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Rapid Paper

Conservation and Diversification of Meristem Maintenance Mechanism in *Oryza sativa*: Function of the *FLORAL ORGAN NUMBER2* Gene

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To elucidate the genetic mechanism that regulates meristem maintenance in monocots, here we have examined the function of the gene *FLORAL ORGAN NUMBER2* (*FON2*) in *Oryza sativa* (rice). Mutations in *FON2* cause enlargement of the floral meristem, resulting in an increase in the number of floral organs, although the vegetative and inflorescence meristems are largely normal. Molecular cloning reveals that *FON2* encodes a small secreted protein, containing a CLE domain, that is closely related to CLAVATA3 in *Arabidopsis thaliana*. *FON2* transcripts are localized at the apical region in all meristems in the aerial parts of rice plants, showing an expression pattern similar to that of *Arabidopsis CLV3*. Constitutive expression of *FON2* causes a reduction in the number of floral organs and flowers, suggesting that both the flower and inflorescence meristems are reduced in size. This action of *FON2* requires the function of *FON1*, an ortholog of *CLV1*. Constitutive expression of *FON2* also causes premature termination of the shoot apical meristem in *Arabidopsis*, a phenotype similar to that caused by constitutive expression of *CLV3*. Together with our previous study of *FON1*, these results clearly indicate that the FON1–FON2 system in rice corresponds to the CLV signaling system in *Arabidopsis* and suggest that the negative regulation of stem cell identity by these systems may be principally conserved in a wide range of plants within the Angiosperms. In addition, we propose a model of the genetic regulation of meristem maintenance in rice that includes an alternative pathway independent of FON2–FON1.

Keywords: CLAVATA signaling pathway — CLE domain — *FLORAL ORGAN NUMBER* (*FON*) gene — Meristem maintenance — *Oryza sativa* (rice).

Abbreviations: CaMV, cauliflower mosaic virus; LRR, leucine-rich repeat.

Nucleotide sequence data of the *FON2* cDNA have been deposited in the DDBJ/EMBL/GenBank database under accession number AB245090.

Introduction

Plant architecture and the formation of lateral organs depend on the function of the meristem that is produced in the embryo. The importance of post-embryonic development from the meristems is a characteristic feature of plant development that is distinct from animal development. The genetic mechanism that regulates meristem maintenance is well understood in *Arabidopsis thaliana*; however, it is largely unknown whether the genetic mechanism of meristem maintenance deduced from *Arabidopsis* research is conserved in a wide range of plants. In the meristems that regulate vegetative and reproductive development in the aerial parts of plants, two activities are carefully balanced in order to maintain meristem activity and continuously to form lateral organs. The first activity is the maintenance of undifferentiated stem cells at the summit of the central zone. The second is the recruitment of their descendants as precursor cells for the initiation of organ primordia in the peripheral zone (for a review, see Steeves and Sussex 1989).

Molecular genetic studies have revealed key regulators that control meristem maintenance in *Arabidopsis* (for reviews, see Carles and Fletcher 2003, Gross-Hardt and Laux 2003). Stem cell population is positively regulated by *WUSCHEL* (*WUS*), which encodes a homeodomain protein belonging to the WOX family and is expressed in a small region underneath the stem cells termed the 'organizing center' (Mayer et al. 1998, Schoof et al. 2000, Haecker et al. 2004). *WUS* activity is negatively regulated by the CLAVATA (CLV) signaling pathway. Mutations in the *CLAVATA1* (*CLV1*), *CLV2* or *CLV3* gene cause enlargement of the meristems of both the vegetative and reproductive phases, a phenotype opposite to that of the *wus* mutant (Clark et al. 1993, Clark et al. 1995, Kayes and Clark 1998). *CLV1* encodes a leucine-rich repeat (LRR) receptor-like kinase, and *CLV2* encodes a protein that is structurally similar to CLV1 but lacks the intracellular

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kinase domain (Clark et al. 1997, Jeong et al. 1999). *CLV3* encodes a small protein and is thought to be a putative ligand for the CLV1–CLV2 receptor complex (Fletcher et al. 1999, Lenhard and Laux 2003). *CLV3* is expressed in the L1 and L2 cell layers and in a few underlying L3 cells, which correspond to the stem cell region (Fletcher et al. 1999). Whereas *CLV2* is expressed ubiquitously, *CLV1* is expressed mainly in the L3 cells that encompass the organizing center, i.e. the *WUS* expression domain (Clark et al. 1997, Jeong et al. 1999). Mutations in the *CLV* genes fail to repress *WUS* activity, resulting in expansion of the *WUS* expression domain (Schoof et al. 2000). In addition, ectopic expression of *CLV3* causes premature termination of the shoot apical meristem, a phenotype similar to that of the *wus* mutant (Brand et al. 2000). Conversely, ectopic *WUS* expression causes expansion of the *CLV3* expression domain, resulting in enlargement of the stem cell region (Schoof et al. 2000). These findings have led to a model of meristem maintenance in which stem cell accumulation is regulated by a negative feedback loop between *CLV3* and *WUS* that communicates between the stem cells and the organizing center (Brand et al. 2000, Schoof et al. 2000). Excellent studies on the inducible expression of *CLV3* have recently indicated the dynamic control of cell fate specification and cell division in the meristem (Reddy and Meyerowitz 2005, Müller et al. 2006).

Genes homologous to *CLV3* constitute a gene family called the CLE family after *CLV3* in *Arabidopsis* and its relative *Esr* in maize (Opsahl-Ferstad et al. 1997, Fletcher et al. 1999). *CLE1* and *CLE4* are capable of replacing CLV3 function in meristem maintenance in *Arabidopsis* (Ni and Clark 2006, Strabala et al. 2006). Some *CLE* genes play essential roles in plant development, such as xylem cell differentiation and root development, probably by producing signal molecules that act in cell–cell communication (Casamitjana-Martinez et al. 2003, Hobe et al. 2003, Ito et al. 2006). An in vitro root assay has shown that peptides comprised of 15 amino acids corresponding to the CLE domain in CLV3, CLE19 and CLE40 are functional in *Arabidopsis* (Fiers et al. 2005). Recently, two excellent papers have revealed that active mature CLE peptides in vivo are 12 amino acids in length (Ito et al. 2006, Kondo et al. 2006). Application of 12-amino acid mature peptide of CLV3 is sufficient to consume the shoot and root meristems in vitro (Kondo et al. 2006).

Although much progress has been made in our understanding of meristem maintenance in *Arabidopsis*, molecular developmental studies are insufficient in other plants. Recently, genetic studies on two grass species, *Oryza sativa* (rice) and *Zea mays* (maize), have shown that the CLV signaling pathway is probably conserved in monocots. In rice, mutations in either *FLORAL ORGAN NUMBER1* (*FON1*) or *FON2* cause enlargement of the floral meristem, resulting in an increase in the number of floral organs (Nagasawa et al. 1996, Suzaki et al. 2004). Gene isolation has revealed that *FON1* encodes an LRR receptor-like kinase most similar to CLV1 (Suzaki et al. 2004). In maize, mutations in *thick tassel dwarf1* (*td1*) or *fasciated ear2* (*fea2*) cause enormous enlargement of the female inflorescence meristem resulting in fasciation, but modestly affect the floral meristem and the floral organ number (Taguchi-Shiobara et al. 2001, Bommert et al. 2005a). It has been revealed that *td1* and *fea2* encode potential orthologs of CLV1 and CLV2, respectively. Whereas *FON1* is expressed in all aerial meristems, *td1* lacks expression in the vegetative meristem. Unlike *CLV1*, *FON1* and *td1* transcripts are detected not only in the inner layers but also in the outer layers of the meristem. In contrast to *clv* mutants, in which all aerial meristems are severely affected, mutations in *FON1*, *td1* and *fea2* do not affect meristem size or development in the vegetative phase, although *td1* shows weak dwarfism. Even among different grass species, mutations in the same orthologs cause partially different phenotypes: for example, *td1* mutation causes highly fasciated inflorescences (ear) in maize, whereas the inflorescences are normal in rice *fon1* mutants. *td1* and *fea2* do not function exclusively in a single pathway that regulates the size of the female inflorescence meristem in maize (Bommert et al. 2005a). Thus, these observations indicate that meristem maintenance is regulated not only by a conserved mechanism controlled by the CLV signaling pathway that is common to all plants, but also by other pathways specific to each species.

In spite of the recent progress in identifying *CLV*-like genes in grasses, to our knowledge no genetic study has investigated the genes encoding a putative signaling molecule involved in meristem maintenance. In this report, we describe isolation of the *FON2* gene and its function in meristem maintenance in rice. *FON2* encodes a putative secreted peptide that is similar to *Arabidopsis* CLV3. Analyses of *fon2* mutants and *FON2*-overexpressing rice reveal that *FON2* is required for maintaining normal sized floral meristems. Taken together with our previous study of *FON1*, these results indicate that the genetic regulation of the CLV signaling pathway is conserved in rice (Suzaki et al. 2004). Furthermore, our data also suggest the existence of an alternative pathway to regulate the size of inflorescence meristem in rice.

Results

FON2 is responsible for regulating floral organ number

Rice flowers have a pistil, six stamens and two lodicules from the inner to outer whorls. These floral organs are subtended by a palea and a lemma, which altogether

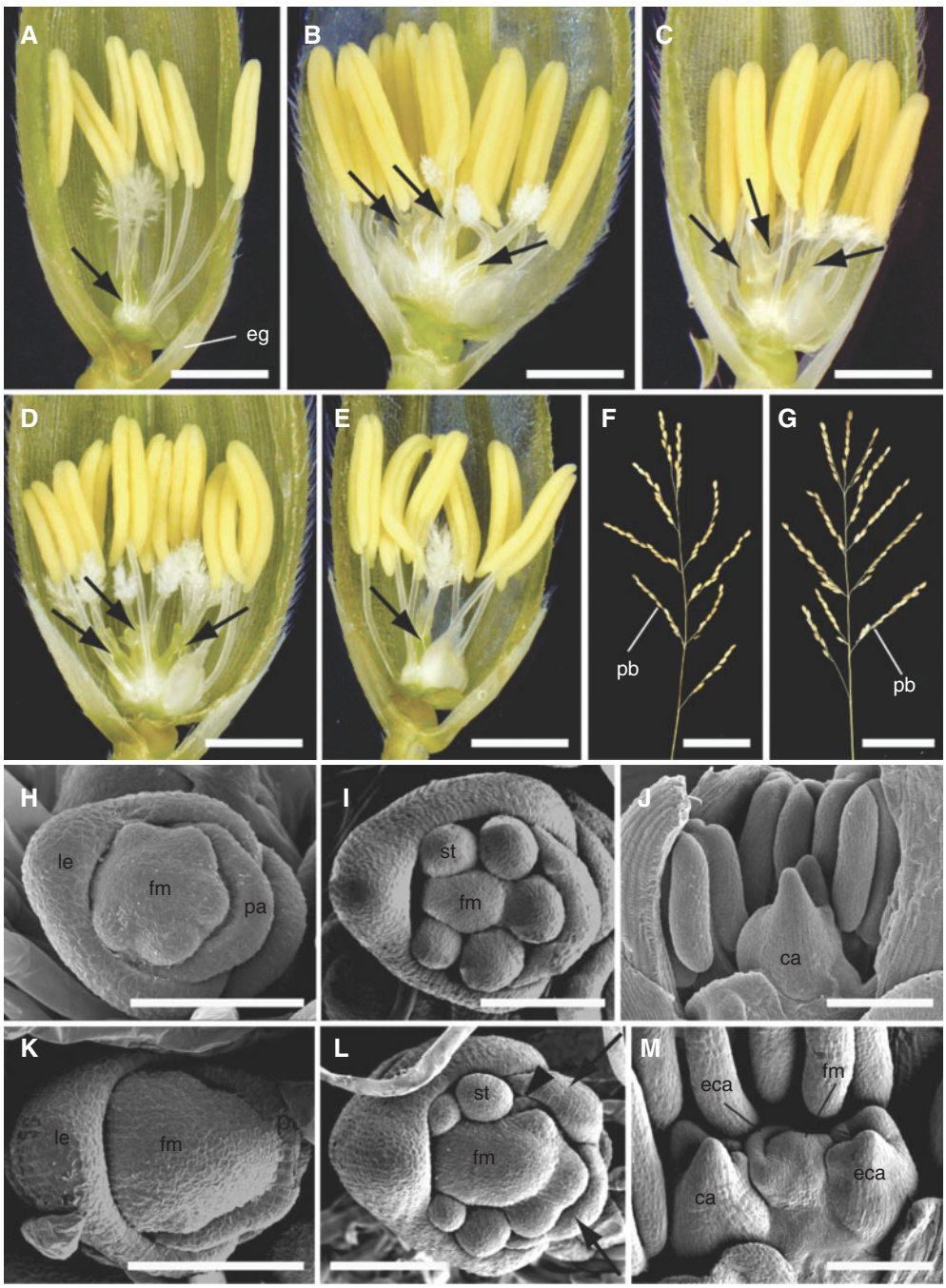

Fig. 1 Mutant phenotypes. (A) Wild-type flower with six stamens and one pistil. (B) *fon2-1* flower with nine stamens and three pistils. (C) *fon2-3* flower with seven stamens and three pistils. (D) *fon2-1 fon1-2* flower with eight stamens and three pistils. (E) *fon2-1* flower rescued by introducing a genomic fragment containing the *FON2* candidate gene. (F) Wild-type inflorescence. (G) *fon2-1* inflorescence. (H–J) Scanning electron micrographs of a wild-type flower. (K–M) Scanning electron micrographs of a *fon2-1* flower. The stages of flower development of *fon2-1* in (K), (L) and (M) correspond to those of the wild type in (H), (I) and (J), respectively. Arrows in A–E indicate pistils. Arrows and arrowheads in (L) indicate palea-like organs and an extra stamen developed from an additional whorl, respectively. ca, carpel; eca, ectopic carpel; eg, empty glume; fm, floral meristem; le, lemma; pa, palea; pb, primary branch; st, stamen. Bars = 2 mm (A–E), 5 cm (F, G), 100 μm (H–M).

Table 1 The number of stamens and pistils

	Stamens					Pistils				
	5	6	7	8	>9	1	2	3	4	>5
Wild type	0	100	0	0	0	100	0	0	0	0
fon2-1	1	30	42	23	4	5	21	59	14	1
fon2-2	4	29	44	21	2	11	65	18	6	0
fon2-3	0	69	31	0	0	17	62	21	0	0
fon1-1	0	89	10	1	0	4	83	13	0	0
fon1-2	3	17	41	23	16	0	12	40	39	9
fon2-1 fon1-1	1	58	34	7	0	0	25	69	6	0
fon2-1 fon1-2	0	25	43	30	2	5	18	48	27	2

For each line, 100 flowers were examined.

constitute a floret. (Fig. 1A, Table 1). Outside the palea and lemma, there are two empty glumes, which are regarded as reduced lemmas, and outside these there are two rudimentary glumes (for a review, see Bommert et al. 2005b, Yamaguchi et al. 2006). Rice spikelets consist of a floret, two empty glumes and two rudimentary glumes.

We found that mutations in the *FON2* locus caused an increase in the number of floral organs. The numbers of pistils in *fon2-1*, *fon2-2* and *fon2-3* increased by about 2.9-, 2.2- and 2.0-fold, respectively, as compared with those in the wild type (Fig. 1B, C, Table 1). In all alleles, there was a very small increase in the number of outer whorl organs (Fig. 1B, C, Table 1, Supplementary Table 1), indicating that the effects of the mutations were more evident in the inner than the outer whorls.

Because the *fon2* and *fon1* mutants showed similar phenotypes, we examined the genetic interaction between *FON2* and *FON1* by generating *fon2 fon1* double mutants. The numbers of pistils in both *fon2-1 fon1-1* and *fon2-1 fon1-2* were very similar to that in the *fon2-1* single mutant (Fig. 1D, Table 1). This result suggests that *FON2* and *FON1* function in the same genetic pathway.

fon2 mutation predominantly affects floral meristem size

To characterize the abnormalities in *fon2* flowers in detail, we examined the flowers of the *fon2-1* mutant in early developmental stages by scanning electron microscopy. At an early stage after initiation of the palea, the floral meristem of *fon2-1* was obviously larger than that of the wild type (Fig. 1H, K). Enlargement of the floral meristem size was also observed in *fon2-2* and *fon2-3* (Supplementary Fig. 1). By the stage that the stamens had developed, the difference in the size of the floral meristem between *fon2-1* and the wild type was more evident (Fig. 1I, L). In wild-type, a palea initiated at the adaxial side of the floral meristem. In *fon2-1*, in contrast, two palea-like organs sometimes developed next to each other in the original whorl (Fig. 1L). Six stamens developed in a concentric whorl in wild-type flowers, whereas extra stamens were produced mainly in the same whorl as the original stamens in *fon2-1*. In rare cases, extra stamens developed from an additional whorl in *fon2-1* (Fig. 1L). In the wild type, the carpel primordia initiate at the flank of the meristem near the lemma (Yamaguchi et al. 2004); subsequently, the primordia elongate towards the opposite side, enclosing the meristem, and eventually fuse to form a pistil (Fig. 1J). In *fon2-1*, the carpel primordia first initiated at the flank of the meristem near the lemma in a manner similar to that of the wild type (data not shown); subsequently, ectopic carpel primordia arose in an alternative phyllotaxy at the time that the third carpel primordia initiated (Fig. 1M). The fourth and fifth carpel primordia initiated irregularly from a more inner region (data not shown). This pattern of early carpel initiation in *fon2-1* is similar to what is observed in *fon1* (Suzaki et al. 2004). In the wild type, floral meristems are consumed when the carpel primordia begin to develop (Suzaki et al. 2004). In *fon2-1*, in contrast, the floral meristem remained morphologically undifferentiated even after some sets of carpels had been produced, suggesting that the regulation of meristem determinacy is disrupted in *fon2-1* (Fig. 1M).

Table 2 Phenotypes of inflorescence

	No. of primary rachis branches	No. of florets	*n*
Wild type	8.7 ± 1.1	91.9 ± 16.5	13
fon2-1	9.9 ± 1.3	88.1 ± 13.8	17
Actin:FON2	4.5 ± 1.2	10.6 ± 3.2	8

The inflorescence phenotypes of *fon2-1* were indistinguishable from those of the wild type. No obvious differences were observed in the number of primary rachis branches and the number of florets between *fon2-1* and the wild type (Fig. 1F, G, Table 2) or in the size of the inflorescence meristem (data not shown). Thus, these results suggest that *FON2* regulates mainly the size of the floral meristem in rice.

FON2 encodes a CLV3-like putative signal protein

To understand the molecular mechanisms underlying the meristem maintenance in rice, we tried to isolate the *FON2* gene. Because the phenotypes of *fon2 fon1* double mutants suggested that *FON2* and *FON1* function in the same genetic pathway, and we previously reported that *FON1* encodes an LRR receptor-like kinase orthologous to CLV1 (Suzaki et al. 2004), we hypothesized that *FON2* is involved in a genetic pathway similar to the CLV signaling system in *Arabidopsis*.

Initially, we identified one *CLV2*-like gene and five *CLV3*-like genes by screening the rice genomic

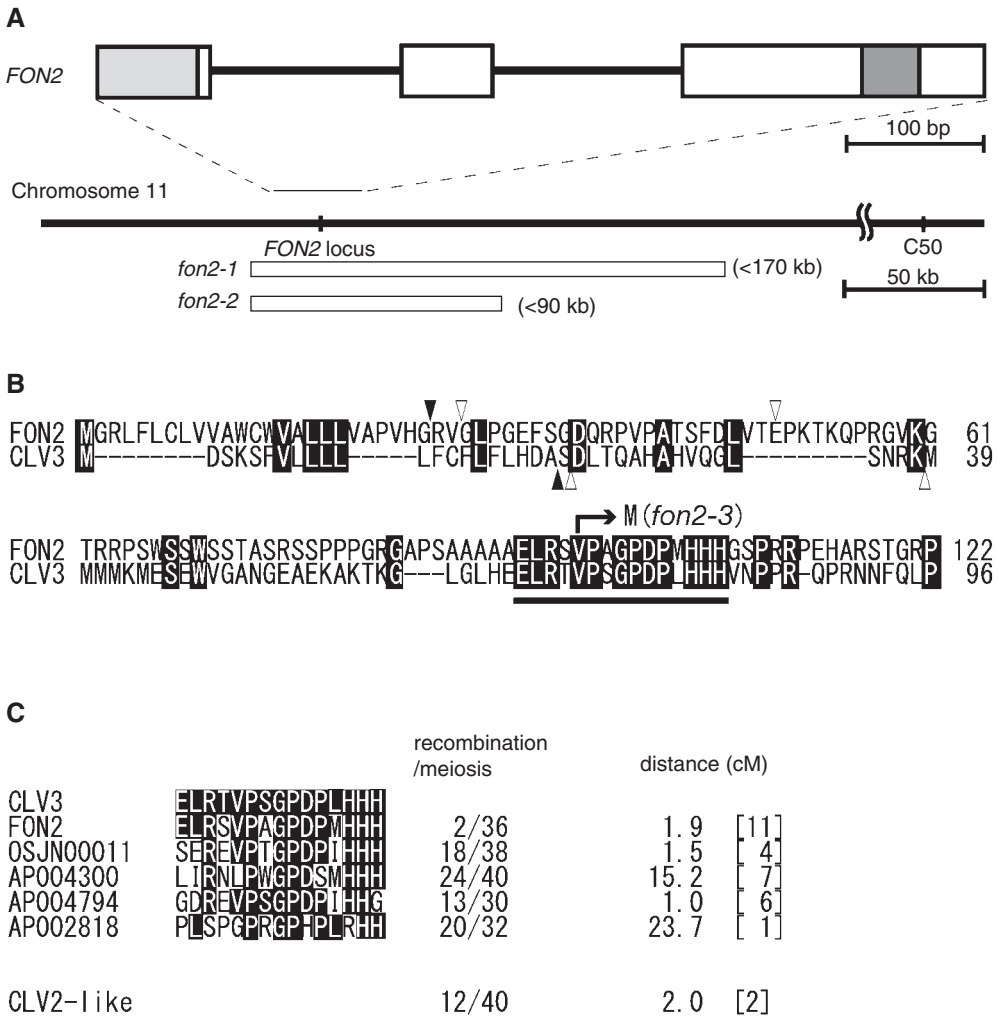

Fig. 2 Isolation of the *FON2* gene and characteristics of the FON2 protein. (A) Top panel: genomic structure of *FON2*. Boxes and thick lines indicate exons and introns, respectively. Light and dark gray box correspond to the putative signal sequence and CLE domain, respectively. Bottom panel: map of the region around the *FON2* locus. Open boxes indicate chromosomal deletions in *fon2-1* and *fon2-2*. (B) Amino acid alignment of FON2 and CLV3. Open triangles indicate the position of the intron. Closed triangle indicates the putative signal sequence cleavage site as predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP; Fletcher et al. 1999). Underline indicates the CLE domain. (C) Amino acid alignment of the CLE domains. The numbers indicate the results of linkage analysis and distances between the *FON2* locus and molecular markers near each *CLE* gene and a *CLV2*-like gene. The numbers of chromosome on which the markers reside are shown in parentheses.

sequence database. Next, we carried out linkage analysis between the *FON2* locus and molecular markers near these six genes (Fig. 2A, C). As a result, the *FON2* locus was roughly linked to a marker near a *CLV3*-like gene on chromosome 11. We therefore tried to find mutations in this candidate gene in the three *fon2* alleles. Southern hybridization and PCR analyses revealed that *fon2-1* and *fon2-2* contained a large deletion (at least 170 and 90 kb, respectively) including the candidate gene (Fig. 2A). A nucleotide change causing substitution of an amino acid was found in *fon2-3* (Fig. 2B). When an 8.1 kb genomic fragment incorporating the candidate gene was introduced into the *fon2-1* and *fon2-2* mutants, the defects in the flowers of both mutants were rescued (Fig. 1E). These results clearly indicated that a gene similar to *CLV3* on chromosome 11 was the *FON2* gene.

Next, we amplified the *FON2* cDNA by reverse transcription–PCR (RT–PCR) using total RNA derived from young panicles. We determined the position of introns in *FON2* by sequencing the RT–PCR product and the predicted open reading frame (Fig. 2A). *FON2* consists of three exons and two introns, which are located at the same positions as those of *CLV3* (Fig. 2A; Fletcher et al. 1999). *FON2* encodes a putative small protein of 122 amino acids

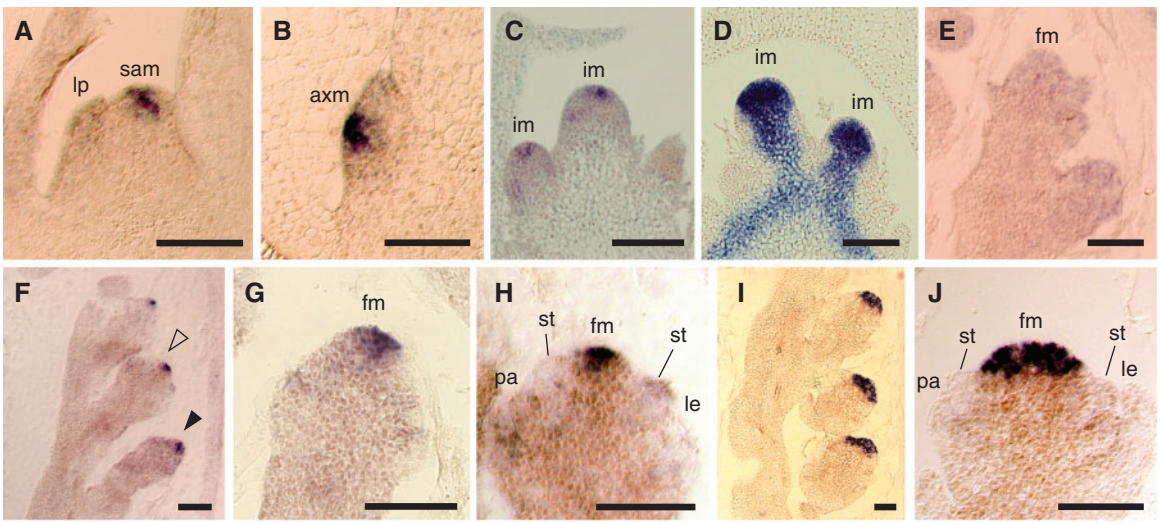

Fig. 3 In situ localization of *FON2* and *OSH1*. (A, B) In situ localization of *FON2* transcripts in the vegetative shoot apical meristem (A) and axillary meristem (B) in the wild type. (C) In situ localization of *FON2* transcripts in the inflorescence meristem at the primary branch stage in the wild type. (D) In situ localization of *OSH1* transcripts in the inflorescence meristem at the primary branch stage in the wild type. (E–H) In situ localization of *FON2* transcripts in the floral meristem in the wild type. (G) Close-up view of the floral meristem, indicated by the closed triangle in (F), which initiates no floral organ. (H) Close-up view of the floral meristem, indicated by the open triangle in (F), which has initiated a lemma, a palea and stamens. (I, J) In situ localization of *FON2* transcripts in the floral meristem in *fon1-2*. The floral meristem has initiated a lemma, a palea and stamens in (J). The stages of flower development of *fon1-2* in (I) and (J) correspond to those of the wild type in (F) and (H), respectively. An antisense probe was used in A–J, whereas a sense probe was used in E. axm, axillary bud meristem; fm, floral meristem; im, inflorescence meristem; le, lemma; lp, leaf primordia; pa, palea; sam, shoot apical meristem; st, stamen. Bars = 100 μm.

with a putative hydrophobic signal peptide at its N-terminus and the CLE domain near its C-terminus (Fig. 2B). Although the overall amino acid identity between FON2 and CLV3 is low, the CLE domain of FON2 shows high homology to that of CLV3 (Fig. 2C). In *fon2-3*, Val97 is replaced by Met in the CLE domain (Fig. 2B). The severity of the mutant phenotype in *fon2-3* is similar to that of phenotypes in the complete loss-of-function mutants *fon2-1* and *fon2-2*, judging from the number of floral organs and the size of floral meristems (Table 1, Supplementary Table 1, Supplementary Fig. 1). This suggests that Val97 is essential for FON2 function and that amino acids in the CLE domains may be crucial in general.

Spatial expression patterns of FON2

We analyzed the spatial and temporal expression patterns of *FON2* by in situ hybridization. In the wild type, *FON2* transcripts were detected in the shoot apical and axillary meristems (Fig. 3A, B), but not in any other vegetative tissues. In both types of meristem, *FON2* transcripts were detected in a group of small cells at the apical region of the central zone. *FON2* transcripts were also detected in the narrow apical region of the inflorescence and floral meristems (Fig. 3C, F–H). These localized expression patterns of *FON2* contrast highly with the expression pattern of *OSH1*, which marks whole undifferentiated cells in the meristem (Fig. 3D). The localized expression patterns of rice *FON2* in the meristems greatly resemble the expression patterns of *Arabidopsis CLV3* (Fletcher et al. 1999). No signal was detected when a sense RNA probe was used (Fig. 3E).

In the severe *fon1-2* mutant, the floral meristems were enlarged and accumulated a large number of cells (Suzaki et al. 2004). In contrast to the wild type, the expression domain of *FON2* was significantly expanded in the enlarged meristems of *fon1-2* (Fig. 3I, J). The width of the *FON2* expression domain was much greater than that of the wild type, whereas the height of the domain was indistinguishable from that of the wild type. No signal was detected in the floral organ primordia in the wild type or *fon1-2*.

Constitutive expression of FON2

To obtain further insight into the functions of *FON2*, we produced transgenic rice plants that constitutively expressed *FON2* under the control of a strong promoter of the rice actin gene. Although their vegetative phenotypes were almost normal, the *Actin:FON2* lines were markedly affected in the reproductive phase. First, the numbers of all floral organs were highly reduced; in the most extreme cases, the severe *Actin:FON2* lines produced spikelets with no florets and with only one or two empty glumes (Fig. 4A).

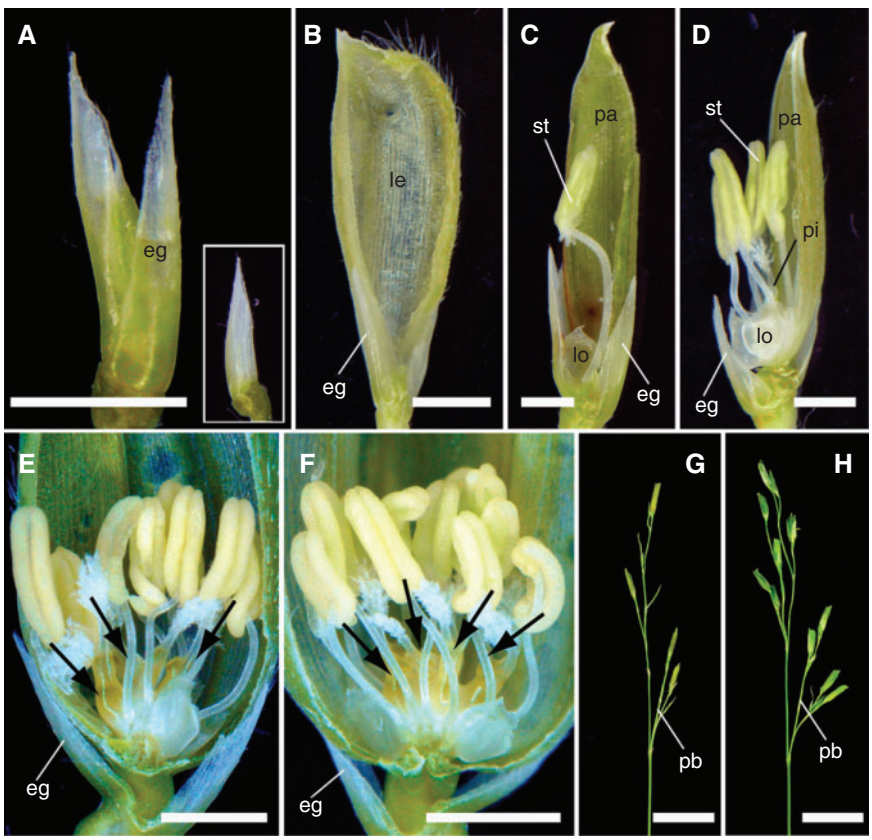

Fig. 4 Phenotypes of *Actin:FON2* transgenic plants. (A) *Actin:FON2* flower with a severe phenotype, which has no floret and only two empty glumes. The inset shows a more severe *Actin:FON2* flower with only one empty glume. (B) *Actin:FON2* flower with an intermediate phenotype, which has one lemma and two empty glumes. (C) *Actin:FON2* flower with an intermediate phenotype, which has one palea, one lodicule and one stamen. For clarity, the lemma has been removed. (D) *Actin:FON2* flower with a weak phenotype, which has one palea, two lodicules, four stamens and one pistil. For clarity, the lemma has been removed. (E) *fon1-2* flower. (F) *Actin:FON2/fon1-2* flower with a phenotype similar to the *fon1-2* flower in E. (G) *Actin:FON2* inflorescence with a severe phenotype, in which the numbers of primary branches and spikelets are highly reduced. (H) *Actin:FON2* inflorescence with an intermediate phenotype, in which the numbers of primary branches and spikelets are moderately reduced. Arrows in E and F indicate pistils. eg, empty glume; le, lemma; lo, lodicule; pa, palea; pb, primary branch; pi, pistil; st, stamen. Bars = 2 mm (A–F), 3 cm (G, H).

This phenotype may be caused by termination of the floral meristem at earlier developmental stages. In intermediate *Actin:FON2* lines, the flowers showed various patterns of reduced floral organs; for example, a flower with only one lemma (Fig. 4B) and a flower with one lemma, one palea, one lodicule and one stamen (Fig. 4C). In weak *Actin:FON2* lines, fertile flowers were produced in spite of a reduced number of stamens because a pistil developed (Fig. 4D). The ratio of transgenic plants in these three classes is roughly even.

Secondly, the numbers of rachis branches and spikelets were considerably reduced in the inflorescences of *Actin:FON2* plants (Fig. 4G, H, Table 2). These results were unexpected because no obvious abnormalities were observed in the inflorescences of the *fon2* mutants. These inflorescence phenotypes suggested that the size of the inflorescence meristem (rachis branch meristem) was highly reduced. The severities of these inflorescence phenotypes correlated with the flower phenotypes (Fig. 4G, H). Thus, constitutive expression of *FON2* may cause a reduction in the size of the flower and inflorescence meristems. The vegetative phenotypes of *Actin:FON2* plants were indistinguishable from those of the wild type (data not shown).

Next, we overexpressed *FON2* in the *fon1-2* mutant. The numbers of floral organs in all transgenic plants were similar to that in the single *fon1-2* mutant (Fig. 4E, F). The numbers of primary rachis branches and florets were also indistinguishable from those in the *fon1-2* mutant. Taken together with phenotypes of the *fon2 fon1* double mutants described above, these results strongly suggest that *FON2* and *FON1* act in the same genetic pathway and that a functional *FON1* gene is required for the action of *FON2*.

Analysis of functional conservation of FON2 in Arabidopsis

The above results indicated that genetic regulation of meristem size by *FON1–FON2* in rice resembles that by *CLV1–CLV3* in *Arabidopsis*. To address the question of whether the FON2 protein has a function similar to CLV3, we introduced a chimeric gene that expressed *FON2* under the 35S promoter of cauliflower mosaic virus (*CaMV35S*) into the wild type and the *clv3* mutant. When introduced into the wild type, shoot apical meristems ceased initiating leaves in the seedlings after the formation of 2–5 leaves (Fig. 5A, B); subsequently, ectopic leaves arose repeatedly after this period of no leaf initiation (about 2 weeks). These phenotypes are reminiscent of those of the *wus* mutant and

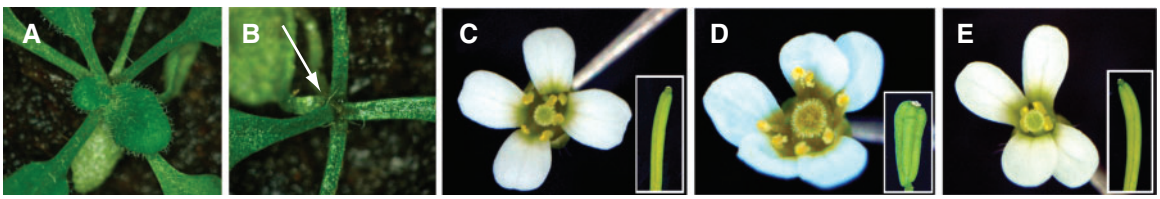

Fig. 5 *FON2* function in transgenic *Arabidopsis* plants. (A) Wild-type seedling producing rosette leaves. (B) *35S:FON2* seedling. The arrow indicates premature termination of the shoot apical meristem. (C) Wild-type flower. (D) *clv3-2* flower with increased number of floral organs. (E) *clv3-2* flower rescued by transformation of *35S:FON2*. The insets in C–E show phenotypes of a silique.

35S:CLV3 plants, suggesting that meristems are consumed by a high expression level of *FON2* (Laux et al. 1996, Brand et al. 2000). When introduced into the *clv3-2* mutant, variable phenotypes, which were roughly classified into three classes, were observed in the transgenic plants. In the first class, the transgenic plants ceased initiating leaves, like the *wus* mutant, as observed in the wild-type hosts. In the second class, the *clv3* mutant phenotypes in the flower were completely rescued in terms of the number of floral organs and the silique phenotype (Fig. 5C–E). In the third class, the mutations were partly rescued in the transgenic plants, for example the silique phenotypes resemble those of the wild type. These results suggest that *FON2* is able to substitute for *CLV3* and that a high expression level of *FON2* can repress *WUS* expression in *Arabidopsis*.

Discussion

We have isolated the *FON2* gene, the loss-of-function mutant of which causes enlargement of the floral meristem and an increase in floral organ number. *FON2* encodes a putative secreted peptide that is most similar to *Arabidopsis* CLV3 and is expressed at the apical region of the meristems. Our molecular genetic studies of *FON2* in rice and *Arabidopsis* have revealed that FON2 regulates the size of the floral meristem in a manner very similar to the function of CLV3 in *Arabidopsis*. Taken together with our previous study of *FON1* (Suzaki et al. 2004), an ortholog of *Arabidopsis CLV1*, the results obtained here strongly suggest that the genetic mechanism underlying the regulation of meristem maintenance by the CLV signaling system in *Arabidopsis* is conserved in rice—a grass species that is distantly related to eudicots within the Angiosperms.

FON2 is a counterpart of CLV3 in rice

It is difficult to construct a phylogenetic tree of the CLE family because the overall amino acid sequence similarities of the proteins are very low. The following structural similarities, however, suggest that *FON2* is an ortholog of *CLV3*. First, *FON2* and *CLV3* consist of three exons and two introns located at similar positions, although the other rice and *Arabidopsis CLE* genes, except for one, have no introns (Fletcher et al. 1999, Hobe et al. 2003, T. Suzaki and H.-Y. Hirano unpublished data). Secondly, the amino acid identity between the CLE domains of FON1 and CLV3 is highest among the CLE proteins in both species.

The spatial expression patterns of *FON2* in the meristems resembled those of *CLV3*, which mark stem cells in the meristems of *Arabidopsis* (Fletcher et al. 1999). *FON2* rescued the *clv3* phenotype when it was transformed into the *clv3-2* mutant. In addition, overexpression of *FON2* in the wild type caused phenotypes similar to those of overexpression of *CLV3* or the *wus* mutant, suggesting that the shoot apical meristem is terminated. Taken together, these results indicate that *FON2* functions in *Arabidopsis* and has activities similar to *CLV3*. Thus, we conclude that *FON2* is a structural and functional counterpart of *CLV3* in rice.

The CLV signaling pathway is conserved in rice

Mutations in the *FON2* locus caused enlargement of the floral meristem, resulting in an increase in the number of floral organs. This phenotype of *fon2* is very similar to that of the *fon1* mutant (Suzaki et al. 2004). Moreover, the phenotypes of the *fon2 fon1* double mutants resembled those of *fon2* or *fon1* single mutants. These results indicate that *FON2* and *FON1* function in the same genetic pathway to regulate floral meristem size in rice. The expression domain of *FON2* was expanded in the floral meristems in the *fon1* mutant, similar to the expansion of the *CLV3*-expressing domain in the *clv1* mutant (Fletcher et al. 1999). If *FON2* marks stem cells in the meristem as does *CLV3*, a large number of stem cells may accumulate in the *fon1* floral meristem. Overexpression of *FON2* caused termination of the floral meristem or a reduction in floral organs in rice. This *FON2* function is dependent on *FON1* activity, because no abnormality was observed after overexpression of *FON2* in the *fon1* mutant. Taking these results together, *FON2* may negatively regulate stem cell proliferation through the activity of *FON1*. Thus, the genetic relationship between *FON2* and *FON1* is very similar to that between *CLV3* and *CLV1*.

FON2 encodes a CLV3-like small peptide and *FON1* encodes an LRR receptor-like kinase most similar to CLV1 (Suzaki et al. 2004). Together with the genetic interaction between *FON2* and *FON1* in rice and the function of *FON2* in transgenic *Arabidopsis*, our results clearly indicate that the FON1–FON2 system in rice corresponds to the CLV signaling system in *Arabidopsis*. FON2, like CLV3, may function as a putative ligand for a receptor complex including FON1. Because rice is a plant in monocots, which are distinct from eudicots such as *Arabidopsis*, the negative regulation of stem cell identity by the CLV signaling system may be principally conserved in a wide range of plants within the Angiosperms.

Meristem maintenance is regulated by a complex genetic network in rice

FON2 transcripts, like *FON1* transcripts (Suzaki et al. 2004), were detected in all of the aerial meristems. Mutations in either *FON2* or *FON1*, however, affect the size of the floral meristem, but not the vegetative or inflorescence meristem (Suzaki et al. 2004). Moreover, constitutive expression of *FON2* caused abnormal flowers and inflorescences, but did not affect the vegetative organs. To overcome this apparent inconsistency, we propose a model to explain meristem regulation in rice in which an alternative pathway also negatively regulates stem cell identity (Fig. 6). For simplicity, here we hypothesize that the alternative pathway is composed of factors similar to those in the FON2–FON1 pathway, because both pathways are supposed to work redundantly.

We can interpret the phenotypes of the *fon1* and *fon2* mutants and those of the *Actin:FON2* transgenic rice lines according to this model. (i) The function of the alternative pathway may mask mutations in either *FON1* or *FON2* in the vegetative and inflorescence meristems. The floral meristem, however, may lack this alternative pathway, and thus a mutation in either *FON1* or *FON2* causes enlargement of the floral meristem. (ii) The constitutive expression of *FON2* causes no changes in the *fon1* mutant, not only in the floral phenotype but also in the vegetative and inflorescence phenotypes, suggesting that FON2 only acts through FON1. In contrast, the constitutive expression of *FON2* in the wild type causes a decrease in the number of rachis branches and flowers, probably due to a reduction in the size of the inflorescence meristem. Therefore, this phenotypic alteration indicates that an excess amount of FON2 acts through the putative receptor FON1 in the transgenic plants constitutively expressing *FON2* in the wild type. (iii) In contrast, FON1 may be inactive in the vegetative meristem because no phenotypic change was observed in this meristem in the transgenic plants constitutively expressing *FON2* in the wild type. It is plausible that this inactivity of FON1 may be due to a lack of either post-transcriptional regulation of FON1 or an additional factor that is required to make up an active receptor complex including FON1. Thus, three kinds of aerial meristems—the vegetative shoot, inflorescence and flower meristems—may be regulated in different ways by a complex genetic network.

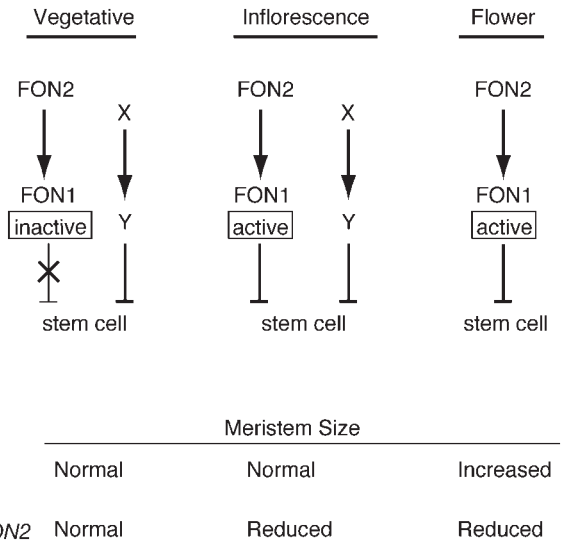

Fig. 6 Model of the regulation of meristem maintenance in rice. See text for details.

What kinds of proteins are involved in the alternative pathway proposed here? It is possible that a combination of another CLE protein and an LRR-type receptor kinase constitutes this pathway. *OsLRK1*, a close relative of *FON1*, may be a candidate for the receptor kinase, because its suppression by antisense RNA causes an increase in floral organs (Kim et al. 2000). In *Arabidopsis*, however, knockout lines of *CLV1*-like genes (*BAM1*, *BAM2* and *BAM3*), which are closely related to *OsLRK1*, show a loss of stem cells, suggesting that these genes have roles opposite to that of *CLV1* in meristem maintenance (DeYoung et al. 2006).

The CLV signaling pathway negatively regulates stem cell identity in the meristem by repressing *WUS*, which promotes stem cell identity in *Arabidopsis* (Brand et al. 2000, Schoof et al. 2000). Mutants similar to *wus* have so far not been found in rice. The most plausible candidate for a gene that promotes stem cell identity in rice is an ortholog of *WUS* (*OsWUS*, Haecker et al. 2004). The protein encoded by *OsWUS*, however, lacks a transcriptional activation domain (data not shown). Thus, in terms of understanding meristem maintenance in rice, it will be essential to identify the genes that promote stem cell identity.

Comparison of meristem regulation

Because rice and maize are closely related, it is possible that a genetic network similar to that in rice regulates meristem maintenance in maize. Unlike the rice inflorescence, however, the maize inflorescence is sensitive to mutations in the genes of the CLV-like pathway such as *fea2* and *td1* (Taguchi-Shiobara et al. 2001, Bommert et al. 2005a). It would be of great interest to know whether the alternative pathway hypothesized in rice (Fig. 6) is functional in the maize inflorescence meristem. Maize has been domesticated from its ancestral species, teosinte (Doebley 1992). During domestication, characters that affect the thickness of ears, i.e. the size of the female inflorescence meristem, may have been selected. An intriguing hypothesis is that the genes involved in the alternative pathway may have been either lost or weakened in function during the domestication of maize.

The CLV1 ortholog *td1* is not expressed in the vegetative meristem in maize, but it is expressed in both the inflorescence and flower meristems (Bommert et al. 2005a). Our model postulates that FON1 may be inactive in the vegetative meristem. Therefore, another unidentified pathway that regulates maintenance of the vegetative meristem may exist in common in the two grass species. It is unlikely, however, that this alternative pathway is involved in *Arabidopsis*, because *clv* mutations cause enlargement of all meristems in the aerial parts of the plant (Clark et al. 1993, Clark et al. 1995, Kayes and Clark 1998).

Rice *FON1* and maize *td1* are both expressed in the floral organ primordia (Suzaki et al. 2004, Bommert et al. 2005a). In contrast, no *FON2* transcripts are detected in the floral organ primordia, suggesting that the FON2–FON1 pathway is not involved in regulating floral organ development. This inference, however, does not necessarily rule out the possibility that *FON1* or *td1* works in floral organ development by interacting with other signaling molecules. Recently, Chu et al. (2006) have reported that *FON4*, loss-of-function mutants of which show phenotypes similar to those of *fon1* and *fon2*, encodes a peptide that contains the CLE domain. Our sequence comparison has revealed that *FON2* and *FON4* encode the same peptide. Therefore, *fon4* mutants reported in the above study are alleles of the *FON2* locus. The results in the study of Chu et al, are largely consistent with our results, except for the following two points. First, the '*fon4*' mutation affected the inflorescence phenotype, although the inflorescence phenotypes of three *fon2* mutants were indistinguishable from that of the wild type. In maize, the severity of the inflorescence phenotype in the *td1* mutants depends on the genetic background (Bommert et al. 2005a). It is plausible that differences of phenotypes among alleles may also be the result of differences in genetic background of the rice strains. Secondly, in vitro application of a 14 amino acid CLE motif peptide caused inhibition of shoot growth, although overexpression of *FON2* induced no phenotypic alterations in the vegetative phase. This inconsistency may be mainly due to differences in experimental approaches. In fact, a very high concentration (50 μM) of peptide is required to reduce the meristem size in rice, whereas 1 μM CLV3 peptide is sufficient to reduce the meristem size in *Arabidopsis* (Kondo et al. 2006).

Materials and Methods

Plant materials

The rice strain used in this study was *O. sativa* L. ssp. *japonica*. The origin of three *fon2* mutants and two *fon1* mutants has been described previously (Nagasawa et al. 1996, Suzaki et al. 2004, Yamaki et al. 2005). Fukei71 and Taichung65 (T65) were used as wild-type strains for comparing phenotypes and in situ hybridization. All *Arabidopsis* plants used in this study were of the Columbia ecotype, except for *clv3-2*, which was the Landsberg ecotype.

Isolation of FON2

Linkage analysis between the *FON2* locus and six molecular markers, which are located near one *CLV2*-like and five *CLV3*-like genes, was carried out by using F_2 plants of *fon2-1* and Kasalath (spp. *indica*). The primers used are listed in Supplementary Table 2. The regions deleted in *fon2-1* and *fon2-2* were mapped by DNA gel blot analysis using DNA fragments amplified by the primers shown in Supplementary Table 2. The genomic sequence of the *FON2* candidate gene in *fon2-3* was determined by direct sequencing of the PCR fragments amplified by the primers shown in Supplementary Table 2. The *FON2* cDNA was synthesized and amplified from poly(A)$^+$ RNA isolated from young panicles. Exon–intron structures were determined by comparing the genomic and cDNA sequences.

In situ hybridization

For the in situ hybridization probe, a 430 bp fragment consisting of the whole coding region, 5′-untranslated region (UTR; 14 bp) and 3′-UTR (47 bp) was amplified with the primers shown in Supplementary Table 2, and cloned into a T-vector (Novagen, Madison, WI, USA). Probe synthesis, the preparation of sections and in situ hybridization were performed as described previously (Suzaki et al. 2004).

Transformation of rice

For complementation, an 8.1 kb fragment including 6.4 kb of sequence directly upstream of the initiation codon was cloned into a binary vector with resistance to hygromycin. For constitutive expression, the *FON2* cDNA was inserted into a binary vector containing a cassette of the rice actin (*Act1*) promoter and the nos terminator (Sentoku et al. 2000). The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain EHA101 and transformed into rice as described previously (Suzaki et al. 2004).

Analyses of FON2 function in transgenic Arabidopsis

For constitutive *FON2* expression, the *FON2* cDNA was cloned into the binary vector pK7WG2 with the *CaMV35S* promoter by using Gateway cloning technology

(see Supplementary Table 2; for a review, see Karimi et al. 2002). The resulting construct was introduced into *A. tumefaciens* strain GV3101 and transformed into the *clv3-2* mutant or the wild type by the floral dip method (Clough and Bent 1998). Transgenic plants were grown at 22°C under continuous white light (20–50 mmol $m^{-2} s^{-1}$). Seedlings at 14 d after germination were used for phenotypic analysis.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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