The gene *FLORAL ORGAN NUMBER1* regulates floral meristem size in rice and encodes a leucine-rich repeat receptor kinase orthologous to *Arabidopsis* CLAVATA1

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Summary

The regulation of floral organ number is closely associated with floral meristem size. Mutations in the gene *FLORAL ORGAN NUMBER1* (*FON1*) cause enlargement of the floral meristem in *Oryza sativa* (rice), resulting in an increase in the number of all floral organs. Ectopic floral organs develop in the whorl of each organ and/or in the additional whorls that form. Inner floral organs are more severely affected than outer floral organs. Many carpel primordia develop indeterminately, and undifferentiated meristematic tissues remain in the center in almost-mature flowers. Consistent with this result, *OSH1*, a molecular marker of meristematic indeterminate cells in rice, continues to be expressed in this region. Although floral meristems are strongly affected by the *fon1-2* mutation, vegetative and inflorescence meristems are largely normal, even in this strong allele. We isolated the *FON1* gene by positional cloning and found that it encodes a leucine-rich repeat receptor-like kinase most similar to CLAVATA1 (CLV1) in *Arabidopsis thaliana*. This suggests that a pathway similar to the CLV signaling system that regulates meristem maintenance in *Arabidopsis* is conserved in the grass family. Unlike *CLV1*, which is predominantly expressed in the L3 layer of the shoot meristem, *FON1* is expressed throughout the whole floral meristem, suggesting that small modifications to the CLV signaling pathway may be required to maintain the floral meristem in rice. In addition, *FON1* transcripts are detected in all meristems responsible for development of the aerial part of rice, suggesting that genes sharing functional redundancy with *FON1* act in the vegetative and inflorescence meristems to mask the effects of the *fon1* mutation.

Key words: CLV signaling, Floral meristem, *FLORAL ORGAN NUMBER1*, Flower development, Rice

Introduction

The floral meristem in angiosperms produces floral organs in numbers and patterns that are characteristic of each species. The number of floral organs, together with their shape and patterns, contributes to the diversification of flowers. Genetic and molecular mechanisms that regulate floral organ number have been well studied in *Arabidopsis thaliana* by means of a number of mutants. Two classes of gene affect floral organ number. The first class includes genes that regulate meristem activity and maintenance, such as *CLAVATA* (*CLV*), *FASCIATA* (*FAS*) and *ULTRAPETALA* (*ULT*) (Clark et al., 1993; Clark et al., 1995; Fletcher, 2001; Kayes and Clark, 1998; Leyser and Furner, 1992); loss of function of these genes causes enlargement of flower meristems and results in an increase in floral organ number. The second class includes genes such as *PERIANTHIA* (*PAN*), of which loss of function give rises to an increase mainly in the number of perianth organs (Running and Meyerowitz, 1996). Mutation of *PAN* does not affect meristem size; instead, *PAN* appears to control the distance between floral primordia initiation events.

The *clv1*, *clv2* and *clv3* mutations cause enlargement of the vegetative and inflorescence meristems, in addition to the floral meristem, leading to abnormal phyllotaxy, inflorescence fasciation and enlargement of floral meristems (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998). Genetic analysis has shown that these three genes act in the same pathway to regulate meristem size. *CLV1* encodes a receptor-like protein consisting of an extracellular leucine-rich repeat (LRR) domain, a small transmembrane domain, and a cytoplasmic serine/threonine kinase domain (Clark et al., 1997). *CLV2* encodes a similar protein that has an LRR domain but lacks the cytoplasmic kinase domain (Jeong et al., 1999). Both proteins are thought to form heterodimers and the LRR domains appear to act in ligand binding. *CLV3* encodes a small peptide that is secreted into the intercellular region and acts as a putative ligand for the CLV1-CLV2 signal transduction complex (Fletcher et al., 1999; Rojo et al., 2002).

This CLV signaling pathway negatively regulates the gene *WUSCHEL* (*WUS*), which controls stem cell fate in the meristem (Brand et al., 2000; Laux et al., 1996; Mayer et al.,

1998; Schoof et al., 2000). Mutations in the *CLV* genes fail to repress *WUS* activity, resulting in expansion of the *WUS* expression domain and enlargement of the meristem. Conversely, *WUS* positively regulates the expression of *CLV3* and maintenance of the stem cell domain. This regulatory feedback system is required to maintain appropriate meristem size throughout *Arabidopsis* development.

In spite of much progress in our understanding of the regulation of floral organ number and maintenance of the meristem in *Arabidopsis*, the regulation of such events in monocots is poorly understood. The *fasciated ear2* (*fea2*) gene is exceptionally well characterized in *Zea mays* (Taguchi-Shiobara et al., 2001). Mutations in *fea2* cause enormous enlargement of the inflorescence meristem but have a more modest effect on floral meristems and organ number. The *fea2* gene encodes an LRR receptor-like protein that is most closely related to *Arabidopsis* CLV2. This finding indicates that the CLV signaling pathway for regulating meristem maintenance is conserved in monocots as well as in dicots. In rice (*Oryza sativa*), two loci responsible for floral meristem size and number have been identified, *FLORAL ORGAN NUMBER1* (*FON1*) and *FON2* (Nagasawa et al., 1996). Floral organ number is also increased in rice by antisense suppression of the genes *OsLRK1* and *OsFOR1*, which encode an LRR receptor kinase-like protein and a polygalacturonase-inhibiting protein, respectively (Kim et al., 2000; Jang et al., 2003).

In this report, we describe in detail a new allele, *fon1-2*, which is stronger than the *fon1-1* (formerly *fon1*) allele previously reported by Nagasawa et al. (Nagasawa et al., 1996). In *fon1-2*, the floral meristem is severely affected and accumulates a large number of cells. Consequently, unlike in *fon1-1*, the number of all organs in the floret increase and the effect of the mutation is more evident in the inner whorls than in the outer whorls in *fon1-2*. We isolated *FON1* by positional cloning and found that this gene encodes an LRR receptor-like kinase that is orthologous to CLV1 of *Arabidopsis*. Our results suggest that the CLV signal transduction pathway for regulating floral meristem size and number is conserved in rice. Although *FON1* is expressed in all of the meristems responsible for development of the aerial part of rice, marked changes are not observed in meristem size or in the phenotypes of the vegetative or inflorescence organs even in the strong *fon1-2* mutant, suggesting that there may be genetic redundancy in the maintenance of both vegetative and inflorescence meristems in rice.

Materials and methods

Plant materials

The rice strains used in this study were *Oryza sativa* L. spp. *japonica*. A new mutant with an increased number of floral organs was found in M_2 plants of a derivative strain from Kinuhikari that had been mutagenized with *N*-methyl-*N*-nitrosourea. An allelism test revealed that this new mutant (*fon1-2*) was allelic to *fon1-1* [previously described as *fon1* by Nagasawa et al. (Nagasawa et al., 1996)]. Kinuhikari and Taichung65 (T65) were used as wild-type strains for comparing phenotypes and for in situ analysis.

Scanning electron microscopy (SEM) and meristem size measurement

For observation by SEM, young panicles and flowers were fixed using the methods of Itoh et al. (Itoh et al., 2000). Samples were dried at their critical point, sputter-coated with platinum, and observed with a scanning electron microscope (model S-4000; Hitachi, Tokyo, Japan) at an accelerating voltage of 10 kV. For observation by Nomarski microscopy, shoot and floral apices were fixed by the methods of Itoh et al. (Itoh et al., 2000). After clearing them in benzyl-benzoate-four-and-a-half fluid, specimens were observed with a microscope equipped with Nomarski differential interference-contrast optics (model M-2; Olympus, Tokyo, Japan). The width and height of the meristems were measured by the methods of Nagasawa et al. (Nagasawa et al., 1996).

Positional cloning of *FON1*

The *fon1* locus was mapped by using an F_2 population of *fon1-1* and Kasalath (spp. *indica*). First, the locus was mapped to a region between CAPS markers R1028 and E10139 on the distal end of the long arm of chromosome 6 by using 119 *fon1* homozygotes. Then, by using 2,419 F2 plants, the locus was narrowed to a region between two closely linked CAPS markers, M11 (5′-AGACCTGATACGATGC-GAAC-3′, 5′-TCCTTCATGGTTGGAACTAG-3′; *Afa*I digestion) and M54 (5′-CACCGCCACCTTCTACGG-3′, GTGGCCGTCACCGT-CACC-3′; *Hha*I digestion), which were designed by comparing genomic sequences of the *japonica* and *indica*. This region of 150 kb in length was present in two YAC contigs, AP003614 and AP003769.

Ten putative genes were identified by using a gene prediction program, Rice Automated Annotation System (http://RiceGAAS.dna.affrc.go.jp). Because a gene highly similar to *Arabidopsis CLV1* was found among these ten putative genes, the genomic sequences of the *FON1* candidate gene in the *fon1-1* and *fon1-2* mutants were determined by a method of direct sequencing after PCR amplification. Primers were selected on the basis of the *CLV1* gene using the database of the rice genomic sequence. A genomic DNA fragment including the *FON1* candidate was isolated from the genomic library. For complementation, a 7.7 kb fragment including 2.2 kb of sequence directly upstream of the initiation codon was cloned into a binary vector and introduced into *fon1-1* by *Agrobacterium*-mediated transformation (Hiei et al., 1994). A *FON1* 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

To detect *FON1* transcripts unambiguously, two probes derived from independent regions of the gene were used: the first region (probe A; 437 bp), consisting of the last 241 bp of the coding region and 196 bp of the 3′ UTR, was amplified with the primers 5′-ACTG-GGTCCGCAAGGTGAC-3′ and 5′-AGATCATTAGCCCCGGAG-3′; the second region (probe B; 396 bp), consisting of 381 bp of the 5′ UTR and 15 bp of the coding region, was amplified with the primers 5′-ACCCCTACTAGTTCAAACG-3′ and 5′-GAGAGTAGGAGGCA-TTGTGA-3′. The amplified DNA fragments were cloned into TA cloning vector (Novagen, Madison) and used for RNA synthesis and labeling. The *FON1* expression patterns detected with these two probes coincided with each other. *OSH1* and *DL* probes were prepared as described in the original papers (Sato et al., 1996; Yamaguchi et al., 2004).

Plant materials were fixed and dehydrated by the methods of Itoh et al. (Itoh et al., 2000). They were embedded in Paraplast Plus (Oxford Labware, St Louis) after replacement with xylene. Microtome sections (8 μm) were mounted on glass slides coated with Vectabond (Vector Laboratories, Burlingame). RNA probes were labeled with digoxigenin using a DIG labeling kit (Roche, Mannheim). In situ hybridization and immunological detection of the signals were carried out by the methods of Kouchi and Hata (Kouchi and Hata, 1993).

Results

FON1 is necessary for regulating floral organ number

Rice flowers have a pistil in the central whorl and six stamens around it. In the next outer whorl, two lodicules corresponding to petals in dicots are produced. These floral organs are subtended by a palea and a lemma (Fig. 1A). Mutations in the *fon1* locus cause an increase in the number of floral organs. In *fon1-1*, the number of pistils increased to two or three in all flowers examined, but there were small or no changes in the number of stamens, lodicules, palea or lemma (Figs 1, 2). However, the *fon1-2* mutation affected all floral organs (Fig. 1C). More than 70% of the *fon1-2* flowers had four or five pistils, and in a few extreme cases the flowers had nine pistils (Fig. 2). Increases in stamen number, varying from seven to 12, were observed in almost all *fon1-2* flowers. Extra lodicules or palea/lemma-like organs were observed in about a third of *fon1-2* flowers. Thus, the *fon1-2* allele seems to have a stronger mutation than the *fon1-1* allele. In both alleles, the effects were more evident in the inner than in the outer floral organs.

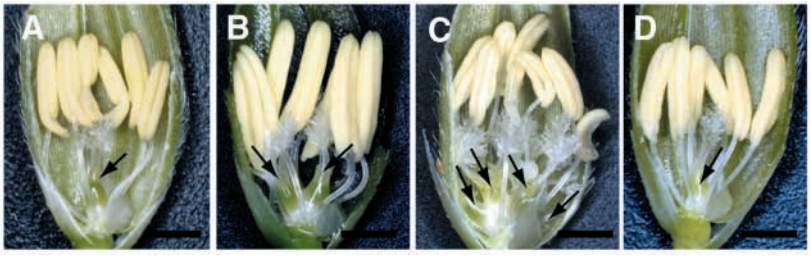

Fig. 1. Flower phenotype. (A) Wild-type flower (T65). (B) *fon1-1* flower with seven stamens and two pistils. (C) *fon1-2* flower with seven stamens and four pistils. (D) *fon1-1* flower rescued by introducing the genomic segments containing the *FON1* candidate gene. Arrows indicate pistils. Scale bars: 2 mm.

Genetic analysis revealed that the *fon1-2* mutation was inherited as a recessive trait. First, the numbers of floral organs in all flowers (n=50) in F1 plants generated between the wild type and *fon1-2* were the same as the number of floral organs in wild-type flowers (i.e. one lemma, one palea, two lodicules, six stamens and one pistil). Second, segregation of wild type and *fon1-2* in the F2 population was in accordance with a 3:1 ratio (36 wild type, 11 *fon1-2*; χ^2=0.064, P>0.7). We found no flowers that showed an increase in the floral organ number out of 400 flowers of the F2 plants examined that were heterozygous for the *fon1* locus.

Flower organ development in earlier stages

To study the abnormalities in *fon1* flowers in detail, we examined the phenotypes of the strong *fon1-2* mutant flowers in the early stages of development by SEM. In wild-type rice, the lemma initiated at the flank of the flower meristem close to the inflorescence axis and the palea initiated at the flank opposite to the lemma (Fig. 3A). By contrast, ectopic palea/lemma-like organs developed on the lateral side (Fig. 3D) or on the palea side of the meristem in *fon1-2* (Fig. 3E). In the former case, a pair of palea/lemma-like organs developed on the inside of the palea, suggesting that an additional whorl had formed (Fig. 3D). In the latter case, an additional palea/lemma-like organ developed on the inside of the palea (data not shown) or two palea-like organs developed next to each other in the original whorl (Fig. 3E). These palea-like organs, which were arranged in parallel, were thinner than wild-type palea in the mature flower. In wild type, two lodicules formed at the flank of the meristem adjacent to the lemma. In *fon1-2*, extra lodicules, usually two, were produced at the flank adjacent to the palea (data not shown). Six stamens developed in a concentric whorl in wild-type flowers. In many of the *fon1-2* flowers, by contrast, extra stamens were produced in the same whorl as the original stamens (Fig. 3D). The maximum number of stamens formed within the same whorl was ten. In other *fon1-2* flowers, extra stamens developed in two concentric whorls, suggesting that an extra whorl for stamens was produced in these flowers (Fig. 3F).

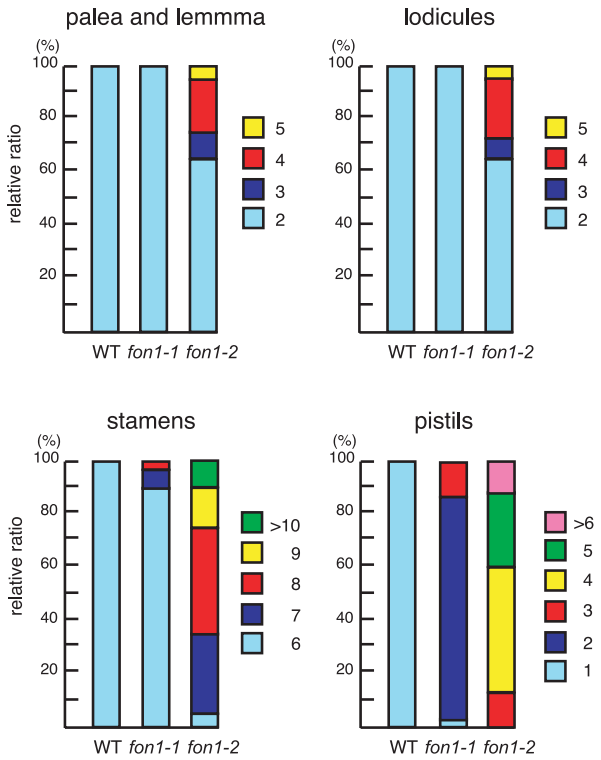

Fig. 2. Number of floral organs in wild type (WT) and *fon1* mutants. For each strain, 100 flowers were examined.

Carpel development was markedly affected by the *fon1* mutations. In wild type, the carpel primordia arose at the flower meristem near the lemma and developed from the flank of the meristem towards the opposite side, enclosing the meristem (Yamaguchi et al., 2004). After the carpel primordia fused at the top, two styles developed (Fig. 3B,C). In *fon1-2*, however, the floral meristem was enlarged and became a long oval dome-like structure before carpel initiation (Fig. 3F). The carpel primordia first arose at the flank of the floral meristem near the lemma, but extra carpel primordia for the second pistil subsequently arose in the opposite flank of the meristem (Fig. 3G). Thus, these two initial sets of ectopic carpel primordia arose in an alternate phyllotaxy, as described previously (Nagasawa et al., 1996). The region between the two independent carpel primordia remained morphologically undifferentiated and maintained meristem activity (see below). Subsequently, carpel primordia arose repeatedly and

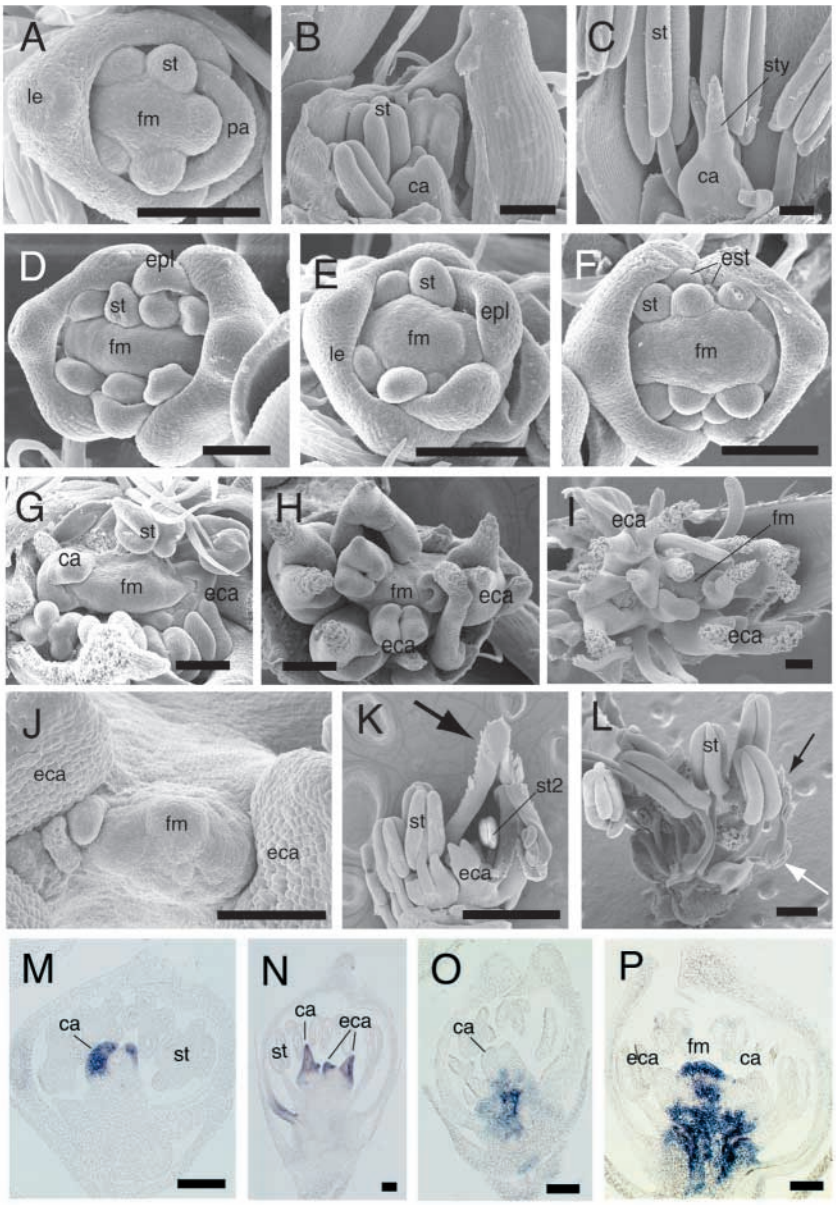

Fig. 3. SEM images and specific expression patterns of *DL* and *OSH1*. (A) Wild-type flower at initiation of stamen primordia. (B) Wild-type flower that has started to form two styles in the pistil. (C) Almost-mature wild-type flower, in which stigmatic hairs are about to begin to grow on the styles. (D) *fon1-2* flower. The floral meristem is enlarged along the palea-lemma axis. Additional stamens form in the same whorl as original stamens, and palea/lemma-like organs form ectopically on the lateral side of the meristem. (E) *fon1-2* flower that develops two ectopic palea-like organs side by side. (F) *fon1-2* flower that develops ectopic stamens in two whorls. (G) *fon1-2* flower that has an enlarged floral meristem and develops two sets of carpels in an alternate phyllotaxy. (H,I) *fon1-2* flowers that produce a number of pistils. (J) Close-up of the central region that remains undifferentiated in almost-mature *fon1-2* flowers. (K) A secondary floret produced in *fon1-2*. The secondary flower (large arrow) has a few stamens and a carpel subtended with palea and lemma (the palea is removed to show the inside of the secondary floret). (L) Partial loss of stamen identity in *fon1-2*. Stigmatic hairs (black arrow) are produced at the top of the stamen instead of the anther and an undifferentiated cell mass has formed at the base of the filament (white arrow). (M,N) Spatial expression of *DL*. (M) Wild type; (N) *fon1-2*. Three sets of carpels develop in *fon1-2*. (O,P) Spatial expression of *OSH1*. (O) Wild type; (P) *fon1-2*. Whereas *OSH1* expression disappears after carpel development in wild type, its expression is maintained in the floral meristem in the center of the flower in *fon1-2*. ca, carpel; eca, ectopic carpel; epl, ectopic palea/lemma-like organ; est, ectopic stamen that develops in an additional third whorl; fm, floral meristem; le, lemma; pa, palea; st, stamen; st2, stamen that develops in the secondary flower; sty, style. Scale bars: 100 μm in A-J,M-P; 500 μm in K,L.

indeterminately. Eventually, many pistils stood together in large numbers (Fig. 3H,I). The pattern of carpel appearance was largely irregular after the third set of carpels emerged, and the floral meristems remained even after the production of many sets of carpels (Fig. 3H-J).

A few *fon1-2* flowers produced a small secondary floret adjacent to the stamens (Fig. 3K). This secondary floret consisted of a pistil, a few stamens, and a pair of palea and lemma that enclosed the reproductive organs. This structure might be associated with a splitting of the flower meristem after the initiation of palea primordia in the primary flower: after separation, the detached meristem could result in the development of a secondary flower independent of the primary flower. Floral organ identity was affected by the *fon1-2* mutation at only a low frequency. For example, lodicules were transformed into palea/lemma-like organs (data not shown) or an anther was partially transformed into stigma-like organs (Fig. 3L).

To verify the results of this phenotype analysis, we examined the expression patterns of two genes, *DROOPING LEAF* (*DL*) and *OSH1*. *DL* regulates carpel identity in rice and is expressed exclusively in carpel primordia (Yamaguchi et al., 2004). *DL* expression in wild type was detected in carpel primordia, which then developed into a pistil enclosing an ovule (Fig. 3M), whereas *DL* transcripts in *fon1-2* were detected in the multiple sets of carpel primordia, which developed into independent pistils (Fig. 3N). *OSH1* is a molecular marker of meristematic indeterminate cells in rice and is expressed in the floral meristem (Sato et al., 1996; Yamaguchi et al., 2004). In wild type, *OSH1* expression was downregulated when the floral organs began to initiate and completely disappeared when the carpel primordia began to develop (Fig. 3O). However, expression of *OSH1* continued in the region around the carpel primordia in *fon1-2* (Fig. 3P). Together with the phenotypic analysis by SEM, this result suggests that the floral meristem maintains its activity and loses its determinacy in *fon1-2*, even after the initiation of several sets of carpel primordia.

fon1 affects predominantly floral meristem size

The control of floral organ number is closely associated with

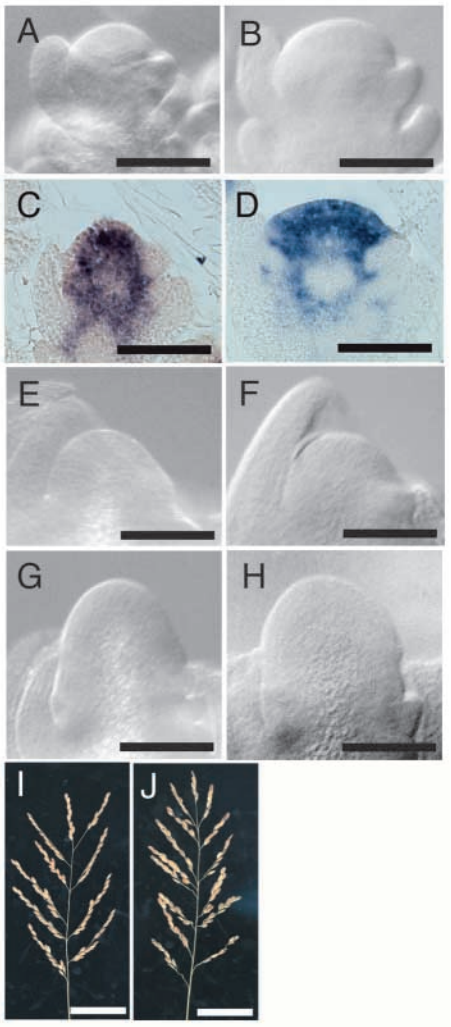

Fig. 4. Meristem sizes and inflorescences in wild-type and *fon1-2*. (A,C,E,G,I) Wild type. (B,D,F,H,J) *fon1-2*. (A,B) Floral meristems. (C,D) Expression of *OSH1* in the floral meristem. The expression domain is expanded in *fon1-2*, but the spatial pattern does not differ from that in wild type. (E,F) Vegetative shoot apical meristems. (G,H) Inflorescence meristems. (I,J) Inflorescence phenotypes after seed production. (A,B,E-H) Micrographs viewed under Nomarski optics. Scale bars: 100 μm in A-H; 5 cm in I,J.

floral meristem size in *Arabidopsis clv* mutants (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998). We examined meristem sizes in the strong *fon1-2* allele. At an early flowering stage, the floral meristem of *fon1-2* was apparently larger than that of wild type (Fig. 4A,B; Table 1). After initiation of the stamens, the *fon1-2* floral meristem became considerably larger and increased in length along the palea-lemma axis (Fig. 3D,F). The expression pattern of *OSH1* suggested that the number of meristematic cells increased in the *fon1-2* floral meristem (Fig. 4C,D). Consistent with the floral phenotype, the floral meristem size of *fon1-2* was larger than that of *fon1-1*.

However, no difference was observed in the size of vegetative meristems of wild type, *fon1-2* and *fon1-1* mutants (Fig. 4E,F; Table 1). The vegetative growth phenotypes of *fon1-2* were indistinguishable from those of wild type. The inflorescence meristem size of *fon1-2* was slightly larger than

Table 1. Sizes of the floral and shoot apical meristems

	Floral meristem size (μm)			Vegetative meristem size (μm)		
Strain	Width	Height	*n*	Width	Height	*n*
T65	97±7	41±6	12	123±9	59±9	11
fon1-1	119±9	44±4	6	118±12	53±12	7
fon1-2	137±5	59±6	13	125±13	59±9	9

that of wild type (Fig. 4G,H). Probably associated with this slight increase in inflorescence meristem size, the number of primary rachis branches increased by about 1.4-fold in *fon1-2* (15.0±0.9), when compared with wild type (10.7±1.7) (Fig. 4I,J). However, marked changes such as fasciation were not observed in *fon1-2*. Thus, the strong *fon1-2* mutation does not profoundly affect the size of vegetative and inflorescence meristems or the phenotypes of the plant bodies derived from them. These results suggest that *FON1* regulates mainly the floral meristem in rice.

Isolation of *FON1*

To elucidate the molecular function of *FON1*, we set out to isolate the gene by positional cloning (Fig. 5A). First, the *fon1* locus was mapped to the distal end of the long arm of rice chromosome 6. Using about 2400 F2 plants, we subsequently confined the *fon1* locus to a region of about 150 kb. Using the rice genomic sequence database and the RiceGAAS program, which efficiently predicts putative genes in rice, we identified ten genes in this region. Among them, we identified a putative gene that encodes an LRR-type receptor kinase similar to *Arabidopsis* CLV1 (Clark et al., 1997).

clv1 mutants share several characteristics with *fon1* mutants, such as an increase in floral organ number and enlargement of the floral meristem. Thus, the *CLV1*-like gene was a strong candidate for the *FON1* gene. We therefore determined the nucleotide sequence of this candidate and identified the nucleotides that cause amino acid substitution in both *fon1-1* and *fon1-2* (Fig. 5B-D). The mutant phenotype of *fon1-1* was rescued when a 7.7 kb genomic fragment containing the candidate gene was introduced into *fon1-1* rice plants (Fig. 1D). These results clearly indicate that the *CLV1*-like gene identified by our positional cloning approach was derived from the *fon1* locus.

FON1 encodes a CLV1-like receptor kinase

We determined the positions of introns in *FON1* by sequencing the RT-PCR product generated with *FON1*-specific primers and the predicted open reading frame. *FON1* encodes a putative protein of 994 amino acids (Fig. 5B), comprising a putative hydrophobic signal peptide, LRRs, a transmembrane domain and a cytoplasmic serine-threonine kinase domain. This overall structure of FON1 closely resembles that of CLV1 (Clark et al., 1997). The number of LRRs (22) and the two cysteine pairs that flank the LRR domain are conserved in both proteins (Fig. 5C). Between FON1 and CLV1, the amino acid identities of the LRR and kinase domains are 55.1 and 73.2%, respectively (Fig. 5B-D). In *fon1-1*, proline (903) is substituted for leucine. This amino acid is located just downstream of subdomain IX of the kinase domain (Die'vart and Clark, 2003), and is shared by other plant LRR-type receptor-like kinases such as CLV1, BRI1 and HAR1 (Clark et al., 1997; Li and Chory, 1997; Nishimura

et al., 2002). In *fon1-2*, glycine (205) is substituted for serine in the sixth repeat of the LRR domain. This amino acid substituted in *fon1-2* is the same amino acid that is altered in *clv1-4* (G201E), one of several strong alleles of *CLV1* that has a dominant-negative effect (Die'vart et al., 2003).

There are numerous LRR-type receptor-like kinases in plant

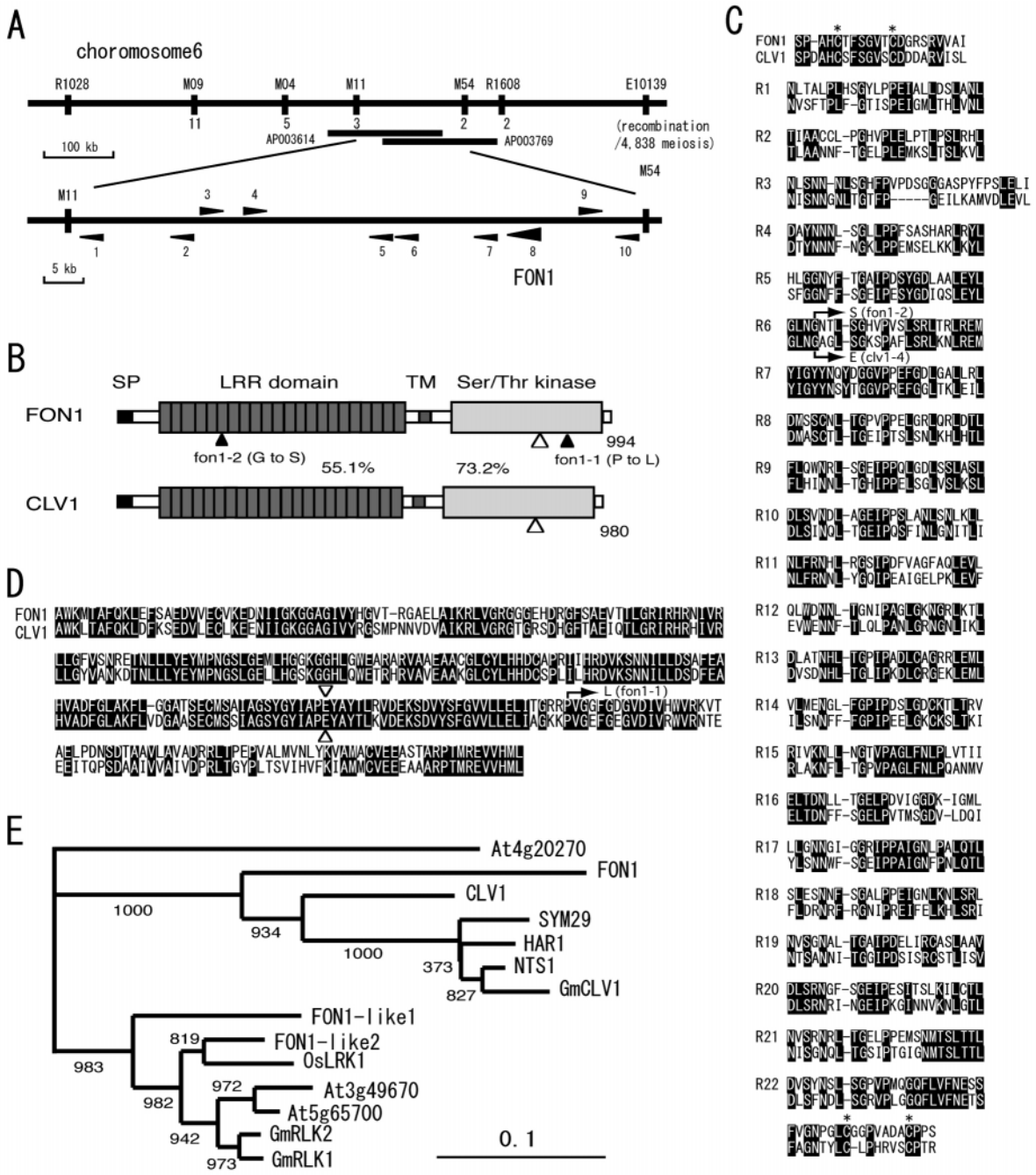

Fig. 5. Isolation of the *FON1* gene and characteristics of the FON1 protein. (A) Positional cloning of *FON1*. (B) Schematic representation of FON1 and CLV1. The LRR and Ser/Thr kinase domains are shown by rectangles, and the amino acid identities of these two domains are indicated. Closed triangles indicate the mutation sites of *fon1-1* and *fon1-2*; open triangle shows the position of the intron. SP, signal peptide; TM, transmembrane domain. (C) Amino acid alignment of the LRR domain and its flanking sequence, with conserved cysteine residues indicated (asterisks). Amino acid numbers: FON1 54-628, CLV1 54-623. Sequences of the cDNA and genomic DNA of *FON1* are deposited in DDBJ under accession numbers AB182388 and AB182389, respectively. (D) Amino acid alignment of the Ser/Thr kinase domain. Amino acid numbers: FON1 684-977, CLV1 672-967. Open triangle shows the position of the intron. (E) Phylogenic tree of LRR-type receptor kinases similar to FON1 and CLV1. Amino acids of the kinase domain (D) were compared and the tree was constructed by neighbor-joining methods (Saitou and Nei, 1987) using At4g20270 as a root. Numbers indicate bootstrap values. The intron positions of uncharacterized rice (FON1-like1, AC099732; FON1-like2, AC092781) and *Arabidopsis* (At4g20270, At3g49670, At5g65700) genes were deduced by comparing amino acid sequences and by the GT-AG rule for spliceosomal introns. Except for OsLRK1 (rice), the other known proteins are legume proteins, including those responsible for hypernodulation (HAR1, SYM29, NTS1).

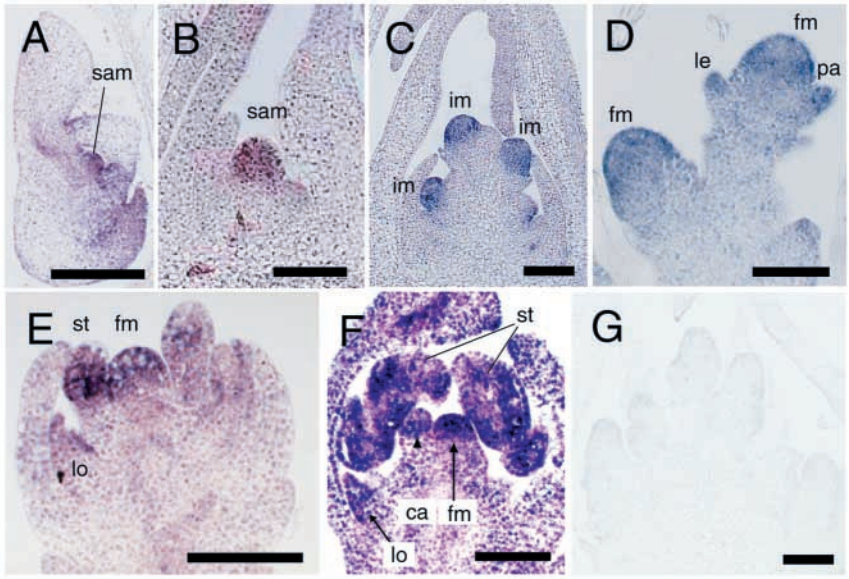

Fig. 6. In situ localization of the *FON1* transcripts in wild-type rice. (A) Embryo. *FON1* transcripts are detected in the shoot apical meristem. (B) Vegetative shoot apical meristem. (C) Inflorescence meristems at the primary branch stage. (D) Spikelets. *FON1* transcripts are distributed throughout the floral meristems (the left meristem is the earliest stage of the floral meristem). In addition to floral meristems, *FON1* transcripts are detected in the palea and lemma primordia. (E) A floret before carpel initiation. *FON1* transcripts are detected in the lodicules, stamen primordia and the floral meristem. (F) A floret at the stage for carpel development. *FON1* transcripts are expressed in the carpel primordia (arrows) and the floral meristem in addition to stamen and lodicules. (G) Inflorescences at the secondary branch stage. No transcripts are observed in the meristems. (A-F) Antisense probe; (G) sense probe. fm, floral meristem; ca, carpel, le, lemma; lo, lodicule; pa, palea; sam, shoot apical meristem; st, stamen. Scale bars: 100 μm.

genomes. To clarify the evolutionary relationship between *FON1* and *CLV1*, we identified genes with high homology to *FON1* and *CLV1* from rice and *Arabidopsis* genomic sequences and predicted the amino acid sequences of these genes. Together with proteins known to belong to this class, the kinase domains were used to construct a phylogenetic tree by the neighbor-joining method (Fig. 5E). The result indicated that *FON1* constitutes a small clade with *CLV1* and other genes encoding receptor-like kinases responsible for hypernodulation in legumes such as *HAR1* (Nishimura et al., 2002). Rice and *Arabidopsis* genes other than *FON1* and *CLV1* are not involved in this clade, which strongly suggests that *FON1* is an ortholog of *CLV1* in rice.

Spatial expression patterns of *FON1*

To elucidate the function of *FON1*, we examined its mRNA expression patterns by in situ hybridization. *FON1* transcripts were initially detected in the shoot meristem in the embryo (Fig. 6A). Transcripts were subsequently detected specifically in the shoot apical meristem, but not in any other regions of the vegetative apex (Fig. 6B). After transition to the reproductive phase, *FON1* transcripts were detected in the primary and secondary rachis branch meristems (Fig. 6C), which correspond to the inflorescence meristems. Subsequently, *FON1* was expressed in the floral meristems from the initial stage of floral development (Fig. 6D). *FON1* continued to be expressed in the floral meristem (Fig. 6E), and its expression persisted even after the initiation of carpel primordia (Fig. 6F). Thus, *FON1* is expressed in all of the meristems responsible for development of the aerial part of rice. *FON1* transcripts were uniformly distributed throughout all types of meristem. This expression pattern differs from that of *CLV1*, which is mainly localized to L3 cells in *Arabidopsis*. Therefore, we analyzed *FON1* expression again using two probes derived from independent regions of the gene and confirmed with both probes that *FON1* was expressed uniformly throughout the meristems.

In addition to the meristems, *FON1* was also expressed in floral organ primordia, such as the palea (Fig. 6D), lemma (Fig. 6D), lodicules (Fig. 6E,F), stamens (Fig. 6E,F), carpels (Fig. 6F) and ovules (data not shown). As a control, no signal was detected in floral meristems and floral organ primordia when sense RNA was used as a probe (Fig. 6G).

Discussion

Mutations at the *fon1* locus cause enlargement of the floral meristem, resulting in an increase in the number of floral organs. Molecular cloning of the causative gene has shown that *FON1* encodes an LRR-type receptor kinase; furthermore, evolutionary analysis clearly indicates that *FON1* is an ortholog of *Arabidopsis CLV1*. Together with the finding that the *CLV2* ortholog *FEA2* regulates female inflorescence size in maize (Taguchi-Shiobara et al., 2001), our results indicate that the *CLV* signaling system that regulates meristem size in *Arabidopsis* is conserved in the grass family.

FON1 regulates maintenance of the floral meristem

The increase in floral organ number in the *fon1* mutants may be closely associated with enlargement of the floral meristem. The meristem size is much larger in *fon1-2* than in *fon1-1*, and the increase in floral organ number is consistently much greater in *fon1-2* than in *fon1-1*. Furthermore, the extent of meristem enlargement in *fon1-2* is much greater at later stages of floral development than at earlier stages. Consistent with this, the increase in floral organ number is more evident in the inner than in the outer whorls.

Floral organs increase in two ways: first, additional floral organs are formed in the same whorl; and second, floral organs develop in the additional whorls that form. In the innermost whorls, carpel primordia arise repeatedly among the carpel primordia that have already been produced. This repetitious production of carpel primordia is associated with the indeterminate nature of floral primordia. For example, *OSH1*, which marks meristematic cells, continues to be expressed in the center of the floral meristem even after the carpel primordia have been produced, confirming that determinacy is lost in the *fon1* floral meristem. Although floral meristem determinacy is affected in loss-of-function mutants of both *DL* and class C MADS-box genes (Yamaguchi et al., 2004) (T. Yamaguchi and

H.-Y.H., unpublished), stamens and carpels, whose identities are regulated by these genes, develop normally in the *fon1* mutants. Therefore, the loss of determinacy in *fon1* seems to be independent of the function of *DL* and class C genes. The continued expression of *OSH1* in the center of floral meristems in the *fon1* mutant and the uniform expression of *FON1* throughout the meristem also suggest that the control of floral meristem determinacy by *FON1* is indirect.

FON1 is expressed in all types of meristem in the aerial part of rice, from the shoot apical meristem in embryos to the floral meristem. Unlike the floral meristem, however, the vegetative and inflorescence meristems show no or only slight abnormalities in *fon1* mutants. Because *fon1* is inherited as a recessive trait, there may be genetic redundancy in the pathways that maintain the vegetative and inflorescence meristems. Because complete loss-of-function mutants of *CLV1* show weak *clv* phenotypes in *Arabidopsis*, the strong *clv* phenotypes are thought to be caused by a dominant-negative effect of the mutated protein, and the existence of an RLK-X protein that functionally overlaps with CLV1 has been assumed in *Arabidopsis* (Die'vart et al., 2003). Analogous to this, it is possible that the gene corresponding RLK-X in rice is not expressed in the floral meristem, but is expressed and functions in the vegetative and inflorescence meristems. In the latter case, the mutation in the FON1 proteins may not cause a dominant-negative effect in the *fon1* mutants. This hypothesis is consistent with both the recessive nature of the *fon1* mutation and the reduced effect of the mutation in the vegetative and inflorescence phases. The phylogenetic tree shows that three genes encoding LRR receptor kinase are close to *FON1* (Fig. 5E). These genes are possible candidates for sharing functional redundancy with *FON1* in the vegetative meristem.

Spatial expression patterns of *FON1*

FON1 transcripts are distributed throughout the whole meristem and in all types of meristem. This pattern of expression is in high contrast to that of *CLV1*, which is predominantly expressed in the L3 cell layer of the meristems (Clark et al., 1997). CLV1 functions on perception of CLV3 ligands that are secreted from stem cells in the L1 and L2 layers (Rojo et al., 2002; Lehard and Laux, 2003). CLV1 transfers a signal that negatively regulates *WUS*, which is expressed in a putative organizing center within the *CLV1* expression domain (Brand et al., 2000; Fletcher et al., 1999; Mayer et al., 1998; Schoof et al., 2000). Thus, CLV1 functions in a signaling pathway that communicates between two distinct domains, the stem cell region and an organizing center in the meristem. Expression of *FON1* throughout the whole meristem would therefore seem to be irrelevant to the communication between two independent domains.

Although there is no evidence that homologs of *CLV3* and *WUS* are expressed and function in rice in a way similar to that in *Arabidopsis*, we can propose two hypotheses for FON1 function on the basis of the *Arabidopsis* CLV signaling system. If LRR-receptor kinases work as heterodimers as proposed by Die'vart et al. (Die'vart et al., 2003), it is possible that a putative protein partner of FON1 may function only in the L3 layer; indeed, whereas *CLV1* is expressed in a domain-specific manner, CLV2, is expressed ubiquitously in *Arabidopsis* (Jeong et al., 1999). Alternatively, mature FON1 proteins may be localized to the L3 layer in rice through post-transcriptional regulation. Both hypotheses would result in the distribution of a functional receptor complex of FON1, similar to the distribution of *CLV1* expression. However, we do not rule out a third hypothesis that the signaling system in rice differs from that in *Arabidopsis*. In this hypothesis, the presence of a *WUS* homolog in an organizing center is not postulated; instead, it is assumed that the FON1 receptor in stem cells transfers the signal from the ligand to a putative factor that suppresses stem cell proliferation in the L1 and L2 layers without passing through an organizer (domain-autonomous regulation). Finally, it is also possible that the difference in the expression pattern between *FON1* and *CLV1* is not essential for the function of both proteins because expression of *CLV1* under the control of the widely expressed *ELECTA* promoter does not cause any changes in the meristem size in *Arabidopsis* (Die'vart et al., 2003).

To understand the genetic mechanism that regulates meristem maintenance in rice, it will be necessary to isolate the genes that function in this pathway. The *fon2* mutant, the flowers of which have an increased number of floral organs similar to those of *fon1* (Nagasawa et al., 1996), may provide clues towards identifying these genes. In addition, it will be essential to determine whether a WUS-like organizer functions in rice meristems.

FON1 transcripts are expressed in the primordia of all floral organs including lemma, palea, lodicules, stamens and carpels, from their inception to later floral stages. This expression of *FON1* seems to be specific to rice, because *CLV1* is not expressed in the floral organ primordia of *Arabidopsis* (Clark et al., 1997). The sizes and identities of floral organs were almost normal, even in the strong *fon1-2* mutant. Therefore, even if *FON1* regulates the development of floral organs, the *fon1* mutation may be masked by other genes that share functional redundancy with *FON1*. We observed slight abnormalities in the floral organs at low frequency. These defects seem to be side-effects of the enlargement of the meristem and do not seem to be associated with the function of *FON1* expressed in these floral organs, because the weaker allele *fon1-1* also produces flowers with abnormal floral organ identities at a similar frequency (Nagasawa et al., 1996). It will be of great interest to examine whether *FON1* is involved in the regulation of floral organ size.

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