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The *OsTB1* gene negatively regulates lateral branching in rice

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Summary

Although the shoot apical meristem (SAM) is ultimately responsible for post-embryonic development in higher plants, lateral meristems also play an important role in determining the final morphology of the above-ground part. Axillary buds developing at the axils of leaves produce additional shoot systems, lateral branches. The rice *TB1* gene (*OsTB1*) was first identified based on its sequence similarity with maize *TEOSINTE BRANCHED 1* (*TB1*), which is involved in lateral branching in maize. Both genes encode putative transcription factors carrying a basic helix–loop–helix type of DNA-binding motif, named the TCP domain. The genetic locus of *OsTB1* suggested that *OsTB1* is a real counterpart of maize *TB1*. Transgenic rice plants overexpressing *OsTB1* exhibited markedly reduced lateral branching without the propagation of axillary buds being affected. We also demonstrated that a rice strain carrying a classical morphological marker mutation, *fine culm 1* (*fc1*), contain the loss-of-function mutation of *OsTB1* and exhibits enhanced lateral branching. Expression of *OsTB1*, as examined with a putative promoter–glucuronidase (*GUS*) gene fusion, was observed throughout the axillary bud, as well as the basal part of the shoot apical meristem, vascular tissues in the pith and the lamina joint. Taking these data together, we concluded that OsTB1 functions as a negative regulator for lateral branching in rice, presumably through expression in axillary buds.

Keywords: lateral branching, TCP family, transcription factor, axillary bud.

Introduction

In flowering plants, the shoot apical meristem (SAM) is ultimately responsible for post-embryonic development of the primary shoot architecture. However, lateral meristems also play an important role in determining the final morphology of the above-ground part of plants. Axillary buds developing at the axils of leaves produce additional shoot systems, lateral branches, which grow in a similar manner to the primary shoot. Although the growth of lateral branches is affected by several environmental conditions, their number and growth are genetically controlled in each plant species.

Recent intensive genetic studies revealed the molecular mechanism as well as the genes underlying the formation and maintenance of SAM (Bowman and Eshed, 2000). However, little is known about the generation and growth control of axillary buds. A series of physiological studies have revealed that two phytohormones, auxin and cytokinin, are mainly responsible for the growth control of axillary buds. Generally known as apical dominance, auxin provided by the primary shoot apex inhibits the growth of axillary buds, whereas cytokinin relieves the inhibition, resulting in the development of lateral branches (Taiz and Zeiger, 1998). For *Arabidopsis*, tomato and maize, several mutants affecting the process of lateral branching have been isolated. The mutants fall into two classes based on the observed phenotypes. One class affects the propagation of meristems at lateral positions. The *REVOLUTA* (*REV*) gene of *Arabidopsis* appears to be involved in the initiation of axillary bud formation because axillary buds were not formed in the mutant (Otsuga *et al.*, 2001; Talbert *et al.*, 1995). The *Blind* (*Bl*) and *Lateral suppressor* (*Ls*) genes of tomato have been shown to have the same function (Mapelli and Kinet, 1992; Schmitz *et al.*, 2002; Schumacher *et al.*, 1999). The outgrowth of axillary buds is affected by

another class of genes including maize *TEOSINTE BRANCHED 1* (*TB1*) (Doebley *et al.*, 1995).

Certain lesions in the maize *TB1* gene cause enhanced lateral branching, suggesting that TB1 functions as a negative regulator for the growth of axillary buds. The *TB1* gene was cloned and appeared to encode a putative transcription factor carrying a basic helix–loop–helix (bHLH)-type DNA-binding motif (Doebley *et al.*, 1997). The primary sequence of the DNA-binding motif, named the TCP domain, was then revealed to be conserved in several genes in a wide variety of higher plants including monocot and dicot species (Cubas *et al.*, 1999). The name TCP stands for *TB1* in maize, *CYCLOIDEA* (*CYC*) in *Antirrhinum* and PCF proteins in rice. The *Antirrhinum CYC* gene, together with the homologous gene, *DICHOTOMA* (*DICH*), is involved in the dorsoventral asymmetry of flowers (Luo *et al.*, 1995, 1999). On yeast one-hybrid screening, rice PCF1 and 2 were isolated according to their ability to bind to a promoter element that is essential for meristematic tissue-specific expression of the rice proliferating cell nuclear antigen (*PCNA*) gene (Kosugi and Ohashi, 1997). Although their precise molecular functions have not been elucidated sufficiently, they are implicated in the growth and development of lateral organs.

In this study, we describe a genetic study for elucidation of the relevant biological function of the rice *TB1* gene, a homolog of the maize *TB1* gene. Rice *TB1* (referred to as *OsTB1* below) was first identified based on its structural similarity to the maize *TB1* (Lukens and Doebley, 2001). The OsTB1 protein contains three significant sequence motifs, the SP, TCP and R domains. The R domain contains basic amino acid residues and is conserved in subpopulations of the TCP family. The SP domain contains a number of serine and proline residues, and is found in a limited number of members whose primary structures entirely match that of TB1. Although the precise molecular functions of these domains except for the TCP domain remain unknown, the close resemblance of the primary structures of OsTB1 and maize TB1 together with the entire sequences strongly suggests that the biological function of OsTB1 is similar to that of maize TB1. A series of genetic and reverse-genetic analyses thus conducted indicated that OsTB1 is a negative regulator for lateral branching in rice.

Results

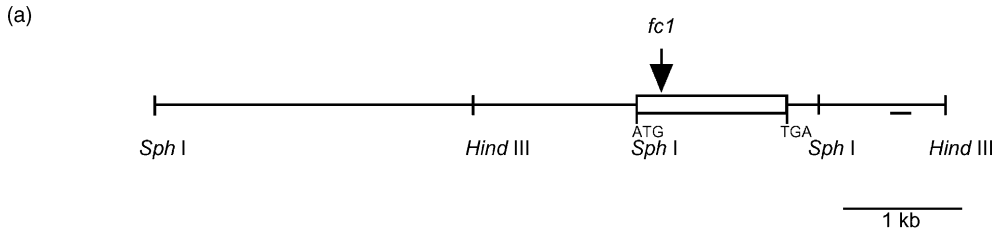
Genetic mapping of the OsTB1 *gene*

The *OsTB1* gene was first identified based on the close sequence similarity to the maize *TB1* gene, which plays an important role in the apical dominance (Doebley *et al.*, 1995; Lukens and Doebley, 2001). To address the issue of whether or not *OsTB1* is a real counterpart of maize *TB1* in terms of the biological function, we first determined the genetic locus of *OsTB1* on the chromosome. Because the local gene content as well as the gene order is well conserved between the rice and maize genomes (Ahn and Tanksley, 1993), so-called genome synteny, the rice gene orthologous to maize *TB1* would share the same genomic location as that of maize *TB1*.

According to the previously reported sequence of the *OsTB1* open-reading frame (ORF), we obtained a genomic fragment encompassing *OsTB1* by PCR-based screening of a DNA library constructed from a *japonica* cultivar, Nipponbare (Figure 1a). Sequencing analysis of the resultant DNA fragment revealed a 3626-bp sequence (Figure 1b). The deduced amino acid sequence of the *OsTB1* ORF comprises 388 amino acid residues. Note that the in-frame stop codon was found two codons upstream of the deduced first methionine, suggesting that the methionine is used as an initiation codon. The DNA fragment also contains 1261- and 1198-bp 5′ and 3′-non-coding regions, respectively. We also determined the nucleotide sequence of the corresponding region of an *indica* cultivar, Kasalath, and identified a sequence polymorphism between the two cultivars. A 20-bp segment within the 3′-non-coding region of *OsTB1* is deleted in Kasalath (Figure 1a,b, underlined nucleotides). PCR-based mapping with a back-crossed recombinant inbred line between the two cultivars precisely mapped the *OsTB1* gene to the bottom end of chromosome 3, with tight linkage to restriction fragment length polymorphism (RFLP) marker C944 (Rice Genome Project, Tsukuba, Japan). The C944 RFLP marker is closely linked to the *OSH1* gene encoding a homeobox protein, which is known to be a functional ortholog of maize *KNOTTED1* (*KN1*) (Matsuoka *et al.*, 1993). The bottom end of rice chromosome 3 including *OSH1* as well as *OsTB1* is closely related, with regard to genome synteny, to the bottom end of maize chromosome 1 including *KN1* and *TB1* (Ahn and Tanksley, 1993). The close genetic linkage between the *OsTB1* and *OSH1* loci is thus consistent with the view that the *OsTB1* gene is a functional ortholog of maize *TB1*. It should be noted that the *OsTB1* gene is the most homologous gene to maize *TB1* in a whole rice genome reported during the course of this study (Chen *et al.*, 2002).

Ectopic overproduction of OsTB1 *significantly reduced lateral branching*

Certain lesions of maize *TB1* cause enhanced lateral branching, suggesting that the TB1 protein has the ability to repress the outgrowth of axillary buds (Doebley *et al.*, 1995, 1997). This led us to the idea that the overexpression of *OsTB1* would result in reduced lateral branching in rice. To address this issue experimentally, the *OsTB1* ORF was cloned under the control of the rice actin promoter, a strong and constitutive promoter (Zhang *et al.*, 1991), and then the resultant construct was introduced into a wild-type rice



(b)

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1     AAGCTTCTGATTCCAATTCACATGAATAAAACTTTTTCAATCCATCAGGTGCTTTGGAGGGGAATTCGAGCAAGTCAGAATTTGGCTGATGAGGTGTCGGAAAGAGAATCTTTTCCTTAT
121   CCTCAATCTTTTTGCTGAACAACTGGAGTAGTATAGAGATGTGTATATCTAGGAATGGAGGGAGTATGTTTTGTTGAACGAGTGAACGACACTTTTTTTTCATCAAGGGTACCTAGCTAGC
241   TAGGGACCATGTGGTTTTGTGCCAATATGTATGTTGATGTTGATGAGCTTTATATCACAACTCACCAAGGCCCCACGTCGAGTAGGGTTCAGAGGACTTACACAACACATATGGCAAATG
361   GTCCTTACCCCAGAGAAACGCAGAAATGTTCAATGTATGCATATATACACACACGCGATCGATCGAATTCCTGTGTGCTGAGCTGAGACTGGGAGAGGATATATATACAAAAATACAAGT
481   CAGTCGTTTCCTGAAGAAGAGGATTACAAAGGAGGCAGATCCGTTTCCAGCCCCCCCTCCCCTCCCATTCAGCCAATAATAAATGCCCTAGGTCACTTGTAGCCAATAGCGATTCAACAT
601   ACACACATGTACTACCACAGCTTTTGCCCCAACAGTAGTGTCCTTTGCCTTGTATTCTCCTCTCCTCTCCTCTTGGTGATGACTGATCGATGACCACCAGCTGCTGCACATATCA
721   TCCCCTTTCACTCCCTCTCCCTCTCTCTCTCTCTATAAGATGGCAACACCCTGATCTCTAGCTTAGCTGCAGAGGGGAGAGGAACCTCACATCCAAACTCCTAGCTACAACTTGTACTAG
841   CATCCTAAGCAACCAAGCACAACCAAAGCAAGCAAGCACGAACAATTCTTTCTTCCTCTCTACCTCTAGCTGCTGCCTGCCTCCTAATCCTCCTACCCACCACTCCACATGAGCCCATGC
961   TGTGTGCCTGTGTCTGTGTGTGTGTTCTACTCCTACCATGAGAGAAGAGACCAAGCATCAACCAAGCTAGCTAGCTCGTCCTCTCCTCGATCTCTACTTCTCTCTCCCACACAAGCTGAG
1081  CGCCCAGGTAGGCTGCCTGCTAGGTCTCGTGCATGGCCGGACACATCTGATCATAGCCCACTACGGCACTATTCCCCCCTTCCGCCTCGCACGCTGAGAGGTGGCCGGAGAGGGAGGGAG
1201  GCCAGCGAGCAGCAGTAGCAGCAGCAACGCGGCTAGGAGTAAGGAGTCCCATCAGTAAAGCATGCTTCCTTTCTTCGATTCCCCAAGCCCCATGGACATACCGCTTTACCAACAGCTTCA
                                                                  M  L  P  F  F  D  S  P  S  P  M  D  I  P  L  Y  Q  Q  L  Q
1321  GCTCACCCCTCCCTCTCCAAAGCCCGACCACCACCACCACCACCATTCCACCTTCTTCTACTACCACCACCCACCTCCCTCCCCTTCCTTCCCCTCCTTCCCCTCCCCCGCCGCCGC
       L  T  P  P  S  P  K  P  D  H  H  H  H  H  H  S  T  F  F  Y  Y  H  H  H  P  P  P  S  P  S  F  P  S  F  P  S  P  A  A  A
                                                                                                                          ▼
1441  CACGATCGCCTCGCCGTCGCCGGCCATGCACCCCTTCATGGACTTGGAGTTGGAGCCGCATGGGCAGCAGCTGGCGGCGGCGGAGGAGGACGGGGCAGGCGGGCAAGGCGTCGACGCCGG
       T  I  A  S  P  S  P  A  M  H  P  F  M  D  L  E  L  E  P  H  G  Q  Q  L  A  A  A  E  E  D  G  A  G  G  Q  G  V  D  A  G
                                                                                                                          T  P  G
1561  GGTGCCCTTCGGCGTCGACGGAGCGGCGGCGGCCGCGGCGGCGAGGAAGGACCGGCACAGCAAGATAAGCACCGCCGGCGGGATGAGGGACCGGCGGATGCGGCTGTCCCTCGACGTCGC
       V  P  F  G  V  D  G  A  A  A  A  A  A  A  A  R  K  D  R  H  S  K  I  S  T  A  G  G  M  R  D  R  R  M  R  L  S  L  D  V  A
          C  P  S  A  S  T  E  R  R  R  P  R  R  R  G  R  T  G  T  A  R  *
1681  CCGCAAGTTCTTCGCGCTCCAGGACATGCTCGGCTTCGACAAGGCCAGCAAGACGGTGCAATGGCTCCTCAACATGTCCAAGGCCGCCATCCGGGAGATCATGAGCGACGACGCCTCCTC
       R  K  F  F  A  L  Q  D  M  L  G  F  D  K  A  S  K  T  V  Q  W  L  L  N  M  S  K  A  A  I  R  E  I  M  S  D  D  A  S  S
1801  CGTCTGCGAGGAGGACGGCTCCAGCAGCCTCTCCGTCGACGGCAAGCAGCAGCAGCACAGCAACCCGGCGGATCGGGGCGGCGGCGCCGGGGACCACAAGGGCGCCGCTCACGGCCACAG
       V  C  E  E  D  G  S  S  S  L  S  V  D  G  K  Q  Q  Q  H  S  N  P  A  D  R  G  G  G  A  G  D  H  K  G  A  A  H  G  H  S
1921  CGACGGGAAGAAGCCGGCCAAGCCGAGAAGGGCAGCGGCCAACCCGAAGCCACCGCGGCGGCTGGCCAATGCGCACCCCGTCCCCGACAAGGAGTCGCGCGCCAAGGCGAGGGAGCGGGC
       D  G  K  K  P  A  K  P  R  R  A  A  A  N  P  K  P  P  R  R  L  A  N  A  H  P  V  P  D  K  E  S  R  A  K  A  R  E  R  A
2041  GCGGGAGCGGACCAAGGAGAAGAACCGGATGCGGTGGGTCACCCTCGCCTCGGCAATCAGCGTCGAGGCGGCCACCGCGGCGGCGGCCGCGGGGGAGGACAAGTCGCCGACGAGCCCCAG
       R  E  R  T  K  E  K  N  R  M  R  W  V  T  L  A  S  A  I  S  V  E  A  A  T  A  A  A  A  A  G  E  D  K  S  P  T  S  P  S
2161  CAACAACCTGAACCACTCATCGTCCACCAATCTTGTGAGCACCGAATTGGAGGACGGCTCCTCGTCAACGCGCCACAACGGCGTCGGCGTCAGCGGCGGCCGGATGCAAGAAATCTCGGC
       N  N  L  N  H  S  S  S  T  N  L  V  S  T  E  L  E  D  G  S  S  S  T  R  H  N  G  V  G  V  S  G  G  R  M  Q  E  I  S  A
2281  GGCTAGCGAGGCGAGCGACGTGATCATGGCGTTCGCCAACGGCGGCGCGTACGGCGACAGCGGCAGCTACTACCTGCAGCAGCAGCATCAGCAGGATCAGTGGGAGCTCGGCGGCGTCGT
       A  S  E  A  S  D  V  I  M  A  F  A  N  G  G  A  Y  G  D  S  G  S  Y  Y  L  Q  Q  Q  H  Q  Q  D  Q  W  E  L  G  G  V  V

2401  CTACGCCAATTCGCGGCACTACTGCTGATGTGATCATCCATCCACACACGAACGAACGAACGAACGGTACGGCACTAAGATCGAACTCCTGCAGCTACATAATTATCCTTTGCTTCTCAA
       Y  A  N  S  R  H  Y  C  *
2521  GAGTAATAATTCTTGACGTGTTAATTAATCCGGGTGTGTATTAATTCCCTCTTTATTATTTTTTCTCGCGTTTATCCGGAGTTGACTGTGGTGAAGACGAACTTTGGTTTGGTCATCGCA
2641  TGGTGTGCATTGCATATATAGCTAGCACTATCGTCTGATCGATGATTCATCATCAATGGAGTCGTCTAGTATTAATCTGTGTGCATGTTCAGATGCTCTCTGTAAACTGATAAGCTGGAT
2761  ACCTTGATCGCGAGCAGGTAGATATGCCTCTCCTGAATGAGTGAAAAACAGCTGGTTTATAGTTTGCAAAAGTTGGAGACTTGTTACTGTATTAGATCAGATCTGAAGCTAGGACACTAA
2881  CTTTCCAATTTCAATCATAAATAAGAGGGAGCATATGGTGGATACGACCCAGACTTGCAGTAGTGTTTTAGTTCATCATCATCAGATGTTCATAAAATAATTACACTTGGCATGCATGTA
3001  AGATGAATTTCTGTACCAACTCCAGCTTAACAATTGATGTGCCTCTGGAAAAGGTAGTTGATCATTTCATTGATTGGTTCACATGGTAAGTTCCACGATTCAGCTAGTTTTTTCTCCTGA
3121  TTCCTCACTAATTTCCTGATGAGTGGTGGTAAAATTACATCGAATTTTCTTCACTACTGTAAGTAAGTAAGTAAGAATGTTTGAAAGTAAGTAAGAATGTTTGAAAGTAAGGTCATGGT
3241  ACTCATATATGTACAACTCACTAGATGCATATTCCATAAAGTACATTATCCATTATGCATATATGCTATTCCACGCATTGATGGACACACATCATATAGCATCTGTTTGCTGAATGGTAA
3361  TTAAATATTAAGCTAGCACAAACACATCAATGTTTTGATGTTTATTTTGCCAACAGATCGATGCAGAACACTTCAAATTGATGTAGGAAGCACAATGGAAACTAAAATACTTCATTGGTA
3481  ATTAAAAGCCAAATGGACGTGTTTTGTAAGTTGTAACTATATACACACAAGTCTTCCAGCCAACTTATAATGACAACTGAAGTGTGGTATACTATAAAACTGAAGTATGGAAGTACTGCC
3601  CTTAATTAAAAAAAAAGAATAAAGCTT
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Figure 1. Genomic structure of *OsTB1*.

(a) The structure of the chromosomal region encompassing the *OsTB1* gene is schematically shown. The open box represents the *OsTB1* open-reading frame (ORF) with presumed initiation and termination codons. The arrow and underline indicate the position of the mutation found in *fc1* mutants and the sequence polymorphism used for genetic mapping, respectively. Restriction sites used for constructing plasmids are indicated.

(b) Nucleotide sequence of the *Hind*III fragment encompassing the *OsTB1* gene. The nucleotide sequence as well as the deduced amino acid sequence of *OsTB1* are shown. The arrowhead indicates the base deleted in *fc1* and the amino acid sequence changed by the mutation is shown below the correct sequence. Underlined amino acid residues indicate TCP domain. The underlined nucleotides indicate the sequence deleted in Kasalath. The nucleotide sequence has been deposited in the DDBJ/ EMBL/GenBank databases (accession no. AB088343).

plant, Taichung 65, by the *Agrobacterium*-mediated transformation method (Hiei *et al.*, 1994). Seedlings harboring the transgene regenerated from antibiotic-resistant calli were transferred to soil and then grown under standard growth conditions.

We obtained seven independent lines of transgenic plants and named them OP1-7. All of the resultant transgenic plants exhibited severe phenotypes as to lateral branching. Three lines (OP-3, 5 and 7) no longer generated a tiller, a lateral branch in rice, and three (OP-1, 4 and 6) and one (OP-2) only generated one and two tillers, respectively, at 70 days after soil growing, even though a control plant transformed with an empty vector generated six to seven tillers under the same conditions (Figure 2a,b). Although the number of tillers was decreased, axillary buds were still observed at the correct positions, the axils of the leaves at each node of the stem, even in the transgenic plants (Figure 2c). Reverse transcriptase–PCR (RT–PCR) analysis revealed that the *OsTB1* message was increased two to threefold in the transformants compared with that in the wild-type plant (Figure 2d). These results, therefore, suggested that the ectopic overexpression of *OsTB1* results in reduced lateral branching. In addition, the *OsTB1* overproducers exhibited another significant phenotype, the

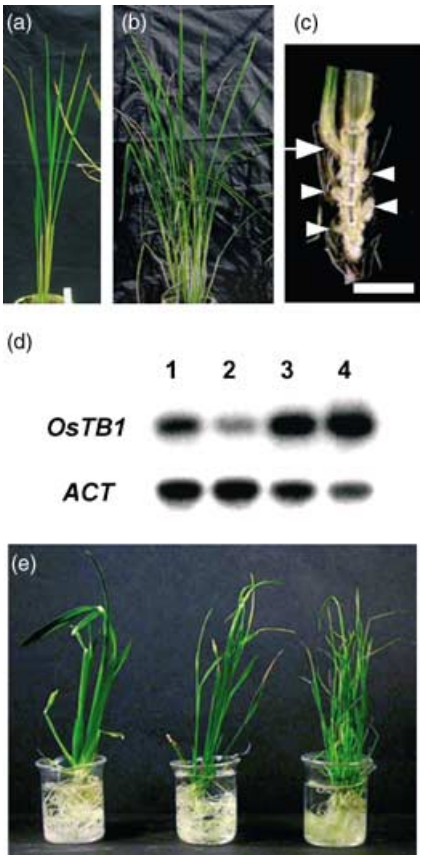

Figure 2. Effect of ectopic overproduction of *OsTB1* in a wild-type rice plant.
(a) Gross morphology of a Taichung 65 plant overproducing *OsTB1* (OP-5)
(b) The control plant transformed with an empty vector. The transgenic plants were grown on soil for 70 days.
(c) Close-up view of the stem of the *OsTB1* overproducer (OP-6). After the leaves had been removed, the stem was longitudinally sectioned. The arrow and arrowheads indicate a uniquely developed tiller and axillary buds, respectively. Bar, 10 mm.
(d) RT–PCR analysis of *OsTB1* expression. Total RNA was prepared from lamina joints and then subjected to RT-PCR analysis followed by Southern hybridization. Lane 1, a wild-type strain (Taichung 65); lane 2, an *fc1* mutant (M56); lanes 3, OP-5; and 4, OP-6.
(e) Morphology of young seedlings of the transgenic plants. OP-5 (left), OP-6 (center), and Taichung 65 transformed with an empty vector (right).

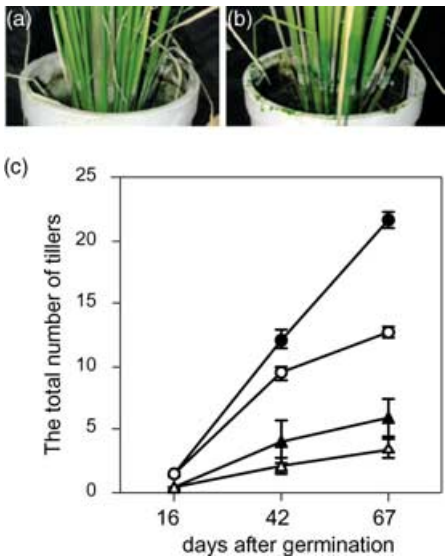

Figure 3. Phenotypes of an *fc1* mutant. Close-up view of the lower part of mature seedlings.
(a,b) An *fc1* mutant (M56, a) and the wild-type strain (Taichung 65, b) were grown on soil for 70 days after germination.
(c) The number of total tillers of the *fc1* and wild-type strains. The *fc1* mutant (M56, ●, ○) and wild-type strain (Taichung 65, ▲, △) were grown at one plant per pot (●, ▲), and three plants per pot (○, △). The number of total tillers were determined on the indicated days after germination. Each value represents the average with the SD for 10 plants.

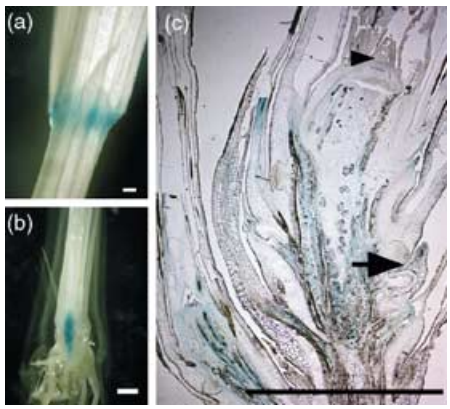

Figure 4. *OsTB1* expression monitored as to *GUS* expression in the wild-type strain.
(a) Close-up view of a lamina joint.
(b) Close-up view of a stem.
(c) Longitudinal section of the stem. SAM and an axillary bud are indicated by thin and thick arrows, respectively. Bars, 10 mm.

young seedlings seemed thick as compared with those of the control transgenic plants (Figure 2e).

An fc1 *mutant exhibits enhanced lateral branching*

The reduced lateral branching in the transgenic plants suggested that a loss-of-function of *OsTB1* would result in enhanced lateral branching in a similar manner to in the case of the maize *tb1* mutations. Similarly, the additional phenotype of the transgenic plants, thick seedlings, also suggested that the mutation might cause thin seedlings. This particular phenotype has been found in several rice marker strains, such as *fine culm 1* (*fc1*) (Figure 3a,b). The fact that *fc1* shares the same genetic locus as *OsTB1* on the genome led us to the idea that *fc1* is allelic to *OsTB1*.

To examine this idea, we first carefully examined the *fc1* mutant phenotype with respect to its lateral branching. An *fc1* mutant strain, M56, exhibited a bushy morphology as to enhanced lateral branching. Quantitative analysis showed that the *fc1* mutant generated a threefold higher number of

tillers than the wild-type strain did (Figure 3c). It is well known that lateral branching in rice is modulated by several environmental conditions, such as the planting density (Hoshikawa 1989). When the wild-type strain was grown at one plant per pot, it generated twofold more tillers than at three plants per pot (Figure 3c). Similarly, the number of tillers in the *fc1* mutant at the low planting density was greater than at the high density (Figure 3c), suggesting that even in the *fc1* mutant the lateral branching is modulated by the planting density. These data indicated that the *fc1* mutation results in enhanced lateral branching although its regulation by a certain environmental condition remains.

The fc1 *mutants contain loss-of-function mutation of* OsTB1

To determine whether or not *fc1* is allelic to *OsTB1*, we next determined the nucleotide sequence of the *OsTB1* gene in the *fc1* mutant, M56. Sequencing analysis of the PCR-amplified *OsTB1* ORF from the *fc1* genome revealed one nucleotide deletion in *OsTB1*. The C-base at the 327th nucleotide in the ORF was deleted in the *fc1* mutant, resulting in a frame shift of the ORF generating a stop codon just downstream of the mutation (Figure 1b). The truncated polypeptide deduced has a markedly small molecular weight and completely lacks the TCP domain, which is implicated in its DNA-binding activity (Figure 1b). The *OsTB1* message examined by RT-PCR analysis appeared to be reduced to one-half of that in the wild-type, presumably through mRNA destabilization caused by abrupt interruption of the translation (Figure 2d). Therefore, the *OsTB1* allele should be regarded as a null mutation.

We also sequenced the *OsTB1* locus of another *fc1* strain, FL253, and found that the strain also contains homogeneously the identical mutational allele of *OsTB1* found in M56. Because FL253 strain was constructed by genetic cross between the original *fc1* and the non-related strains, the fact that the both strains contain the same mutational allele in *OsTB1* strongly suggested that the *OsTB1* mutation is tightly linked to *fc1* phenotype through several recombination events.

OsTB1 *is expressed in axillary buds*

Finally, we examined the expression of *OsTB1* in living organisms by using an *OsTB1* promoter-glucuronidase (*GUS*) fusion gene. The 4.5-kb 5′-non-coding region of the *OsTB1* genomic sequence encompassing the putative promoter was fused to the *GUS* gene and then introduced into the wild-type strain, Taichung 65. The resultant young seedlings harboring the fusion gene were stained and analyzed. The GUS staining was observed in the stem region and lamina joints (Figure 4a,b). In a longitudinal section of the former part, the staining was visible in the basal part of SAM, vascular tissues in the pith and an axillary bud (Figure 4c). It is noteworthy that the staining was observed in the almost entire axillary bud, whereas it was restricted to within a relatively narrow region of the basal part of the primary SAM.

Discussion

Lateral branching is one of the most important processes that determine the shoot architecture in flowering plants. In this study, we found that the total number of tillers is significantly reduced by the overexpression of *OsTB1*, but increased in an *fc1* mutant containing a loss-of-function mutation of *OsTB1*. These results thus strongly suggested that OsTB1 functions as a negative regulator for lateral branching in rice, similar to maize TB1. Lateral branching apparently involves two developmental steps, the formation and outgrowth of axillary buds. Several genes, such as tomato *Bl* and *Ls*, and *Arabidopsis REV*, have been shown to be involved in the former step (Mapelli and Kinet, 1992; Otsuga *et al*., 2001; Schmitz *et al*., 2002; Schumacher *et al*., 1999; Talbert *et al*., 1995). *OsTB1* may rather play an important role in the latter step, because primordia for tillers were still propagated even in the *OsTB1*-overproducing transgenic plants. As shown by the promoter-*GUS* fusion gene, *OsTB1* is expressed in an entire axillary bud and may severely inhibit its subsequent outgrowth.

OsTB1 regulates the growth of lateral buds, but not of SAM. A similar situation is also seen in mutants affecting the formation of lateral buds. The mutational effect of the *REV, Bl* and *Ls* genes is observed solely in axillary buds (Mapelli and Kinet, 1992; Otsuga *et al*., 2001; Schmitz *et al*., 2002; Schumacher *et al*., 1999; Talbert *et al*., 1995). The different response to the mutations between SAM and lateral buds can be explained by the organ-specific expression of these factors. Indeed, the *OsTB1* gene is expressed in an entire axillary bud, but only in a relatively limited area of SAM. However, this explanation is not consistent with the observed phenotype of the transgenic plants in which *OsTB1* is overexpressed. The SAM activity of the transgenic plant seemed to be normal, whereas the outgrowth of axillary buds was severely inhibited. Of course, the possibility that *OsTB1* is not sufficiently overexpressed in SAM cannot be excluded *a priori*, but it is rather likely that the sensitivity to OsTB1 is somehow different between the two types of meristems.

The number of tillers in rice is regulated by several environmental cues, such as the planting density, at the level of outgrowth, but not that of formation of axillary buds (Hoshikawa, 1989). Even in the *fc1* mutant, the planting density modulated the number of tillers. Under high planting density conditions, several axillary buds remain dormant. Thus, the regulatory circuit that modulates lateral

branching coordinately with planting density does not involve OsTB1. Moreover, the result also suggests that OsTB1 is not a unique factor for the control of lateral branching in rice. There may be an additional factor (s) other than OsTB1 that negatively regulates lateral branching. Alternatively, rice may have a positive regulator(s) that promotes the outgrowth of axillary buds. Either or both factors may be involved in the regulatory mechanism for lateral branching involving planting density. Because rice has a number of genes carrying the TCP domain (Kosugi and Ohashi, 1997, 2002), it is possible that such genes function as putative negative and/or positive regulators in lateral branching. In any event, further genetic analysis is necessary to comprehensively elucidate the molecular mechanism of lateral branching in rice.

Cell division is essential for the outgrowth of axillary buds. Active cell proliferation is inevitably inhibited in dormant buds. It is thus likely that OsTB1 inhibits the growth of axillary buds directly or indirectly through cell division activity. Intensive physiological studies have revealed that the outgrowth of axillary buds is regulated positively and negatively by cytokinin and auxin, respectively (Taiz and Zeiger, 1998). It is possible that OsTB1 eliminates the cytokinin action, or enhances the inhibitory effect of auxin on the meristematic activity of axillary buds. Alternatively, a more plausible idea is that OsTB1 may inhibit cell proliferation in a more direct manner. OsTB1 is a member of a novel, plant-specific gene family, named the TCP family, which is widespread in angiosperms (Cubas *et al*., 1999). The TCP family carries the TCP domain, a bHLH motif of putative transcription factors. In *Antirrhinum* flowers, *CYC*, one of the most characterized *TCP* genes, is postulated to inhibit stamen development at the dorsal position by repressing expression of the *cyclin D3b* gene, one of the key factors for progression through the G_1 phase in the cell division cycle (Gaudin *et al*., 2000). It is also known that rice PCF1 and 2 are able to bind to a putative promoter region of the *PCNA* gene, which also plays an important role in DNA synthesis (Kosugi and Ohashi, 1997). One might thus imagine that the TCP family is deeply involved in cell-cycle regulation at the G_1/S transition. Therefore, the idea that OsTB1 negatively controls cell proliferation through transcriptional regulation of genes involved in the cell-division cycle may be worth investigating further.

Lateral branching is one of the important factors that determine the final shape of flowering plants. The number of lateral branches is very important as to the amount of seeds in rice. Therefore, the control of lateral branching should be a subject for research from not only the scientific, but also the agricultural point of view. Enhancement of the maize *TB1* function is implicated to have been decisive on the evolution of a maize ancestor to a cultivated plant (Doebley *et al*., 1995, 1997). The results described here suggest that the morphology of rice can be improved by modification of the *OsTB1* gene function to obtain high-yield seeds.

Experimental procedures

Plant materials and growth conditions

Rice cultivar *Oryza sativa* L. cv. Taichung 65 was used as a wild-type strain. Rice mutants M56 and FL253 carrying an *fc1* mutation were also used for the genetic study. Rice plants were mainly grown on soil in cylindrical pots (157 m in diameter and 190 mm in height) in a greenhouse at 30°C (day) and 24°C (night) under long day (16 h light and 8 h dark) conditions. Transgenic rice plants were grown in a safety cabinet under the same conditions as above.

Isolation of genomic DNA and total RNA from rice plants

Rice genomic DNA was isolated from leaves using an ISOPLANT DNA isolation kit (Nippon GENE Co., Toyama, Japan). Total RNA was isolated by means of the sodium dodecyl sulfate (SDS)–phenol method (Palmiter, 1974).

Cloning and genetic mapping of the OsTB1 *gene*

A BAC clone harboring the *OsTB1* gene was identified in a library by PCR screening using a pair of primers; 5′-GACGGGG-CAGGCGGGCAAGG-3′ and 5′-TGGTGGACGATGAGTGGTTC-3′. Note that the BAC library (Chen *et al*., 2002) was constructed from genomic DNA fragments of a *japonica* cultivar, Nipponbare. The resultant BAC clone was then subjected to the Southern hybridization assay to identify an appropriate DNA fragment containing the *OsTB1* gene. The probe DNA used was prepared by PCR using the above primer pair and genomic DNA of Nipponbare as a template. The 3.6-kb *Hin*dIII fragment thus identified was cloned into the *Hin*dIII site of pUC119 (Vieira and Messing, 1987) to yield pCUA196, and then subjected to sequencing analysis. To identify a sequence polymorphism between the *japonica* and *indica* cultivars, several genomic DNA fragments of the *OsTB1* locus were amplified from Kasalath genomic DNA by PCR using a series of appropriate primer sets and then subjected to sequencing reactions. To determine the genetic locus of *OsTB1*, a back-crossed recombinant inbred line derived from the cross between Nipponbare and Kasalath (*japonica* and *indica* cultivars, respectively) (Lin *et al*., 1998) was used. Using genomic DNA prepared from each individual line as a template, a series of PCR reactions with primers, 5′-TCACATGG-TAAGTTCCACGA-3′ and 5′-TGGAATATGCATCTAGTGAG-3′ was carried out. The genotype-data obtained for the recombinant inbred lines were calculated with mapmaker program (Lander *et al*., 1987).

Plasmid construction

For the overproduction of *OsTB1* in rice, the 1166-bp *OsTB1* open-reading frame (ORF) was first amplified by PCR using primers, 5′-CAGTCTAGAATGCTTCCTTTCTTCGATT-3′ and 5′-ATGCCCGGGT-CAGCAGTAGTGCCGCGAA-3′ (note that the underlined nucleotides were added to create restriction sites). The resultant DNA fragment was treated with *Xba*I and *Sma*I, and then inserted

between the *Xba*I and *Sma*I sites of pUC119. After confirmation of the sequence, the DNA fragment encompassing the entire *OsTB1* ORF was purified and transferred to the pActnos/Hm2 vector (Zhang *et al.*, 1991) carrying a rice actin promoter, yielding pCUA202.

To construct an *OsTB1* promoter-glucuronidase (*GUS*) fusion gene, the about 4.5-kb *Sph*I fragment encompassing a putative promoter region of *OsTB1* was first isolated from the BAC clone described above and then cloned into the *Sph*I site of pUC119, yielding pCUA197. The *Sph*I fragment was blunt-ended with T4 DNA polymerase, and then inserted into the *Sma*I site in the multi-cloning sites of pCU101, which contains the *GUS* gene and a *nos* terminater (Tnos) fragment following the multi-cloning sites. The details of the construction of pCU101 will appear elsewhere. The *Sal*I/*Not*I fragment encompassing the promoter-*GUS*-Tnos construct was isolated, and then inserted between the *Sal*I and *Kpn*I sites of pBIB-Hm (Becker, 1990) (note that the *Not*I and *Kpn*I sites were previously blunt-ended with T4 DNA polymerase). The resultant plasmid was designated as pCUA203.

Transformation of rice plants

Agrobacterium tumefacience-mediated transformation of rice was carried out according to the method of Hiei *et al.* (1994)

GUS staining

Plant materials were infiltrated with a staining solution [100 M $NaPO_4$ (pH 7.0), 100 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide] under vacuum for 15 min and then incubated at 37°C for 12 h. Chorophyll and other pigments were removed by incubation in ethanol at room temperature. Plant tissues were observed under an SZH10 dissecting microscope (Olympus Co., Tokyo). FAA-fixed and dehydrated material was embedded in Paraplast Plus (Oxford Labware, USA) and sectioned using a rotary microtome. Ten micrometer sections were placed on slide glasses, dewaxed, and mounted. Images were acquired with a Leitz DMRBE microscope (Leica, Germany).

Reverse transcriptase (RT)–PCR analysis

Total RNA was isolated from lamina joints of adult plants followed by treatment with RNase-free DNase I. The reverse transcription reaction was carried out with a Superscript II kit (Invitrogen, USA) and a poly-dT primer. The resultant cDNA sample was used as a template for the PCR reaction, with 35–50 cycles of 0.5 min each at 94 and 55°C, and 1 min at 72°C. The following primers were used to amplify the *OsTB1* and *OsACT* cDNAs: 5′-GCCGGATGCAAGAAATC-3′ and 5′-TCAGCAGTAGTGCCGCGAA-3′for *OsTB1*, and 5′-TCCATCTTGGCATCTCTCAG-3′and 5′-GTACCCGCATCAGGCATCTG-3′ for *OsACT*. The predicted size of the amplified fragment was 169 bp for *OsTB1* or 350 bp for *OsACT*. The PCR products were separated by agarose gel electrophoresis and then transferred to a Hybond-N+ nylon membrane (Amersham). Hybridization was carried out with ^{32}P-labeled DNA fragments as probes in a buffer comprising 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, and 1 mM EDTA at 65°C for 20 h. The membrane was washed with 40 mM Na_2HPO_4 (pH 7.2) and 1% SDS for 10 min at 65°C twice, and then with 0.2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) and 0.1% SDS for 30 min at 65°C, and finally visualized with a BAS-2000 (Fuji-Xerox, Tokyo).

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