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

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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) (Suzuki et al., 2004) and FON4/FON2 (Chu et al., 2006; Suzuki et al., 2006) in rice, and fascinated ear2 (feu2; orthologue of CLV2) (Taguchi-Shiobara et al., 2001) and thick tassel dwarf1 (tdl; 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; Suzuki 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 (Suzuki 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 (Shiminger, 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 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 (Suzuki 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 9322 background (Oryza sativa L. ssp. Japonica) were germinated in liquid medium containing half-strength Murashige and Skoog (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 HindIII and SacI, 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 SotII and SpeI, and then cloned into pER12 between the XhoI and SpeI 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 2 h for QHB-GUS, 8 h for H272, and 4 h 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% LMP agarose (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 J03087F22 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, 8 mm 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 36 h at room temperature.

BrdU incorporation immunofluorescence staining
To investigate the cell division activity of the QC, germinated seeds were cultured for 7 d, then 10 mM bromodeoxyuridine (BrdU; Sigma-Aldrich) was added and the plants were incubated for a further 24 h. 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). 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^(-ΔCt) 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; Suzuki 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 (Suzuki 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 (Suzuki 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 (Suzuki 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 within a 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
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...
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?id=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.

**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.

**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 17β-oestradiol for 12 h, and then transferred to a medium containing both 20 µM 17β-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 17β-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 17β-oestradiol and 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 17β-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 17β-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
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. **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 down-regulated, either constitutively or inducibly, 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 (Suzuki et al., 2008). FCP2p appeared to induce changes in QHB expression in the QC of rice roots (Fig. 3C4, C′) 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,
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

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**References**


