (Kikuchi *et al.*, 2003). Sense and antisense probes were transcribed *in vitro* from the T7 or T3 promoter with the respective RNA polymerases using the digoxigenin RNA-labelling kit (Roche).

Samples were fixed and embedded in Steedman's wax according to the protocol of Vitha *et al.* (2000). Microtome sections, 8mm thick, were applied to glass slides (Sigma). RNA hybridization and immunological detection of the hybridized probes were performed according to the protocol of Kouchi and Hata (1993). All slides were histochemically stained for 36h at room temperature.

BrdU incorporation immunofluorescence staining

To investigate the cell division activity of the QC, germinated seeds were cultured for 7 d, then 10mM bromodeoxyuridine (BrdU; Sigma-Aldrich) was added and the plants were incubated for a further 24h. The root tips of the plants were then excised, fixed, and embedded using Steedman's wax, and sectioned as described (Vitha *et al.*, 2000). Immunofluorescence staining was performed following the method described by Kerk and Feldman (1995) and observed with the LSM 510 laser scanning system (Zeiss).

Real-time quantitative reverse transcription–PCR (qRT–PCR)

Total RNA was isolated using Trizol reagent (Generay, Shanghai). After treatment with DNase (Promega), 0.3 μg of RNA was used to synthesize the oligo(dT)-primed first-strand cDNA using the ReverTra Ace-α-First Strand cDNA synthesis kit (TOYOBO). The real time qRT–PCR was performed according to the protocol of Yang *et al.* (2005). All the primers and Taqman probes for real-time qRT–PCR are listed in Supplementary Table S1 at *JXB* online. All samples were analysed three times. Samples were normalized using *ACTIN* expression; relative expression levels were determined compared with each other using the $2^{(-\Delta Ct)}$ analysis method.

Results

Exogenous application of FCP2p and overexpression of FCP2 *lead to reduced RAM size and root growth*

Previous studies showed that exogenous application of the synthetic peptides CLV3p, FCP1p, and OsCLE202p, corresponding to the CLE domain of CLV3, FCP1, and OsCLE202, respectively, led to a reduction of RAM size (Chu *et al.*, 2006; Kinoshita *et al.*, 2007; Suzaki *et al.*, 2008). However, transgenic rice plants in which endogenous *FCP1* gene expression was repressed by RNA interference (RNAi) did not exhibit any obvious root phenotypes when compared with the wild-type control (Suzaki *et al.*, 2008), indicating functional redundancy among rice *CLE* gene(s) in the regulation of RAM maintenance. Indeed, *in situ* hybrid analysis showed that *FCP1* and its close homologue *FCP2* were preferentially expressed in the RAM (Suzaki *et al.*, 2008), and simultaneous knock-down of *FCP1* and *FCP2* resulted in plants with no root (Ohmori *et al.*, 2013). To overcome problems of functional redundancy and reveal new *CLE* genes functioning in the rice root in a relatively high-throughput manner, 11 CLE peptides representing the CLE motifs of 13 rice CLEs and *Arabidopsis* CLV3 were synthesized (Supplementary Fig. S1 at *JXB* online), and the root phenotypes of wild-type rice seedlings grown in liquid medium containing one of these CLE peptide at the concentration of 1 μM were investigated. It was found that, among all these peptides, only CLV3p, FCP2p, and CLE15p, had an evident effect on root development (Supplementary Figs S1, S2 at *JXB* online). FCP2p severely impaired root growth, which is very similar to the impairment caused by CLV3p (Fig. 1A; Chu *et al.*, 2006) and FCP1p (Suzaki *et al.*, 2008), whereas CLE15p had a milder effect on root growth (Supplementary Fig. S2). Compared with the mock treatment, the length of the primary root of those seedlings treated with 1 μM FCP2p became significantly shorter after 4 d (Fig. 1B), suggesting that FCP2 is functional in rice root development. In agreement with this proposition, overexpression of *FCP2* in rice led to severe defects in root development (Fig. 1C, D) resembling those observed after FCP2p treatment (Fig. 1A).

Next experiments were carried out to examine how FCP2p treatment and *FCP2* overexpression interfered with root growth in rice. It was found that the size of the RAM decreased progressively after 1–3 d treatment with 1 μM FCP2p (Fig. 1F1–F3, compared with Fig. 1E). Moreover, when a higher concentration of FCP2p (10 μM) was applied, further reduction of the RAM size was observed after a 2 d treatment, which was comparable with that seen witha 3 d treatment with 1 μM FCP2p (Fig. 1G1–G2 compared with Fig. 1F3). These results suggest that FCP2p abolishes the activity of the RAM in a dosage-dependent manner. Consistently, a reduction of RAM size was also observed in *FCP2-OE* transgenic lines (Fig. 1I compared with Fig. 1H).

Exogenous application of FCP2p and overexpression of FCP2 *lead to defects in QC and stem cell maintenance*

A reduced RAM size has been reported in *Arabidopsis* mutants with defects in the QC (Ortega-Martinez *et al.*, 2007), which is mitotically inactive and has the ability to inhibit the differentiation of surrounding stem cells (van den

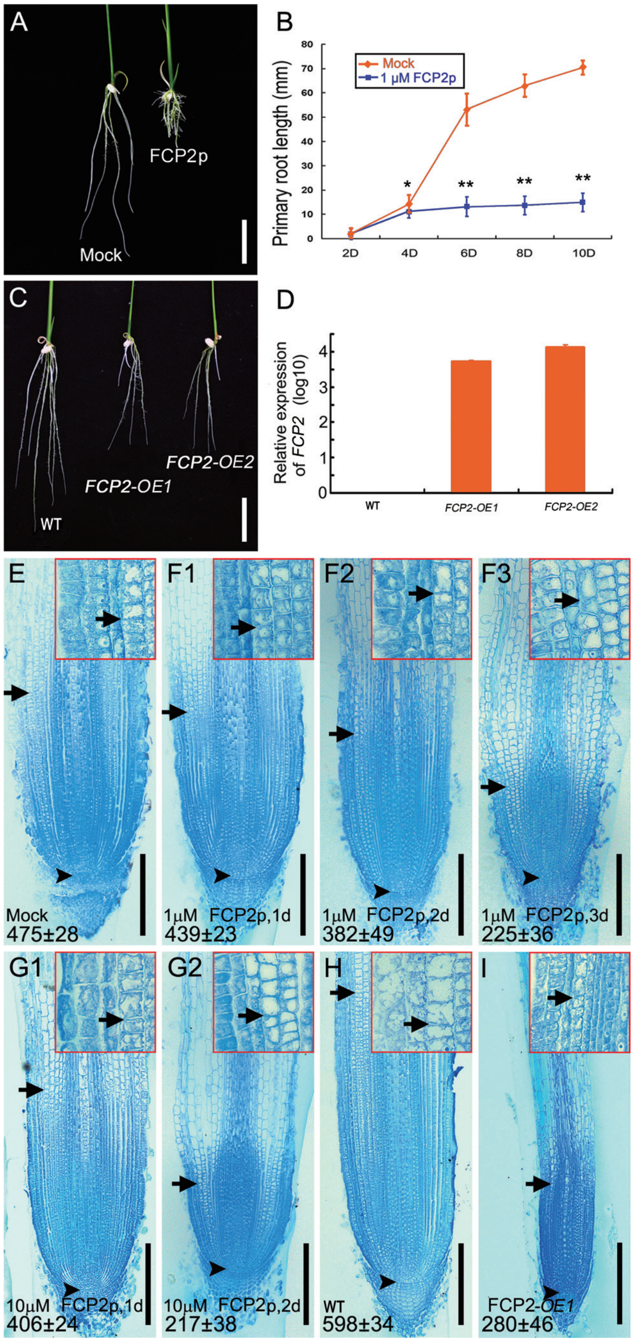

Fig. 1. Rice root phenotypes caused by exogenous application of FCP2p and overexpression of *FCP2*. (A) Root phenotypes of 10-day-old wild-type (WT) seedlings treated without (left) or with (right) 1 μM FCP2p. (B) Primary root lengths of WT seedlings treated with or without 1 μM FCP2p and analysed over time. Graph values are means ±SD, *n*=12; **t*-test *P*-value < 0.05; ***t*-test *P*-value < 0.01. (C) Root phenotypes of 10-day-old WT (left) and *FCP2-OE* plants (T_1 lines, middle and right). (D) qRT–PCR analysis of *FCP2* in roots of two independent lines *FCP2-OE1* and *FCP2-OE2* (T_1 lines). The relative expression levels were quantified as fold changes normalized to the WT *FCP2* transcription level, followed by log10 transformation. (E) Longitudinal view of a RAM of a 10-day-old mock-treated WT plant. (F1–F3) Longitudinal views of RAMs of WT plants treated with 1 μM FCP2p for 1 d (F1), 2 d (F2), and 3 d (F3). (G1 and G2) Longitudinal views of the RAM of WT plants treated with 10 μM FCP2p for 1 d (G1) and 2 d (G2). (H and I) Longitudinal views of RAMs of 10-day-old WT (H) and *FCP2-OE* (I) plants. A and C: bar=2 cm; E–I: bar=200 μm. Arrows and arrowheads in E–I indicate the transition zone (TZ) of the mesodermis (me) and the QC, respectively. Insets are an enlargement of the TZs. Numbers in E–I represent the distance between the TZ and QC (μm), values are means ±SD, *n*=6.

Berg *et al.*, 1997). It was thus reasoned that FCP2p application and *FCP2* overexpression might cause defects in QC specification and loss of stem cell activity in rice roots, and as a result the consumption of RAM. To test this hypothesis, a BrdU incorporation assay was performed in rice roots to monitor the mitotic activity of cells in the root tip (Kerk and Feldman, 1995). It was found that no immunofluorescent signals could be detected in the QC and surrounding stem cells in mock-treated wild-type roots (Fig. 2A). In contrast, strong immunofluorescent signals were observed in the QC and surrounding cells in rice seedlings treated with 10 μM FCP2p for 1 d (Fig. 2B) and in transgenic rice plants constitutively overexpressing the *FCP2* gene (Fig. 2C). This result suggests that both overexpression of *FCP2* and short-term exposure to excess FCP2p alter the mitotic activity and identity of the QC and stem cells.

FCP2p negatively regulates QHB *expression in QC and late metaxylem cells*

It was next asked how the FCP2p treatment and *FCP2* overexpression influenced QC and stem cell maintenance in rice. It was reasoned that *WOX* gene(s) might be involved, and rice transgenic lines (*QHB-GUS*) carrying a *QHB* promoter–*GUS* fusion were generated. *QHB* was previously described to be specifically expressed in rice QC cells (Kamiya *et al.*, 2003), and was shown to be down-regulated by application of FCP1p (Suzaki *et al.*, 2008). Unexpectedly, additional GUS expression signal was detected in late metaxylem cells of the root vascular bundle in the transgenic rice lines (Fig. 3A1–A4) and was confirmed by *in situ* hybridization (Fig. 3A1′–A4′), suggesting a role for *QHB* in late metaxylem cells.

In wild-type and *QHB-GUS* plants treated with 10 μM FCP2p for 1 d, *QHB* transcript and GUS signal became greatly reduced in the late metaxylem and QC cells (Fig. 3B1–B4, 3B1′–B4′) compared with the mock-treated controls (Fig. 3A1–A4, 3A1′–A4′). After a 2 d treatment with 10 μM FCP2p, the *QHB* expression was almost undetectable (Fig. 3C1–C4, 3C1′–C4′), suggesting that *FCP2* negatively regulates *QHB* expression. Notably, ectopic GUS staining and hybridization signals were observed in cells proximal to the QC after a 1 d treatment (Fig. 3B1, B4, B1′, B4′), indicating defects in QC identity.

FCP2p mis-specifies the late metaxylem identity

Late metaxylem cells in wild-type rice roots exhibit a cell morphology and anticlinal division pattern distinct from

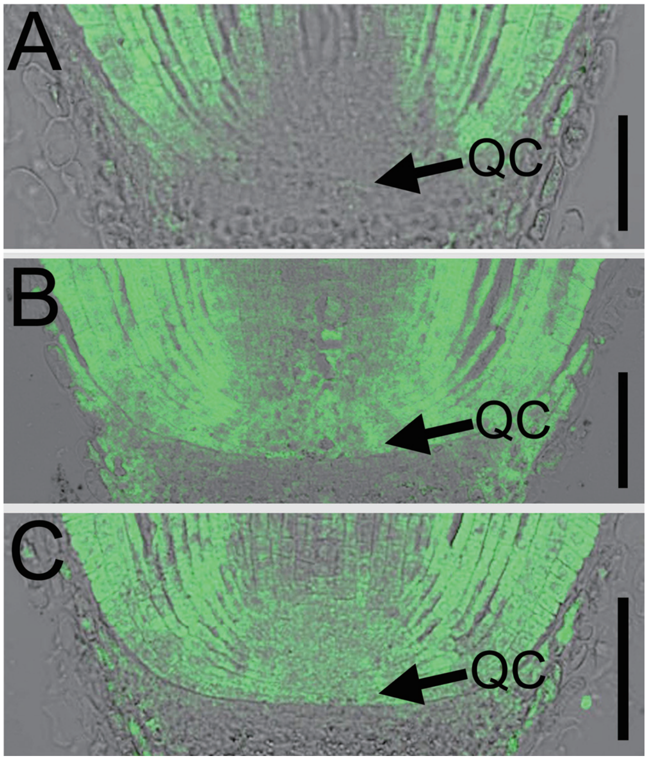

Fig. 2. BrdU incorporation assay of mitotic activity in the QC region. (A) BrdU incorporation assay in mock-treated wild-type (WT) roots. No obvious BrdU signals were detected in the region of the QC and surrounding stem cells. (B) BrdU incorporation assay in WT roots treated with 10 μM FCP2p for 1 d. BrdU signals were detectable in the QC and surrounding stem cells, indicating altered mitotic activity. (C) BrdU incorporation assay in *FCP2-OE* transgenic line. BrdU signals were detectable in the QC and surrounding stem cells, showing similar mitotic activity to that of (B). Bar = 50 μm.

that of surrounding cells (Fig. 3A2, A3, A2′, A3′) (Kawata *et al.*, 1978; Scarpella *et al.*, 2000). When wild-type plants were treated with 10 μM FCP2p for 2 d, aberrant cell divisions were observed in the cells at the position of late metaxylem adjacent to the QC (Fig. 3C3, C3′). It was suspected that these aberrant divisions might be because of mis-specification of cell identity at the position of late metaxylem. To explore this, T-DNA enhancer trap lines from the Rice Mutant Database (RMD, http://www.ncpgr.cn/web/detail.jsp?i_=420) were screened for QC- and vascular-specific marker lines (Zhang *et al.*, 2006). Despite no marker line for the QC being obtained, an enhancer trap line, H729, with early metaxylem-specific *GUS* expression was identified (Fig. 4A, D, E). This line was treated with 10 μM FCP2p and the GUS expression pattern was analysed after 1–2 d treatments. In agreement with the assumption, ectopic GUS staining signal was observed in the cells at the position of late metaxylem adjacent to the QC (Fig. 4B, F), and early metaxylem-like cell divisions could be found in these cells when exposed to FCP2p for 2 d (Fig. 4C, G). Taken together, these results strongly suggest that the FCP2 peptide plays a key role in regulating metaxylem development in rice roots.

Inducible overexpression of QHB *reduced the sensitivity of rice roots to FCP2p treatment*

Previous studies on *Arabidopsis* showed that stem cell homeostasis in the SAM is maintained through a CLV–WUS negative feedback loop (Brand *et al.*, 2000; Schoof *et al.*, 2000). Therefore experiments were carried out to determine whether a similar feedback loop operated in rice root development. Using an oestrogen receptor-based chemical-inducible system (Zuo *et al.*, 2000), it was investigated whether inducible overexpression of *QHB* could rescue the RAM defects caused by exogenous FCP2p application.

No obvious difference was observed between wild-type and *QHB*-inducible (*QHB-ind*) lines grown on the non-inductive medium, and, as expected, reduction of RAM size and defects of vascular tissue patterning became evident in these *QHB-ind* lines when treated with 10 μM FCP2p for 2 d (Fig. 5A1–A3). In contrast, when the *QHB-ind* lines were treated with 20 μM 17-β-oestradiol for 12 h, and then transferred to a medium containing both 20 μM 17-β-oestradiol and 10 μM FCP2p, an apparently functional RAM remained after 2 d (Fig. 5B1–B3), and even after prolonged incubation (Fig. 5D). In contrast, the RAM of *QHB-ind* lines treated solely with 10 μM FCP2p was completely consumed and lateral root primordia appeared near the root tip (Fig. 5C). Moreover, the BrdU incorporation assay indicated that *QHB-ind* lines grown on the medium with 20 μM 17-β-oestradiol had an expanded QC and surrounding stem cell areas (Fig. 5F) as compared with the wild type (Fig. 2A) and *QHB-ind* lines grown on non-inductive media (Fig. 5E). These observations suggest that overexpression of *QHB* could repress the differentiation of stem cells surrounding the QC. In addition, unlike the detectable mitotic activity which appeared in the wild-type QC after 24 h treatment with 10 μM FCP2p (Fig. 2B), no BrdU signal was observed in the QC of the *QHB-ind* lines induced with 20 μM 17-β-oestradiol for 12 h, and followed by an additional 24 h treatment with 20 μM 17-β-oestradiol plus 10 μM FCP2p (Fig. 5G), suggesting the existence of a functional QC. Consistently, the time course analysis showed that FCP2p-induced suppression of root growth of *QHB-ind* plants was reduced in the presence of 17-β-oestradiol (Fig. 5H).Notably, real-time qRT–PCR analysis showed that an increase in *FCP2* transcription in rice roots was associated with the induction of *QHB*. In *QHB-ind* lines treated with 20 μM 17-β-oestradiol, *QHB* transcription gradually increased and peaked at 36 h after treatment, whereas elevated expression of *FCP2* was observed from 24 h after *QHB* induction (Fig. 6). Thus, these results suggest that a negative feedback regulation between *QHB* and *FCP2* may exist and regulate RAM activity and vascular tissue identity in rice roots.

FCP2p exerts evolutionarily conserved and species-specific roles in Arabidopsis roots

In *Arabidopsis*, overexpression of *CLV3*, *CLE19*, and *CLE40*, and exogenous application of the corresponding CLE peptides, has been shown to trigger the consumption of

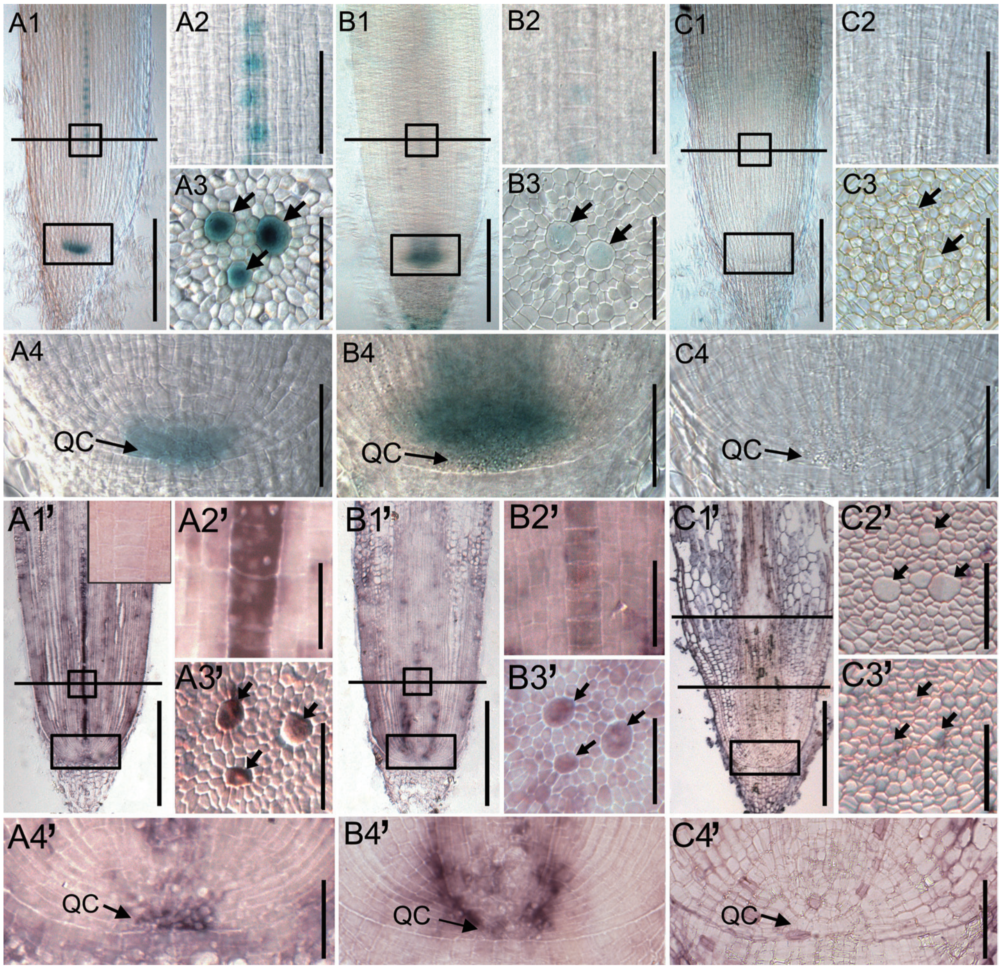

Fig. 3. FCP2p negatively regulates *QHB* expression in rice roots. (A1–A4) GUS expression in the mock-treated *QHB-GUS* line. (A2 and A4) Enlargements of the upper and lower boxed regions in A1, showing the GUS signal in the late metaxylem and QC cells, respectively. (A3) Transverse section at the position indicated by the black line in A1, showing the GUS signal in late metaxylem cells. (B1–B4) GUS expression in the *QHB-GUS* line treated with 10 μM FCP2p for 1 d. (B2 and B4) Enlargements of the upper and lower boxed regions in B1, showing decreased GUS signal in late metaxylem cells (B2), and a diffuse GUS signal around the QC (B4) compared with the mock-treated line. (B3) Transverse section at the position indicated by the black line in B1, showing decreased GUS signal. (C1–C4) GUS expression in the *QHB-GUS* line treated with 10 μM FCP2p for 2 d. No obvious GUS signal was detected in the root tip. (C2 and C4) Enlargements of the upper and lower boxed regions in C1, respectively. (C3) Transverse section at the position indicated by the black line in C1. Aberrant periclinal cell divisions at the position of late metaxylem cells were observed in C3. (A1′–A4′) *In situ* analysis of *QHB* expression in mock-treated rice root using an antisense cDNA probe of *QHB*. (A2′ and A4′) Enlargements of the upper and lower boxed regions in A1′, showing *QHB* transcripts in late metaxylem and QC cells, respectively. (A3′) Transverse section at the position indicated by the black line in A1′, showing *QHB* transcripts in late metaxylem cells. The inset in A1 is the sense probe control which had no detectable signal in late metaxylem cells. (B1′–B4′) *In situ* analysis of *QHB* in rice root treated with 10 μM FCP2p for 1 d using an antisense cDNA probe of *QHB*. (B2′ and B4′) Enlargements of the upper and lower boxed regions in B1′, showing a decreased expression level of *QHB* in late metaxylem cells (B2′) and diffuse expression of *QHB* around the QC (B4′) compared with the mock-treated control. (B3′) Transverse section at the position indicated by the black line in B1′. Note that *QHB* transcripts were decreased compared with the mock-treated control. (C1′–C4′) *In situ* analysis of *QHB* expression in rice root treated with 10 μM FCP2p for 2 d using an antisense cDNA probe of *QHB*. No obvious signal was detected. (C2′ and C4′) Enlargements of the upper and lower boxed regions in C1′, respectively. (C3′) Transverse section at the position indicated by the black line in C1′. Aberrant periclinal cell divisions at the position of late metaxylem cells were observed in (C3′). A1, B1, C1, A1′, B1′, and C1′: bar=200 μm; A2–A4, B2–B4, C2–C4, A2′–A4′, B2′–B4′, and C2′–C4′: bar=50 μm. Seven-day-old seedlings with different periods of peptide treatment were examined. Arrows in A3, B3, C3, A3′, B3′, C2′, and C3′ indicate late metaxylem cells or cells at the positions of late metaxylem cells.

the RAM (Casamitjana-Martinez *et al.*, 2003; Hobe *et al.*, 2003; Fiers *et al.*, 2004, 2005); and Kinoshita *et al.* (2007) found that among 26 *Arabidopsis* CLE peptides examined, 19 had an inhibitory effect on the rice RAM (Kinoshita *et al.*, 2007), suggesting that the molecular mechanism underlying the regulation of RAM maintenance is conserved between monocot and dicot species. Consistent with this notion, *Arabidopsis* CLE peptide CLV3p is able to trigger meristem

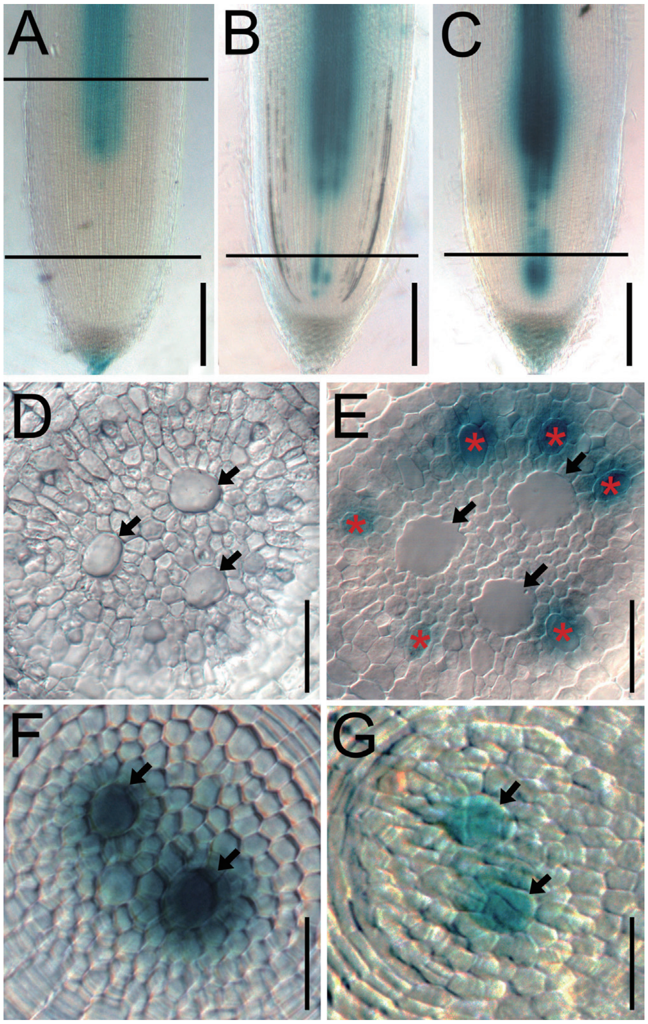

Fig. 4. Effect of exogenously applied FCP2p on the enhancer trap line H729. (A) GUS signals in the vascular tissue of mock-treated H729 root. (B and C) GUS expression in H729 treated with 10 µM FCP2p for 1 d (B) and 2 d (C), respectively. Note that ectopic GUS expression appeared in the procabium of the vascular tissue. (D and E) Transverse sections at the positions indicated by the lower and upper lines in A, respectively. Red asterisks indicate GUS expression in the early metaxylem cells. Black arrows indicate the late metaxylem cells, showing no detectable GUS signal in this region. (F and G) Transverse sections at the positions indicated by the lines in B and C, respectively, showing ectopic GUS expression at the position of late metaxylem cells. A–C: bar=200 µm; D–G: bar=20 µm. Seven-day-old seedlings with different periods of peptide treatment were examined. Arrows in D–G indicate late metaxylem cells or cells at the positions of late metaxylem cells.

termination, as reported by Kinoshita *et al.* (2007), and vascular defects in rice roots, which resembled those caused by FCP2p application (Supplementary Fig. S3 at *JXB* online), whereas FCP2p application led to the consumption of the RAM in *Arabidopsis* (Fig. 7A, B) and suppressed the QC-specific expression of *WOX5-GUS* and QC46 (Fig. 7C, D; Sarkar *et al.*, 2007). Compared with the mock control, cells at the QC position became enlarged after FCP2p treatment (Fig. 7C, D), resembling those of *wox5* mutants (Sarkar *et al.*, 2007), and ectopic starch granule accumulation was observed in columella stem cells (Fig. 7C, D), indicating loss of QC and stem cell activities in FCP2p-treated roots. The expression of *ACR4*, encoding a receptor-like kinase of the CRINKLY4 family (Tanaka *et al.*, 2002), was increased in the QC and distal cells after FCP2p treatment, which was similar to the case of CLE40p treatment (Stahl *et al.*, 2009) (Fig. 7E). Taken together, the results provide strong evidence for an evolutionarily conserved role for CLE signalling in root development.

It was next asked whether FCP2p could regulate xylem differentiation in *Arabidopsis*. Vascular morphological changes were monitored in *Arabidopsis* seedlings treated with FCP2p, CLV3p, or TDIF. TDIF affected xylem differentiation in the leaf (Supplementary Fig. S4A, B at *JXB* online), as reported previously (Hirakawa *et al.*, 2008; Whitford *et al.*, 2008), but FCP2p and CLV3p did not (data not shown), suggesting a unique role for TDIF in leaf vascular development. Interestingly, none of these three peptides caused visible phenotypic changes in protoxylem and metaxylem cells in *Arabidopsis* roots (Supplementary Fig. S4D–F compared with the mock-treated wild type in Supplementary Fig. S4C), indicating that the regulatory mechanism controlling root xylem development in *Arabidopsis* is probably distinct from that of rice.

Discussion

CLE genes have been found from a variety of dicot and monocot plants such as *Arabidopsis*, soybean, rice, wheat, and maize (Cock and McCormick, 2001). In *Arabidopsis*, *CLE* genes have been categorized into A-type and B-type classes based on their ability to promote terminal differentiation of the SAM and RAM. Overexpression of A-type *CLE* genes or exogenous application of A-type CLE peptides triggers consumption of the RAM and SAM, whereas B-type *CLE* genes such as those encoding TDIF (*CLE41* and *CLE44*) are known to promote procambial cell proliferation while suppressing xylem differentiation (Whitford *et al.*, 2008). A synergistic effect of A-type and B-type CLE peptides on vascular development was also observed (Whitford *et al.*, 2008), but the underlying mechanism was not understood. Here, it is shown that overexpression of the rice *FCP2* gene and exogenous application of FCP2p or *Arabidopsis* CLV3p suppresses the RAM activity in both rice and *Arabidopsis*, and that FCP2p or CLV3p influences late metaxylem development in rice roots, but not in *Arabidopsis* roots. The observations suggest that *CLE* genes have evolutionarily conserved and species-specific roles in root development, and indicate that *FCP2* and probably other rice *CLE* genes have a dual function in rice roots, regulating both meristem activity and vascular cell identity.

Both *FCP1* and *FCP2* were previously shown to be expressed in the rice root tip, and exogenous application of FCP1p could also cause patterns of changes in root morphology and expression of *QHB* similar to those seen with FCP2p

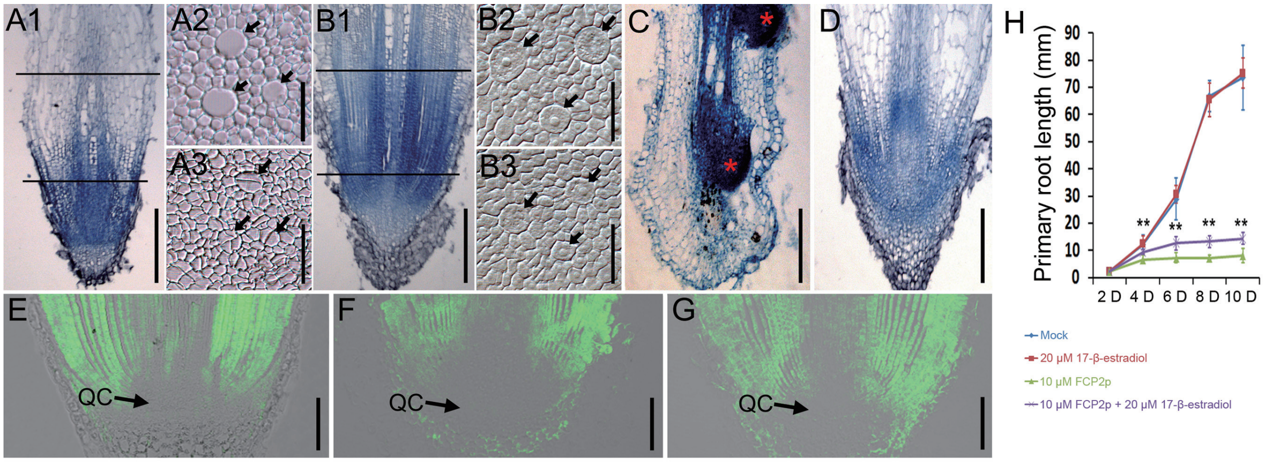

Fig. 5. Ectopic expression of *QHB* rescues the defects caused by exogenously applied FCP2p. (A1–A3) Root tip of a 7-day-old *QHB-ind* line treated with 10 μM FCP2p for 2 d. (A2, A3) Transverse sections at the positions indicated by the upper and lower lines in A1, respectively. (B1–B3) Root tip of a 7-day-old *QHB-ind* line treated with 20 μM 17-β-oestradiol for 12 h, and then treated with 10 μM FCP2p for 2 d in the medium containing 20 μM 17-β-oestradiol. (B2, B3) Transverse sections at the positions indicated by the upper and lower lines in B1, respectively. (C) Root tip of a 7-day-old *QHB-ind* line treated with 10 μM FCP2p for 4 d. Red asterisks indicate lateral root primordia which appeared near the root tip. (D) Root tip of the 7-day-old *QHB-ind* line treated with 20 μM 17-β-oestradiol for 12 h, and then treated with 10 μM FCP2p and 20 μM 17-β-oestradiol for 4 d. (E) BrdU incorporation assay in the mock-treated *QHB-ind* line. (F) BrdU incorporation assay in the *QHB-ind* line treated with 20 μM 17-β-oestradiol for 12 h. (G) BrdU incorporation assay in the *QHB-ind* line treated with 20 μM 17-β-oestradiol for 12 h, and then treated with 10 μM FCP2p and 20 μM 17-β-oestradiol for 1 d. (H) Time course analysis of primary root lengths of *QHB-ind* seedlings grown in the indicated conditions. Graph values are means ±SD, *n*=12. ***t*-test, *P*-value <0.01. A1, B1, C, and D: bar=200 μm; A2, A3, B2, and B3: bar=20 μm, E–G: bar=40 μm. Arrows in A2, A3, B2, B3) indicate late metaxylem cells or cells at the positions of late metaxylem cells.

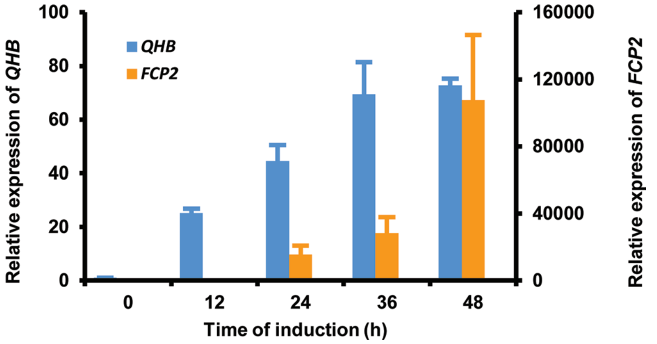

Fig. 6. Expression analyses of *QHB* and *FCP2* in *QHB-ind* lines treated with 20 μM 17-β-oestradiol. The relative expression levels of *QHB* and *FCP2* were quantified as fold changes normalized to those of the WT, respectively. qRT–PCR analysis showed that the *QHB* transcript was increased in *QHB-ind* lines at 12 h after induction by 20 μM 17-β-oestradiol, and peaked at 36 h after induction. An increased expression of *FCP2* was observed after 24 h of induction.

(Suzaki *et al.*, 2008), suggesting that *FCP1* and *FCP2* are functionally redundant. In agreement with this, repression of the endogenous gene activity of *FCP1* (Suzaki *et al.*, 2008) or *FCP2* (this study, data not shown) by RNAi led to no obvious defect in rice roots. When both *FCP1* and *FCP2* were downregulated, either constitutively or inducibily, however, no transgenic plants could be obtained from calli (Suzaki *et al.*, 2008) or no roots could be formed after induction (Ohmori *et al.*, 2013), further supporting the notion that *FCP1* and *FCP2* have redundant functions in the rice root.

While a better understanding on the *in planta* functions of CLEs has long been hindered due to the lack of loss-of-function mutants or analysable knockdown or knockout phenotypes, the use of synthetic CLE peptides and gain-of-function studies have proved to be extremely useful for determining the regulatory relationships among the components of CLE signalling. Using these approaches, it is shown that exogenous application FCP2p eliminates the expression of *QHB*, whereas overexpression of *QHB* is sufficient to induce *FCP2* transcription and stem cell fate, and reduce the defects caused by FCP2p application. Together, these data allow a potential negative feedback loop mechanism to be proposed that fine-tunes the expression level and distribution of *FCP2* and *QHB*, and regulates RAM activity and root vascular tissue identity in rice.

Interestingly, similar to CLE40 (Stahl *et al.*, 2009), FCP2 is also expressed in the columella root cap cells (Suzaki *et al.*, 2008). FCP2p appeared to induce changes in *QHB* expression in the QC of rice roots (Fig. 3C4, C4′) and in *WOX5* expression in the QC of *Arabidopsis* roots (Fig. 7C), similar to those induced by CLE40p in *WOX5* expression in *Arabidopsis* (Stahl *et al.*, 2009). These similarities indicate that RAM activity in rice and *Arabidopsis* is controlled by parallel CLE–WOX signalling modules.

The findings presented here, together with previous reports on TDIF–WOX4 signalling in *Arabidopsis* (Hirakawa *et al.*, 2008), strongly suggest that in both *Arabidopsis* and rice,

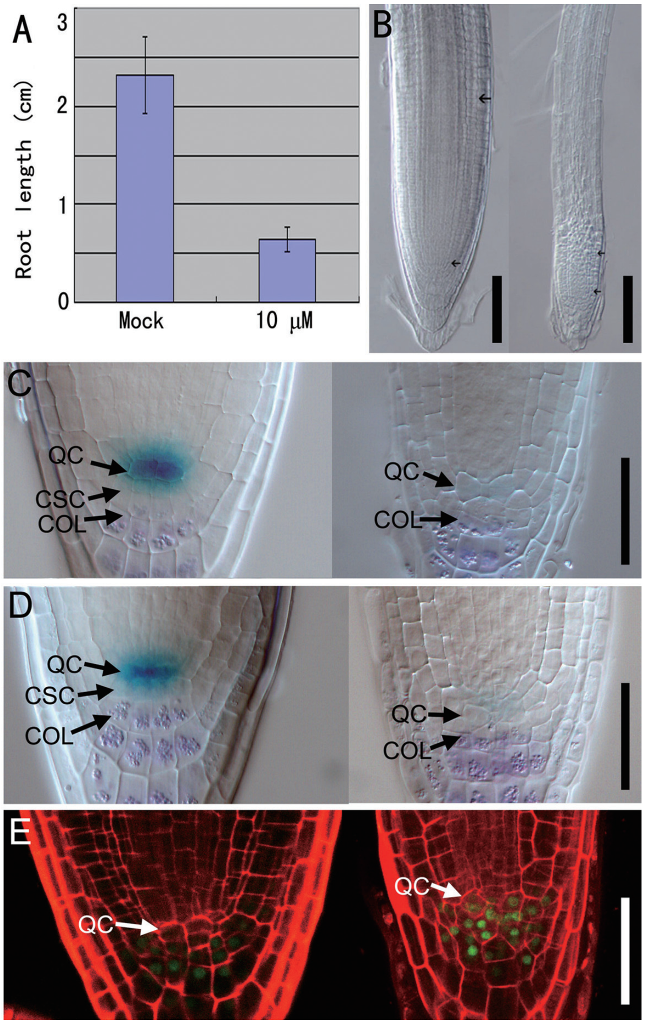

Fig. 7. Effect of FCP2p on the RAM of *Arabidopsis*. (A) Root lengths of 7-day-old seedlings treated with (right) or without (left) 10 µM FCP2p. (B) DIC images of roots treated with (right) or without (left) 10 µM FCP2p. Arrows indicate the distal and proximal ends of the RAM. (C) *WOX5-GUS* expression in roots treated with (right) or without (left) 10 µM FCP2p for 7 d. (D) *QC46* expression in roots treated with (right) or without (left) 10 µM FCP2p for 7 d. (E) *ACR4-H2B-YFP* expression in roots treated with (right) or without (left) 10 µM FCP2p for 7 d. Violet colour in C and D indicates starch granules visualized by lugol staining, indicating differentiated columella root cap cells. B: bar=100 µm; C–E bar=50 µm. QC, quiescent centre; CSC, columella stem cell; COL, columella.

vascular development is controlled by signalling through CLEs/WOXs. TDIF appears to act through increasing the expression of *WOX4* to promote the proliferation of procambial/cambial cells but suppressing their differentiation into xylem in a WOX4-independent manner (Hirakawa *et al.*, 2010), whereas FCP2p exerts its inhibitory effects on late metaxylem differentiation through repressing the expression of *QHB*, suggesting that the regulation of CLEs on WOXs is complex and associated with different stages of vascular development. Future studies will be important to reveal whether other CLE–WOX signalling modules in rice roots (if any) are able to induce the proliferation of different types of vascular cells such as procambium and protoxylem. These should shed more light on the factors and signalling pathways that establish and maintain vascular tissue identity, as well as on the mechanism shared by vascular and meristem development.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Amino acid sequences of 11 CLE peptides and their effects on RAM.

Figure S2. Root phenotypes caused by *in vitro* application of CLE peptides.

Figure S3. Root phenotypes caused by treatment with 10 µM CLV3p.

Figure S4. Effects of CLE peptides on vascular development in *Arabidopsis*.

Table S1. Primers and TaqMan probes used in this study

Acknowledgements

We thank Hongquan Yang, Nam-Hai Chua, and Bo Zhou for providing and modifying pHB and pER12 vectors, respectively. We also thank the Rice Genome Resource Center (Japan) for providing the rice *QHB* cDNA clone, and Zhijin Luo and Mingjiao Chen for growing rice plants. This work was funded by the National Key Basic Research Developments Program of the Ministry of Science and Technology, PR China (2009CB941500), National Natural Science Foundation of China (30725022, 90717109, and 30600347), Shanghai Leading Academic Discipline Project (B205), Huazhong Agricultural University, and the National Key Laboratory of Crop Genetic Improvement; and was supported by the AcRF Tier 2 grant (MOE2009-T2-1–060) from the Ministry of Education of Singapore and by the National Research Foundation Singapore under its Competitive Research Programme (CRP Award no. NRF2010NRF-CRP002-018).

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