New Phytologist

Overexpression of a NAC-domain protein promotes shoot branching in rice

Chuanzao Mao, Wona Ding, Yunrong Wu, Jie Yu, Xiaowei He, Huixai Shou and Ping Wu

State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou, 310058, P. R. China

Author for correspondence:
Ping Wu
Tel: +86 571 88206412
Fax: +86 571 88206617
Email: clspwu@zju.edu.cn

Received: *8 April 2007*
Accepted: *31 May 2007*

Summary

• For a better understanding of shoot branching in rice (*Oryza sativa*), a rice activation-tagging library was screened for mutations in tiller development. Here, an activation-tagging mutant *Ostil1* (*Oryza sativa tillering1*) was characterized, which showed increased tillers, enlarged tiller angle and semidwarf phenotype.

• Flanking sequence was obtained by plasmid rescue. RNA-interfering and overexpression transgenic rice plants were produced using *Agrobacterium*-mediated transformation.

• The mutant phenotype was cosegregated with the reallocation of Ds element, and the flanking region of the reallocated Ds element was identified as part of the *OsNAC2* gene. Northern analysis showed that expression of *OsNAC2* was greatly induced in the mutant plants. Transgenic rice overexpressing the *OsNAC2* resulted in recapture of the mutant phenotype, while downregulation of *OsNAC2* in the *Ostil1* mutant through RNA interfering (RNAi) complemented the mutant phenotype, confirming that the *Ostil1* was caused by overexpression of *OsNAC2*.

• Overexpression of *OsNAC2* regulates shoot branching in rice. Overexpression of *OsNAC2* contributes tiller bud outgrowth, but does not affect tiller bud initiation. This suggests that *OsNAC2* has potential utility for improving plant structure for higher light-use efficiency and higher yield potential in rice.

Key words: activation-tagging, axillary bud, NAC (NAM, ATAF1, 2, CUC2) family, rice (*Oryza sativa*), shoot branching, transcription factor.

New Phytologist (2007) **176**: 288–298

doi: 10.1111/j.1469-8137.2007.02177.x

Introduction

Tiller is an important agronomic trait for rice production, and also a model system to study shoot branching in monocotyledonous plants. Shoot branching is regulated by two distinct steps: the establishment of axillary meristems and the outgrowth of axillary buds. After formation, axillary buds often remain dormant and require one or more of a wide range of cues before outgrowth ensues. Several genes, including rice *MOC1*, tomato *BL* and *LS*, and *Arabidopsis REV*, have been reported to be involved in the initiation of axillary buds (Talbert *et al.*, 1995; Schumacher *et al.*, 1999; Schmitz *et al.*, 2002; Li *et al.*, 2003), while some other genes, such as *Arabidopsis MAX1-4*, pea *RMS1* and *Petunia hybrida DAD1*, were involved in axillary bud outgrowth (Sorefan *et al.*, 2003; Booker *et al.*, 2004, 2005; Snowden *et al.*, 2005). Despite the encouraging progress made in the molecular cloning of genes, knowledge of the molecular mechanism for shoot branching is still fragmentary, especially in the monocotyledonous plants.

Several genes affecting the shoot-branching process have been described in rice. MOC1, a GRAS family transcription factor, was reported to control axillary meristem initiation and axillary bud outgrowth (Li *et al.*, 2003). OsTB1, a TCP

The GenBank accession number for the *OSTIL1* sequence is DQ520641.

domain protein, functions in the repression of bud activity. The dormancy of tiller buds was weakened in the *fc1* mutant, a loss-of-function mutant of *OsTB1*. Transgenic rice ectopically expressing *OsTB1* led to the suppression of tiller development (Takeda *et al.*, 2003). Several other mutants associated with tiller bud outgrowth were also reported. Among these, two genes were fine-mapped, designated as *d3* and *htd-1*, and suggested to be the rice homologues of *MAX2* and *MAX3*, respectively, in *Arabidopsis* (Ishikawa *et al.*, 2005; Zou *et al.*, 2005, 2006). Although the homologous relationship was found between MOC1 and LS, HTD-1 and MAX3, D3 and MAX2, further studies to characterize more tillering genes are required to elucidate whether monocot and eudicot plants share similar molecular mechanisms controlling shoot branching.

The NAC (NAM, ATAF1, 2, CUC2) family proteins constitute one of the largest plant-specific families of transcription factors with > 100 members in *Arabidopsis* (Riechmann *et al.*, 2000; Olsen *et al.*, 2005) and rice (http://www.tigr.org), respectively. The N-terminal region of NAC proteins contains a highly conserved NAC domain, which may form a helix-turn-helix structure that specifically binds target DNA (Aida *et al.*, 1997; Xie *et al.*, 2000; Duval *et al.*, 2002). The C-terminal regions of NAC proteins, on the other hand, are highly divergent, and are thought to act as transcription-activation regions (Xie *et al.*, 2000; Takada *et al.*, 2001; Duval *et al.*, 2002).

Although NAC family proteins are involved in various processes (Olsen *et al.*, 2005), only a small proportion of the NAC proteins have been characterized. One of the best characterized functions of NAC genes is delimiting organs during embryonic, floral and vegetative development (Souer *et al.*, 1996; Aida *et al.*, 1997; Sablowski & Meyerowitz, 1998; Takada *et al.*, 2001; Vroemen *et al.*, 2003; Mitsuda *et al.*, 2005). NAC genes were also reported to be involved in auxin and abscisic acid signal transduction (Xie *et al.*, 2000; Aida *et al.*, 2002) and plant responses to biotic and abiotic stress (Xie *et al.*, 1999; Ren *et al.*, 2000; He *et al.*, 2005; Selth *et al.*, 2005; Hu *et al.*, 2006). Recent reports indicated that NAC family genes are also involved in regulation of senescence (Guo & Gan, 2006; Uauy *et al.*, 2006). However, no NAC gene has been reported to be involved in lateral shoot branching to date.

Activation tagging produces dominant mutations by means of the overexpression of endogenous genes with transcriptional enhancers or promoters that cause the ectopic expression of genes in the vicinity of the T-DNA insertion site (Suzuki *et al.*, 2001). However, no gene was cloned in rice using the activation-tagging system. In this study, an activation-tagging transposon system, which can activate transcription of neighbouring genes by two 35S promoters and/or by four tandem repeats of the enhancer fragment of this promoter (Suzuki *et al.*, 2001), was used to produce activation-tagging T-DNA insertion mutagenesis lines. An activation-tagging mutant with extensive tiller number and enlarged tiller angle was identified and characterized.

Materials and Methods

Isolation of *Ostil1* mutant

The rice activation-tagging library of the Nipponbare (*Nip*) genotype was constructed using the vector pAD100 designed for obtaining gain-of-function mutations (Suzuki *et al.*, 2001). The library was screened for mutations in tiller development. The *Ostil1* mutant was found to have increased tiller number and tiller angle, and semidwarf phenotype.

Plant materials and growth conditions

Wild-type (WT) rice (*Oryza sativa* L.) cv. Nipponbare and mutant *Ostil1* were used for the genetic study. Rice plants were mainly grown on soil in a glasshouse at 30°C (day) and 24°C (night) under long-day (15 h light and 9 h dark) conditions. Transgenic rice plants were grown in a safety cabinet under the same conditions as previously described.

At maturity, plants were characterized for the phenotype alteration between the WT and the mutant, and the tiller buds were investigated at different growth stages.

Cosegregation analysis and cloning of *OsNAC2*

The original T-DNA insertion, excision and reinsertion of the Ds element were analysed as described (Suzuki *et al.*, 2001). The hygromycin-resistant gene located in the Ds element was used for analysis of the cosegregation of the Ds insertion with the mutant phenotype. The Ds insertion was determined by PCR and Southern blot. The primers were designed as follows: GCTTCTGCGGGCGATTTGTGTA; CGGTCG-CGGAGGCTATGGATG. To confirm the Ds insertion, a pair of primers was designed across the insert site: pres-up: ACCTGGTGTCTGCTTCCCTAACAG; pres-down: ATGGC-TGAGATGTGAATACGGTT. Each of the primers combined with 35S primer was used to confirm the existence of Ds insertion.

The 1192-bp cDNA sequence containing the predicted ORF of *OsNAC2* was amplified using RT–PCR with the primers of 5′-AGGAGCAGTTAGCCAGGTAAAG-3′ and 5′-ATTAAGCGAACCTTGGTAGATG-3′. PCR conditions were 94°C for 5 min, followed by 31 cycles, 94°C for 30 s, 60°C for 30 s and 72°C for 1.5 min. The PCR product was cloned into the pUC-T vector and sequenced.

Plasmid rescue

Genomic DNA (20 μg) was digested with *Hin*dIII and purified. The digested DNA pellet was then dissolved in water (at a concentration of 20 μg ml^{-1}) and autoligated. After ligation, DNA was purified and dissolved in water at a concentration of 100 μg ml^{-1}. Ligated DNA (1 μl) was transferred into *Escherichia coli* DH5α cells and plated onto LB plates containing 100 mg ml^{-1} ampicillin. The positive clones were

sequenced using the 35S and M13 primers in the plasmid construct, as described by Suzuki *et al.* (2001). Sequence was analysed using CLUSTALX ver. 1.81.

Construction of vectors and plant transformation

The overexpression vectors were constructed as indicated. First, the CaMV 35S promoter was subcloned between the *Eco*RI and *Sac*I sites of pCAMBIA1301; second, the poly(A) addition sequence of pea ribulose 1,5-bisphosphate carboxylase small-subunit rbcS-E9 was also inserted into the site between *Hin*dIII and *Pst*I. The resulting plasmid was named 35S-pCAMBIA1301. Then the ORF of *OsNAC2* was introduced into 35S-pCAMBIA1301 using the *Bam*HI, *Eco*RV site.

A 2203-bp promoter was obtained by PCR using primers: TCAGTCGACAACACAGCACTAACCGAGGATTCAG (containing the *Sal*I recognition site) and TCACCCGGG TAGCTAGAGCTTTACCTGGCTAACTG (containing the *Sma*I recognition site). The resulting DNA fragment was inserted into the 5′ end of the GUS gene (*gusA*) in pCAMBIA1391Z to create the OsNAC2 promoter::GUS construct.

For the RNAi construct, 192 bp of *OsNAC2* cDNA in sense and antisense orientation was constructed into both sides of the second intron of the maize *NIR1* gene. The fragment was then inserted into the multiple cloning site of 35S-pCAMBIA2301 (constructed just as 35S-pCAMBIA1301) to construct the OsNAC2 RNAi vector.

The above constructs were used for *Agrobacterium*-mediated rice transformation of WT or mutant materials (Chen *et al.*, 2003).

Histochemical analysis and GUS assay

GUS analysis was performed as follows. Transgenic plant samples were incubated with GUS staining solution (100 mmol l^{-1} NaH_2PO_4 buffer pH 7.0, 0.5% Triton X-100, 0.5 mg ml^{-1} X-Gluc and 20% methanol) overnight at 37°C. After staining, tissues were rinsed and fixed in formalin acetic acid (FAA) ethanol fixation solution at 4°C overnight, then mounted on slides and photographed (Leica MZ95, Nussloch, Germany).

Shoot bases of 5-d-old seedlings were fixed with FAA fixation solution at 4°C overnight, followed by dehydration and embedding in paraffin (Paraplast Plus, Sigma, MO, USA). Sections (thickness 8 μm) were cut with a microtome (Microm HM325, Walldorf, Germany) and stained with hematoxylin. Sections were observed under bright-field through a microscope (Zeiss AxioCam HRC, Oberkochen, Germany).

Subcellular localization of OsNAC2

CaMV35S-OsNAC2-mGFP4 was subcloned into the binary vector pCAMBIA 1300. The resulting construct was sequenced to verify in-frame fusion and used for transient transformation of onion epidermis using a gene gun (Bio-Rad, Hercules, CA, USA). The GFP was visualized by LSM 510 Laser Scanning Microscope (ZEISS, Oberkochen, Germany).

Yeast system analysis

The Matchmaker Yeast Two-hybrid System (cat. # K1615-1; Clontech, CA, USA) was used for transactivation analysis. The deduced amino acid sequence of OsNAC2 was inserted into the vectors pGBKT7 (BD) and pGADT7 (AD). Two subclones with N-terminal 190 amino acids (1–570 bp) and C-terminal 156 amino acids (562–1029 bp) of OsNAC2 were inserted into pGBKT7 (BD), resulting in fusions with the GAL4 DNA-binding domain and activation domain. The fusion plasmids, BD-OsNAC2, BD-OsNAC2 (1–190 aa) and BD-OsNAC2 (188–343 aa), were transformed into yeast stain AH109. The transformation mixture was plated on synthetic defined media (SD)/-Trp/-His/-Ade medium plates for examination of growth. Quantitative α-galactosidase assays were performed using the method described in the Clontech protocol. The interaction between the pGBKT7 (BD)-p53 and pGADT7 (AD)-SV40 large T-antigen served as a positive control. The empty pGBKT7 (BD) vector was used as a negative control.

RT–PCR analysis

Total RNA was extracted from tissues of 20-d-old seedlings of WT, *Ostil1* mutant and transgenic plants, and used for reverse transcription using Superscript II according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was synthesized from total RNA and used as RT–PCR templates. RT–PCR was performed using gene-specific primers for *OsNAC2*, forward (5′-AGATCGCCATGTCGTCCGTC-3′) and reverse (5′-GCTGCCCGTACTGCGTGAAG-3′). Amplification of actin cDNA was performed as a control. The PCR products were analysed on 1% agarose gel.

Quantitative real-time RT–PCR was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the SYBR green I master mix (Applied Biosystems) containing optimized buffer, dNTP and Taq DNA polymerase, and manufactured as described in the user manual.

Southern blot analysis

Genomic DNA was isolated from transgenic plants, WT and mutant plants using the cetyl trimethyl ammonium bromide (CTAB) method. Genomic DNA (5 μg) was digested with restriction enzymes and separated on 0.8% agarose gel. After electrophoresis, the digested DNA was transferred to a Hybond-N+ Nylon membrane (Amersham Pharmacia, Little Chalfont, UK). ^{32}P-dCTP-labelled DNA of hygromycin-resistant gene was used as a probe. The blots were hybridized and washed at 65°C under stringent conditions, and analysed using Typhoon-8600.

Northern blotting

Northern blotting was performed as described previously (Mao *et al.*, 2004).

Computational analysis

The sequence of the full-length cDNA was analysed using BLAST and MOTIFSCAN (http://hits.isb-sib.ch/cgi-bin/PFSCAN). Genome annotation was done using RGP (RICE GENOME RESEARCH PROGRAM, http://rgp.dna.affrc.go.jp) and TIGR (http://www.tigr.org/tdb/e2k1/osa1/pseudomolecules/info.shtml), and blocks of NAC domain were made using BLOCK MAKER (http://blocks.fhcrc.org/blocks/make_blocks.html).

Multiple sequence alignment of the known NAC family genes was conducted using the CLUSTALX ver. 1.81 program using the default multiple alignment parameters and the weight matrix BLOSUM series. The phylogenetic analysis was carried out by the neighbour-joining method, and no treatment was done for the alignment and gaps. The phylogenetic tree was constructed using PHYLIP ver. 3.5c (http://bioweb.pasteur.fr) with a bootstrap analysis of 1000 resampling replications.

Results

Phenotypic analysis of the *Ostil1* mutant

A mutant exhibiting increased number of tillers and enlarged tiller angle was isolated from *c.* 1000 activation-tagged rice lines of Nipponbare (Nip) (as described in Materials and Methods). According to the phenotype, the mutant was named *Ostil1* (*Oryza sativa tillering1*). To investigate the genetic basis of the mutant, *Ostil1* was backcrossed with the WT Nip. The BC_1F_1 showed the phenotype between the mutant and the WT plant. The phenotype of 108 BC_1F_2 lines showed a ratio of 1 : 2 : 1 of WT phenotype lines : middle-type phenotype lines : mutant phenotype lines ($\chi^2 = 0.264 < \chi^2_{0.05} = 5.99$), indicating that the mutant phenotype is controlled by a single semidominant gene.

The *Ostil1* mutant (the homozygous mutant) showed an increased number of tillers, enlarged tiller angle and reduced plant height. Under field conditions, the average tiller number in *Ostil1* plants was 27.5 per plant, more than twice that of the WT plants (11.5 per plant) (Fig. 1; Table 1). Furthermore, the tiller angle of *Ostil1* was approx. 41.3°, about fivefold that of the WT (7.4°) (Fig. 1; Table 1). The *Ostil1* mutant showed shorter plant height (75% of WT), relatively smaller leaf size and slightly shorter panicle length than the WT plants (Fig. 1; Table 1). Slimmer stem and smaller seed size were also observed in the *Ostil1* mutant (data not shown). However, no significant difference was observed in root traits between *Ostil1* and WT plants (data not shown).

Ostil1 promotes tiller bud outgrowth but does not affect tiller bud initiation

Shoot branching is regulated by two distinct steps: the establishment of axillary meristems, and the outgrowth of axillary buds. To investigate which step was promoted in the

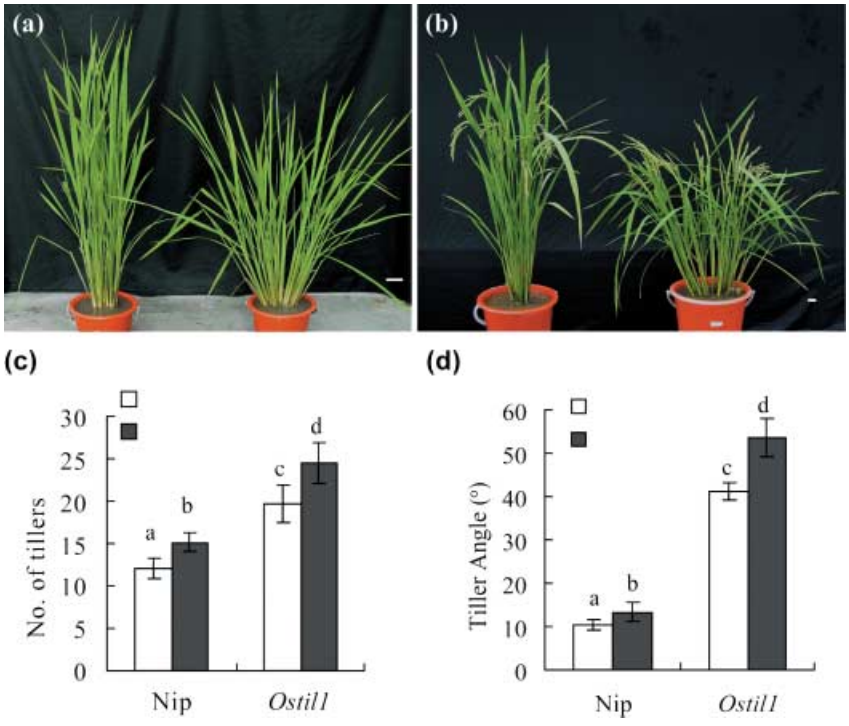

Fig. 1 Phenotype of *Ostil1* mutant. (a,b) Phenotype of *Ostil1* (right) and wild-type (WT, left) rice (*Oryza sativa*) at (a) maximum tillering stage (70 d); (b) mature stage (90 d). (c) Tiller number; (d) tiller angle of *Ostil1* and the WT (Nip) under different planting densities (open bars, 20×20 cm^2; closed bars, 30×30 cm^2). Error bars, SD of 15 plants. Different letters (a–d) at the top of each column indicate significant difference.

Table 1 Phenotype of *Ostil1* and the wild-type rice plant Nip

Line	Tillers	Tiller angle (°)	Plant height (cm)	Length of sword leaf (cm)	Width of sword leaf (cm)	Panicle length (cm)
Nip	11.5 ± 2.02	7.4 ± 1.46	83.2 ± 3.45	34.8 ± 4.1	1.5 ± 0.1	21.9 ± 0.9
Ostil1	27.5 ± 2.83	41.3 ± 3.28	62.8 ± 2.43	27.9 ± 3.0	1.1 ± 0.1	19.7 ± 0.7

Data are average ± SD of 15 seedlings.

Ostil1 mutant, tiller bud initiation and its outgrowth were investigated. Compared with WT plants, the *Ostil1* mutant showed no extra axillary buds at each of its leaf axils (Fig. 2a–f). Longitudinal sections showed that the initiation of tiller buds was normal in the mutant (Fig. 2a,b).

At the six-leaf stage, the outgrowth of tiller buds was observed at the first node on the main tiller (stem) in *Ostil1* mutant plants, but not in WT plants (Fig. 2e). Altered patterns of tiller development were also found at higher nodes (Fig. 2f). In WT plants, tillers appeared from the second to the seventh nodes. The tiller buds at the upper three to four nodes become arrested. By contrast, tiller buds up to the second node from top could grow out in the *Ostil1* mutant (Fig. 2f). The same pattern of enhanced tiller bud outgrowth was observed on secondary and higher-order tillers (data not shown). At the mature stage (90 d), the tiller number of each *Ostil1* plant is more than twice that of the WT plant (Fig. 1; Table 1). The tiller number of WT plants reached maximum level at 50 d after germination (Fig. 2g). Nevertheless, the *Ostil1* mutant is capable of developing tillers through all its life stages, even at the mature stage (Fig. 2g). As shown in Fig. 2(g), visible tiller buds were almost the same in *Ostil1* and WT plants before 30 d after germination. After that, more tiller buds were observed in the mutant than that in the WT. These results suggest that the enhanced tillering capacity in the *Ostil1* mutant results from the release of axillary buds from their dormant status, but not from the increase of axillary bud numbers. The tiller bud at the top node was still suppressed in the mutant, indicating that the suppression of tiller bud outgrowth was weakened, but developmental control of tiller bud activity was still functional in the mutant.

It is well known that lateral branching in rice is modulated by planting density (Hoshikawa, 1989). When grown at a density of 30 × 30 cm, the WT plants generated significantly more tillers, and tiller angles were larger compared with plants grown at a density of 20 × 20 cm (Fig. 1c,d). Similarly, the number of tillers and tiller angle in the *Ostil1* mutant at low planting density were greater than at high planting density (Fig. 1c,d), suggesting that lateral branching in *Ostil1* is also modulated by planting density. These data indicate that the *Ostil1* mutation results in enhanced lateral branching, but its regulation by environmental conditions remains, which is in accordance with other reported tillering mutants (Takeda *et al.*, 2003; Ishikawa *et al.*, 2005).

Ostil1 is caused by activation-tagging of *OsNAC2*

Among a population of 108 BC_1F_2 plants, all plants with the *Ostil1* phenotype showed the hygromycin-resistance gene detected by PCR and Southern analysis, while the WT phenotype progenies showed no hygromycin-resistance gene (data not shown). Southern hybridization of *Ostil1* genomic DNA probed by the hygromycin-resistance gene (located in the Ds element) showed that the mutant contained a single-copy Ds insertion (Fig. 3c). According to the tagging system, the Ds element carried by the T-DNA can jump and insert into a new position and then activate the gene(s) nearby (Suzuki *et al.*, 2001). If the Ds did not jump, amplification products of 678 and 771 bp from the original T-DNA would be obtained by PCR with primer sets a (Ac primer and 35S primer) and b (35S primer and Sp primer), respectively. If the Ds jumped, no PCR products would be obtained. In this case, no PCR product was obtained from the mutants by any of the above two primer combinations and the Ac primer and Sp primer combination (flanking the Ds element). These results indicate that the Ds element jumped and inserted into a new position. Using the spectinomycin-resistance gene in the T-DNA region as a probe, no hybridization band was found in T_2 mutant lines (data not shown). This indicates that *Ostil1* was caused by insertion of the Ds element (DsAT) and the original T-DNA insertion site was lost during propagation.

The 5′ flanking sequence of Ds was obtained by plasmid rescue. Nucleotide sequencing analysis showed that the Ds element (DsAT) is inserted in the 59 064–59 065-bp region of the BAC clone OSJNBa0072F16 of chromosome 4 (Fig. 3a,b). A pair of primers (named 'pres-up' for left of the insert site and 'pres-down' for right of the insert site) were designed to amplify the DNA sequence across the insert site. The 35S primer combining with 'pres-up' and 'pres-down' was used to amplify the end of DsAT and the flanking genome sequences. A 799-bp fragment was amplified in the WT plant, while there were no PCR products in the *Ostil1* mutant using the primer combination of 'pres-up' and 'pres-down'. By contrast, using the primer combination of 35S primer with 'pres-up' or 'pres-down', a fragment of *c.* 500 bp or 1 kb was obtained in the mutant, but not in the WT. The results were also confirmed in the BC_1F_2 population. This confirmed that the mutant phenotype is produced by the insertion of DsAT.

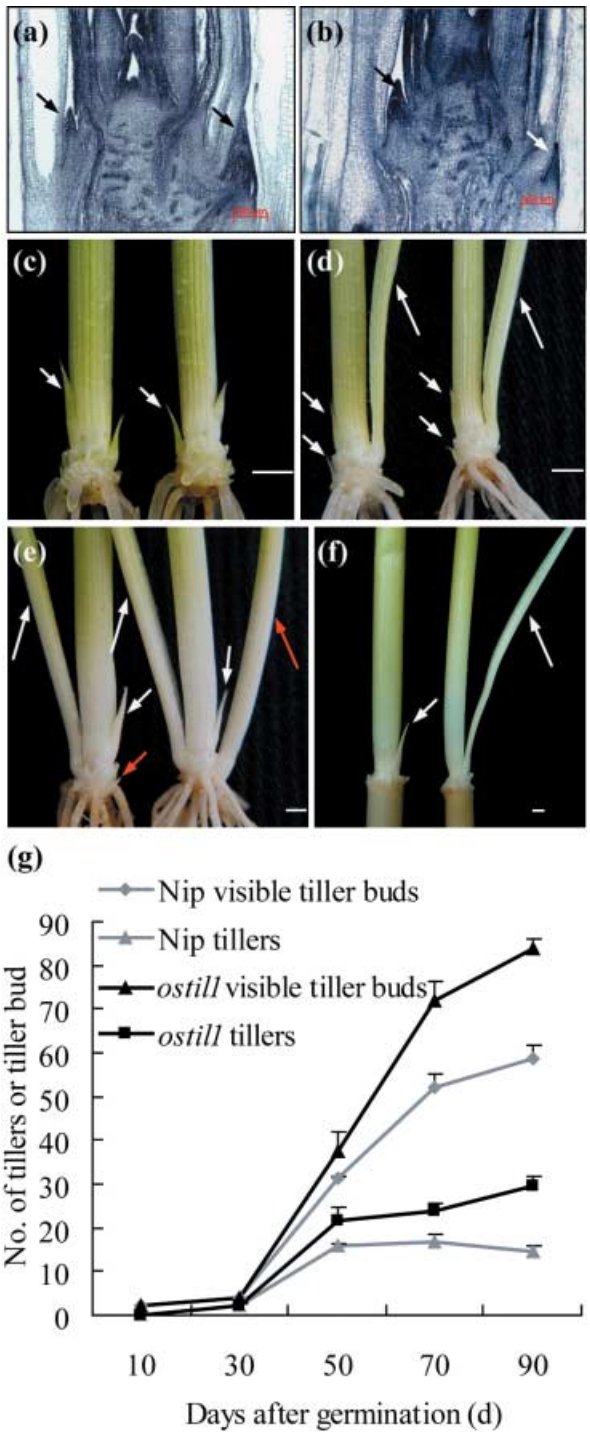

Fig. 2 Tiller bud development of wild-type (WT) and *Ostil1* rice (*Oryza sativa*). (a,b) Longitudinal sections of three-leaf stage shoot apexes of (a) Nip; (b) *Ostil1*. (c–f) Tiller buds of Nip (left) and *Ostil1* (right) at different growth stages. (c,d) Tiller buds from the first to third leaf axils, (c) three-leaf stage; (d) four-leaf stage; (e) six-leaf stage; (f) heading stage, second node from top. (g) Growth curve of visible tiller buds and tillers after different time periods. Short arrows, tiller buds; long arrows, outgrowth tiller buds; red arrows in (e) indicate tiller bud in first node. Bar: (a,b) 100 μm; (c–f) 1 mm. Error bar, SD of 15 plants.

Table 2 Phenotypes of transgenic rice plants

Line	Tiller number	Plant height (cm)	Tiller angle (°)
Nip	11.5 ± 2.02	83.2 ± 3.45	7.4 ± 1.46
Ostil1	27.5 ± 2.83	62.8 ± 2.43	41.3 ± 3.28
Ri1	11.8 ± 1.98	83.0 ± 1.73	10.7 ± 0.85
Ri2	11.4 ± 1.68	83.1 ± 1.63	10.3 ± 0.65
OV1	18.2 ± 2.10	70.0 ± 0.57	29.9 ± 3.04
OV2	18.8 ± 0.54	66.9 ± 0.41	30.8 ± 2.84

Data are average ± SD of 15 seedlings. Ri1, 2, *OsNAC2* RNA-interfering transgenic mutant line1 and 2; OV1, 2, *OsNAC2* overexpression transgenic line1 and 2.

According to the genome annotation provided by the Rice Genome Research Program (RGP, http://rgp.dna.affrc.go.jp) and TIGR (http://www.tigr.org), the DsAT was inserted in the intergenic region between a molybdenum cofactor biosynthesis protein C and an NAC-domain protein (identical to the *OsNAC2* registered in the GeneBank) (Fig. 3a). Northern blotting was performed to investigate if these neighbouring genes were upregulated. Using the 3-kb genome sequence flanking the right end of the DsAT as the probe, an overexpressed transcript can be detected in different tissues of the mutant plants, but not in those of the WT (Fig. 3d). Further analysis using *OsNAC2* as a probe also detected the same transcript, indicating that overexpression of *OsNAC2* may result in the *Ostil1* phenotype.

Recapitulation of the *Ostil1* mutant phenotype

To confirm that the overexpression of *OsNAC2* results in the *Ostil1* phenotype, the *OsNAC2* RNA interfering (RNAi) and overexpressing constructs were transformed to the *Ostil1* mutant line and the WT plant, respectively. The expression level of *OsNAC2* in those transgenic lines was detected by quantitative real-time RT–PCR analysis (Fig. 4e). Six independent RNAi transgenic lines, which the expression of *OsNAC2* reduced almost to the level of WT, recovered the WT phenotype (Fig. 4b,c,e; Table 2). On the other hand, altered tiller development including increased tiller numbers, enlarged tiller angle and reduced plant height was observed in seven independent *OsNAC2* overexpression transgenic lines. Although the severity varied among the seven lines compared with the *Ostil1* mutant, all showed the same phenotypic spectrum (Fig. 4d,e). The correlation between the severity of mutant phenotypes and the level of *OsNAC2* expression confirmed that the observed phenotypes were indeed caused by the ectopic overexpression of *OsNAC2* (Fig. 4e; Table 2).

OsNAC2 is expressed in all tissues

RT–PCR was performed to investigate the spatial expression of *OsNAC2* in rice. The results showed that *OsNAC2* is

New Phytologist

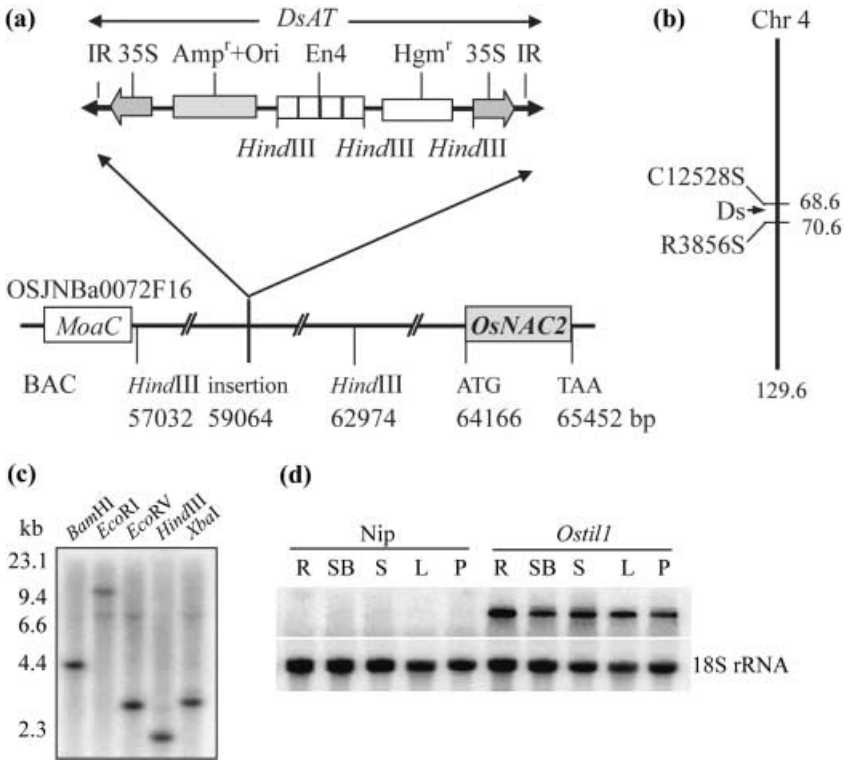

Fig. 3 Molecular characterization of *Ostil1*. (a) Genomic context of Ds insertion in *Ostil1* and the structure of DsAT (the latter redrawn from Suzuki *et al*., 2001). The *Hind*III recognition sites used for plasmid rescue are listed. IR, duplicate sequence recovered with the Ac element inserted in the maize *wx* gene; 35S, CaMV 35S promoter; Amp^r, β-lactamase gene; Ori, *Escherichia coli* ColE1 plasmid origin of replication; En4, four-time repeats of enhancer fragments from CaMV 35S promoter; Hgm^r, hygromycin phosphotransferase gene with a nopaline synthase promoter and a gene 4 polyadenylation signal; MoaC, MoaC family protein. (b) Chromosome location of Ds insertion. Numbers indicate cM. (c) Ds copy number in *Ostil1* revealed by Southern blotting. (d) Northern blotting probed by flanking genome sequence in the right of Ds. R, Root; SB, stem base; S, stem; L, leaf; P, young panicle.

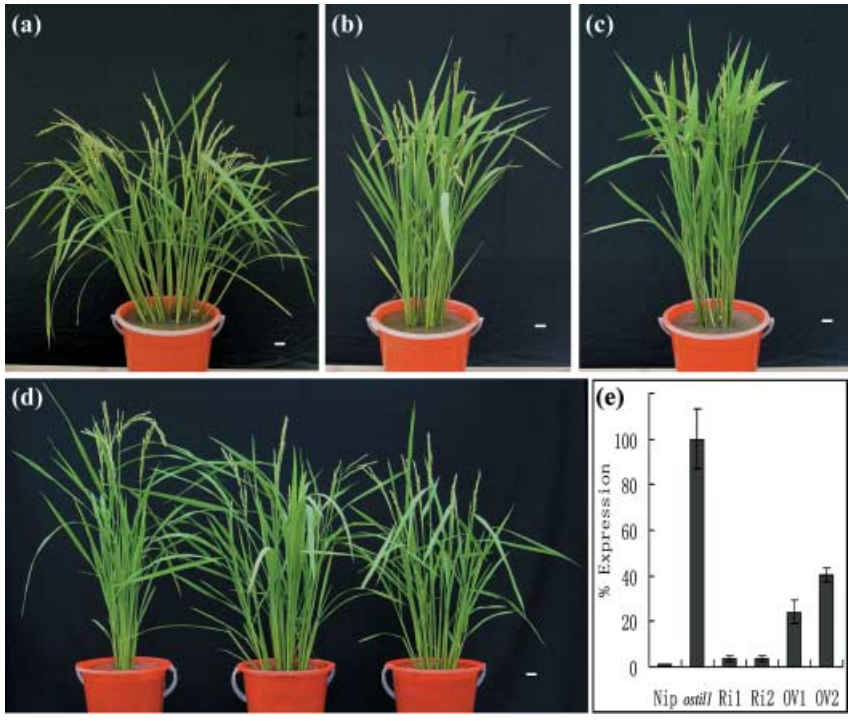

Fig. 4 Recapitulation of the rice *Ostil1* mutant phenotype. (a–c) RNA-interfering transgenic lines from the *Ostil1* mutant. (a) *Ostil1* mutant; (b,c) RNA-interfering transgenic lines 1 and 2 (Ri1 and Ri2). (d) Overexpression of *OsNAC2* in the wild-type (WT) plant recapitulating the mutant phenotype. Left plant, WT; two right plants indicate *OsNAC2* overexpression transgenic lines 1 and 2 (OV1 and OV2). (e) Quantitative RT–PCR analysis of *OsNAC2* in the RNA-interfering and overexpression transgenic lines. Expression levels were normalized with respect to the internal control *ACTIN* and plotted relative to the expression of *Ostil1* mutant. Nip, WT. Bar, 2 cm. Error bar, SD of three replicates.

expressed in all rice tissues tested except for seed, and the transcript accumulated more in roots and stem bases than in other tissues (Fig. 5g). To confirm the expression pattern, an expression vector of *udiA* (β-glucuronidase, GUS protein) driven by the *OsNAC2* promoter was constructed and transformed into the WT plants. The transgenic plants showed GUS staining in the roots, tiller buds, stem, leaf, lamina joint and the young husks (Fig. 5a–f). The expression pattern revealed by GUS staining is similar to that from RT–PCR analysis.

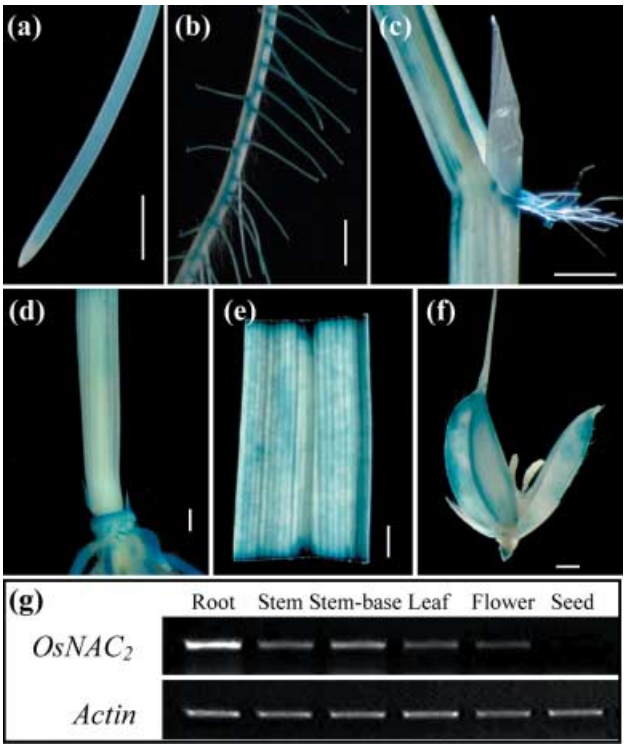

Fig. 5 Expression pattern of *OsNAC2*. (a–f) GUS staining of different tissues from *OsNAC2::GUS* transgenic rice plants, (a) root tip; (b) middle part of root; (c) lamina joint; (d) stem base; (e) leaf blade; (f) young husks; (g) expression pattern revealed by RT–PCR. Bar, 1 mm.

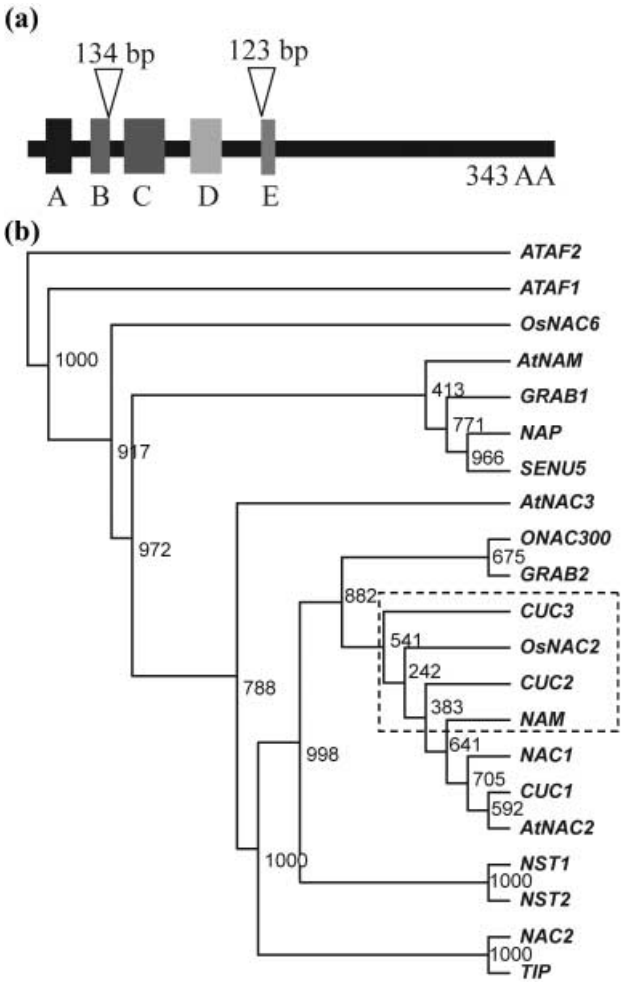

Fig. 6 Structure of OsNAC2 and alignment of its putative translation product with other NAC-domain proteins. (a) Schematic diagram showing the genomic structure of the *OsNAC2* gene. Open triangles, introns; numbers on top, length of intron. Five conserved regions within NAC family members are indicated by A–E. AA, amino acid. (b) Unrooted phylogenetic tree of known NAC transcription factors. Numbers between branches indicate bootstrap values based on 1000 replications. Names and references for other NACs: *Arabidopsis thaliana*, ATAF1, ATAF2 (Aida *et al*., 1997), AtNAC2 (He *et al*., 2005), AtNAC3 (Takada *et al*., 2001), AtNAM (Duval *et al*., 2002), CUC1, CUC2 (Takada *et al*., 2001), CUC3 (Vroemen *et al*., 2003), NAC1 (Xie *et al*., 2000), NAC2, NAP (Sablowski & Meyerowitz, 1998), NST1, NST2 (Mitsuda *et al*., 2005), TIP (Ren *et al*., 2000); rice, OsNAC6, ONAC300 (Kusano *et al*., 2005; Ohnishi *et al*., 2005); petunia, NAM (Souer *et al*., 1996); tomato, SENU5 (John *et al*., 1997); wheat, GRAB1, GRAB2 (Xie *et al*., 1999).

OsNAC2 is a transcription activator

The coding sequence of *OsNAC2* consists of three exons of 175, 296 and 561 bp separated by two introns of 134 and 123 bp (Fig. 6a). The NAC domain, which spans amino acids 12–168, is encoded by most of the first, the second, and the beginning of the third exon. Sequence alignment showed that OsNAC2 is a member of the NAC family protein, which is estimated to have about 105 members in *Arabidopsis* (Riechmann *et al*., 2000) and *c.* 125 members in rice (http://www.tigr.org/tdb/e2k1/osa1/pseudomolecules/info.shtml). A phylogenetic tree of the whole protein of OsNAC2 and function identified NAC-domain proteins revealed that OsNAC2 belongs to a branch involved in the CUC/NAM subclade, and is most similar to the *Arabidopsis* CUC2, CUC3 and petunia NAM (Fig. 6b). The whole protein of OsNAC2 showed 62.4% similarity with CUC2 and 61.3% with CUC3.

It has been reported that NAC proteins are nuclear-located, and the NAC family proteins have been proposed to function as transcription factors (Xie *et al*., 2000; Vroemen *et al*., 2003). To test whether OsNAC2 is localized in the nucleus, OsNAC2 was fused in-frame to the N terminus of the mGFP4 and transiently expressed in onion epidermal cells. The green fluorescent signal was detected only in the nucleus (Fig. 7a–c), suggesting that OsNAC2 is a nuclear protein. To determine whether OsNAC2 has transactivation activity, the OsNAC2 protein, the fragment of OsNAC2 without the NAC domain (from 188 to 343 aa) and the N-terminal fragment from 1 to 190 aa were fused to GAL4 DNA-binding domain and transformed to yeast strain AH109. The result showed that the yeast cells containing pBD-OsNAC2, pBD-OsNAC2-188-343, pBD-OsNAC2-1-190 and the negative control plasmid pBD all grew well on SD medium, while the cells containing pBD-OsNAC2-1-190 and the negative control plasmid pBD

New Phytologist

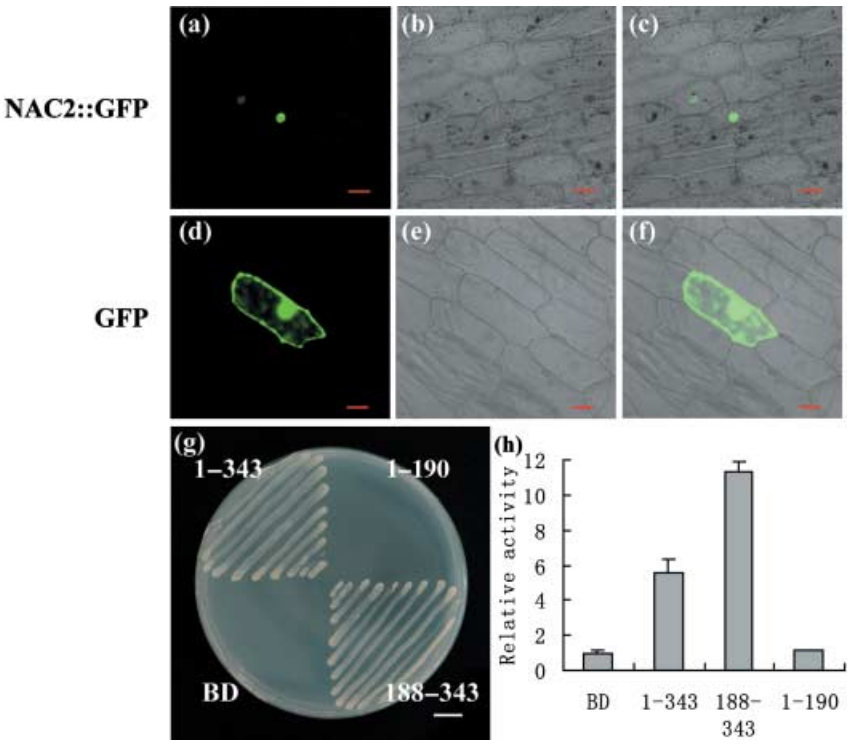

Fig. 7 Nuclear localization and transactivation activity of OsNAC2. (a–f) Nuclear localization of the OsNAC2 protein in onion epidermal cell. Photographs were taken in dark field for green fluorescence (a,d); in bright light for morphology of the cell (b,e); and in combination (c,f). (a–c) Transformed cell expressing OsNAC2–GFP fusion. (d–f) Transformed cell expressing GFP control. (g,h) Localization of the transactivation domain of OsNAC2. (g) Transactivation analysis of OsNAC2 in yeast. (h) α-galactosidase activity assays in the yeast two-hybrid system. The α-galactosidase activity obtained from different constructs was normalized to that obtained from the pBD and plotted to compare transactivation activity in different parts of the OsNAC2 protein. BD, pBD; 1–343, pBD-OsNAC2; 1–190, pBD-OsNAC2 (1–190 aa); 188–343, pBD-OsNAC2 (188–343 aa). Activity values are means of three measurements. Bar: (a–f) 50 μm; (g) 1 cm. Error bar, SD of three replicates.

could not grow in Trp-, His- and Ade-deficient SD medium (Fig. 7g). The α-galactosidase activity of yeast with BD-OsNAC2 (188–343 aa) was more than twice as much as that with BD-OsNAC2 (Fig. 7h). The results indicate that the part of OsNAC2 from 188 to 343 aa is a putative transcriptional activator, and the activation may depend somewhat on the unmasking of a repressor domain (the N-terminal NAC domain). α-galactosidase activities of the yeast with AD-OsNAC2 + BD-OsNAC2-1-190 showed no significant difference from that of the yeast with AD-OsNAC2 + BD, suggesting that OsNAC2 does not form a homodimer, at least in the N-terminal region (data not shown).

Discussion

Overexpression of *OsNAC2* promotes shoot branching in rice

In this study, an activation-tagging tillering mutant *Ostil1* was identified. *OSTIL1* showed pleiotropic phenotypes including increased tiller numbers, enlarged tiller angle and semidwarf phenotype. Molecular biological analysis showed that the mutant was cosegregated with the Ds insertion and the overexpression of *OsNAC2*. The *OsNAC2* overexpression transgenic lines showed increased tiller number, enlarged tiller angle and reduced plant height in a dose-dependent way, which is in accordance with the phenotype of the *Ostil1* mutant (Fig. 4d). Furthermore, the WT phenotype could be recovered by reduction of the expression of *OsNAC2* in the *Ostil1* mutant (Fig. 4b,c). These results indicate that overexpression of *OsNAC2* causes an alteration of shoot branching in rice, suggesting that OsNAC2 has potential utility for improving the rice plant structure for higher light-use efficiency and higher yield potential.

Phylogenetic analysis indicates that OsNAC2 is grouped into the same subclade with the *Arabidopsis* CUC2, CUC3 and petunia NAM proteins (Fig. 6b). CUC2, CUC3 and NAM were known to function in the development of shoot apical meristem (SAM) and cotyledons, and to act in boundary specification and SAM formation (Souer *et al.*, 1996; Vroemen *et al.*, 2003). However, no phenotypic defect in SAM and organ boundary formation is observed in the *Ostil1* mutant and the *OsNAC2* overexpressing transgenic lines. This suggests that overexpressed *OsNAC2* has no abnormal effects on SAM development and the boundary specification. On the other hand, reduction of the expression of *OsNAC2* in WT plants using RNAi showed no significant phenotype alteration (Fig. S1 in Supplementary Material). This could be a reflection of the redundancy of the NAC genes, or could indicate that parts of the accumulated transcripts would be enough to maintain normal development. Further studies are needed to investigate the functional alteration of T-DNA insertional line in which the *OsNAC2* was knocked out.

Overexpression of *OsNAC2* contributes tiller bud outgrowth while not affects tiller bud initiation

Shoot branching is regulated by two distinct steps. The first step is the initiation of axillary meristems in the axils of leaves. Subsequently, the bud either remains active or goes dormant until outgrowth is triggered (Ward & Leyser, 2004). Tiller

buds are normally developed in each leaf axil in the *Ostil1* mutant and the WT plant; no extra tiller bud or axillary meristem was found by longitudinal section in the *Ostil1* mutant or the *OsNAC2* overexpressing transgenic lines (Fig. 2a,b). However, more tiller buds are developed into tillers in the *Ostil1* mutant (Fig. 2e,f). These results indicate that the establishment of axillary meristems is normal, but subsequent suppression of tiller bud activity is weakened in the *Ostil1* mutant. The activity of tiller buds is controlled by a variety of factors, including their position on the axis, the developmental status of the primary SAM, the age of the plant, and various environmental conditions (Hoshikawa, 1989). In WT plants, the growth of the tiller bud at the first node of the main culm is usually suppressed, and the bud often degenerates. Buds on higher nodes, which arise at a later stage of the vegetative phase, also tend to become dormant in the WT plants. In the *Ostil1* mutant, dormancy of the bud at first node and buds at higher-order nodes up to the second node from the top can develop into tillers (Fig. 2e,f). This indicates that overexpression of *OsNAC2* promotes tiller bud activity or weakens bud dormancy.

Overexpression of *OsNAC2* might regulate tiller bud outgrowth independently of known pathways

Three known pathways that regulate shoot branching have been proposed, including repression of cytokinin synthesis by auxin signalling, regulation of auxin transport by the MAX pathway (and orthologous pathways: Ward & Leyser, 2004; Bennett *et al.*, 2006; Beveridge, 2006; Lazar & Goodman, 2006), and the action of Tb1 (Takeda *et al.*, 2003). To investigate if the first two pathways are involved in shoot branching regulated by the *OsNAC2* overexpression, the levels of endogenous auxin IAA and cytokinin zeatin in the junction of root and shoot were measured. No significant difference was observed between the *Ostil1* mutant and WT (Table S1). In addition, the expression of *D3*, *HTD-1* and other rice homologues of *MAX1* to *MAX4* were not changed in the *Ostil1* mutant compared with the WT plant (Fig. S2b). These results suggest that shoot branching regulated by overexpression of *OsNAC2* could be different from the known auxin-regulated pathways.

OsTB1 was a negative regulator of tiller bud outgrowth. The *OsTB1* knockout mutant showed increased tillers by weakened tiller bud outgrowth repression (Takeda *et al.*, 2003). RT–PCR showed that the expression of *OsTB1* was not changed in the *Ostil1* mutant (Fig. S2b). Yeast two-hybrid analysis revealed that OsTb1 and OsNAC2 do not interact physically (Fig. S2a). These results show that overexpression of *OsNAC2* should not regulate shoot branching through *OsTB1*. For the absence of *OsTB1* knockout and overexpression materials, whether OsNAC2 is regulated by OsTB1 was not investigated. However, the enlarged tiller angles were not reported in the OsTB1 knockout material (Takeda *et al.*, 2003). These results suggest that OsNAC2 might not act as a downstream component of OsTB1.

Taken together, overexpression of *OsNAC2* in the regulation of shoot branching in rice might involve a pathway that is independent of the known pathways. Further analysis of upstream and downstream genes is needed for a comprehensive understanding of the control of shoot branching by overexpression of *OsNAC2*.

Acknowledgements

This work was supported by the Special Program of Rice Functional Genomics of China (2002AAZZ1003), Zhejiang Bureau of Science and Technology and Zhejiang Bureau of Education. The authors thank Yoshihito Suzuki for kindly providing the vector pAD100.

References

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M. 1997. Genes involved in organ separation in Arabidopsis: an analysis of the cup-shaped cotyledon mutant. *Plant Cell* **9**: 841–857.

Aida M, Vernoux T, Furutani M, Traas J, Tasaka M. 2002. Roles of PIN-FORMED1 and MONOPTEROS in pattern formation of the apical region of the Arabidopsis embryo. *Development* **129**: 3965–3974.

Bennett T, Sieberer T, Willett B, Booker J, Luschnig C, Leyser O. 2006. The Arabidopsis MAX pathway controls shoot branching by regulating auxin transport. *Current Biology* **16**: 553–563.

Beveridge CA. 2006. Axillary bud outgrowth: sending a message. *Current Opinion in Plant Biology* **9**: 35–40.

Booker J, Auldridge M, Wills S, McCarty D, Klee H, Leyser O. 2004. MAX3/CCD7 is a carotenoid cleavage dioxygenase required for the synthesis of a novel plant signaling molecule. *Current Biology* **14**: 1232–1238.

Booker J, Sieberer T, Wright W, Williamson L, Willett B, Stirnberg P, Turnbull C, Srinivasan M, Goddard P, Leyser O. 2005. MAX1 encodes a cytochrome p450 family member that acts downstream of max3/4 to produce a carotenoid-derived branch-inhibiting hormone. *Developmental Cell* **8**: 443–449.

Chen SY, Jin WZ, Wang MY, Zhang F, Zhou J, Jia QJ, Wu YR, Liu FY, Wu P. 2003. Distribution and characterization of over 1000 T-DNA tags in rice genome. *Plant Journal* **36**: 105–113.

Duval M, Hsieh TF, Kim SY, Thomas TL. 2002. Molecular characterization of AtNAM: a member of the Arabidopsis NAC domain superfamily. *Plant Molecular Biology* **50**: 237–248.

Guo Y, Gan S. 2006. AtNAP, a NAC family transcription factor, has an important role in leaf senescence. *Plant Journal* **46**: 601–612.

He XJ, Mu RL, Cao WH, Zhang ZG, Zhang JS, Chen SY. 2005. AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. *Plant Journal* **44**: 903–916.

Hoshikawa K. 1989. *The growing rice plant. An anatomical monograph.* Tokyo: Nobunkyo.

Hu H, Dai M, Yao J, Xiao B, Li X, Zhang Q, Xiong L. 2006. Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proceedings of the National Academy of Sciences, USA* **103**: 12987–12992.

Ishikawa S, Maekawa M, Arite T, Onishi K, Takamure I, Kyozuka J. 2005. Suppression of tiller bud activity in tillering dwarf mutants of rice. *Plant and Cell Physiology* **46**: 79–86.

John I, Hackett R, Cooper W, Drake R, Farrell A, Grierson D. 1997. Cloning and characterization of tomato leaf senescence-related cDNAs. *Plant Molecular Biology* **33**: 641–651.

Kusano H, Asano T, Shimada H, Kadowaki K. 2005. Molecular

characterization of *ONAC300*, a novel NAC gene specifically expressed at early stages in various developing tissues of rice. *Molecular Genetics and Genomics* **272**: 616–626.

Lazar G, Goodman HM. 2006. MAX1, a regulator of the flavonoid pathway, controls vegetative axillary bud outgrowth in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **103**: 472–476.

Li X, Qian Q, Fu Z, Wang Y, Xiong G, Zeng D, Wang X, Liu X, Teng S, Hiroshi F, Yuan M, Luo D, Han B, Li J. 2003. Control of tillering in rice. *Nature* **422**: 618–621.

Mao C, Yi K, Yang L, Zheng B, Wu Y, Liu F, Wu P. 2004. Identification of aluminium-regulated genes by cDNA-AFLP in rice (*Oryza sativa* L.): aluminium-regulated genes for the metabolism of cell wall components. *Journal of Experimental Botany* **55**: 137–143.

Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M. 2005. The NAC transcription factors NST1 and NST2 of Arabidopsis regulate secondary wall thickenings and are required for anther dehiscence. *Plant Cell* **17**: 2993–3006.

Ohnishi T, Sugahara S, Yamada T, Kikuchi K, Yoshiba Y, Hirano HY, Tsutsumi N. 2005. *OsNAC6*, a member of the NAC gene family, is induced by various stresses in rice. *Genes and Genetic Systems* **80**: 135–139.

Olsen AN, Ernst HA, Leggio LL, Skriver K. 2005. NAC transcription factors: structurally distinct, functionally diverse. *Trends in Plant Science* **10**: 79–87.

Ren T, Qu F, Morris TJ. 2000. *HRT* gene function requires interaction between a NAC protein and viral capsid protein to confer resistance to turnip crinkle virus. *Plant Cell* **12**: 1917–1926.

Riechmann JL, Heard J, Martin G, Reuber L, Jiang C, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu G. 2000. Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. *Science* **290**: 2105–2110.

Sablowski RW, Meyerowitz EM. 1998. A homolog of *NO APICAL MERISTEM* is an immediate target of the floral homeotic genes *Apetala3/PISTILLATA. Cell* **92**: 93–103.

Schmitz G, Tillmann E, Carriero F, Fiore C, Cellini F, Theres K. 2002. The tomato *Blind* gene encodes a myb transcription factor that controls the formation of lateral meristems. *Proceedings of the National Academy of Sciences, USA* **99**: 1064–1069.

Schumacher K, Schmitt T, Rossberg M, Schmitz G, Theres K. 1999. The *Lateral suppressor (Ls)* gene of tomato encodes a new member of the VHIID protein family. *Proceedings of the National Academy of Sciences, USA* **96**: 290–295.

Selth LA, Dogra SC, Rasheed MS, Healy H, Randles JW, Rezaian MA. 2005. A NAC domain protein interacts with tomato leaf curl virus replication accessory protein and enhances viral replication. *Plant Cell* **17**: 311–325.

Snowden KC, Simkin AJ, Janssen BJ, Templeton KR, Loucas HM, Simons JL, Karunairetnam S, Gleave AP, Clark DG, Klee HJ. 2005. The *Decreased Apical Dominance1/Petunia hybrida CAROTENOID CLEAVAGE DIOXYGENASE8* gene affects branch production and plays a role in leaf senescence, root growth, and flower development. *Plant Cell* **17**: 746–759.

Sorefan K, Booker J, Haurogne K, Goussot M, Bainbridge K, Foo E, Chatfield S, Ward S, Beveridge C, Rameau C, Leyser O. 2003. *MAX4* and *RMS1* are orthologous dioxygenase-like genes that regulate shoot branching in *Arabidopsis* and pea. *Genes and Development* **17**: 1469–1474.

Souer E, van Houwelingen A, Kloos D, Mol J, Koes R. 1996. The *NO APICAL MERISTEM* gene of *Petunia* is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. *Cell* **85**: 159–170.

Suzuki Y, Uemura S, Saito Y, Murofushi N, Schmitz G, Theres K, Yamaguchi I. 2001. A novel transposon tagging element for obtaining gain-of-function mutants based on a self-stabilizing ac derivative. *Plant Molecular Biology* **45**: 123–131.

Takada S, Hibara K, Ishida T, Tasaka M. 2001. The *CUP-SHAPED COTYLEDON1* gene of Arabidopsis regulates shoot apical meristem formation. *Development* **128**: 1127–1135.

Takeda T, Suwa Y, Suzuki M, Kitano H, Ueguchi-Tanaka M, Ashikari M, Matsuoka M, Ueguchi C. 2003. The *OsTB1* gene negatively regulates lateral branching in rice. *Plant Journal* **33**: 513–520.

Talbert PB, Adler HT, Parks DW, Comai L. 1995. The *REVOLUTA* gene is necessary for apical meristem development and for limiting cell divisions in the leaves and stems of *Arabidopsis thaliana. Development* **121**: 2723–2735.

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J. 2006. A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. *Science* **314**: 1298–1301.

Vroemen CW, Mordhorst AP, Albrecht C, Kwaaitaal MA, de Vries SC. 2003. The *CUP-SHAPED COTYLEDON3* gene is required for boundary and shoot meristem formation in Arabidopsis. *Plant Cell* **15**: 1563–1577.

Ward SP, Leyser O. 2004. Shoot branching. *Current Opinion in Plant Biology* **7**: 73–78.

Xie Q, Sanz-Burgos AP, Guo H, Garcia JA, Gutierrez C. 1999. GRAB proteins, novel members of the NAC domain family, isolated by their interaction with a geminivirus protein. *Plant Molecular Biology* **39**: 647–656.

Xie Q, Frugis G, Colgan D, Chua NH. 2000. Arabidopsis *NAC1* transduces auxin signal downstream of *TIR1* to promote lateral root development. *Genes and Development* **14**: 3024–3036.

Zou J, Chen Z, Zhang S, Zhang W, Jiang G, Zhao X, Zhai W, Pan X, Zhu L. 2005. Characterizations and fine mapping of a mutant gene for high tillering and dwarf in rice (*Oryza sativa* L.). *Planta* **222**: 604–612.

Zou J, Zhang S, Zhang W, Li G, Chen Z, Zhai W, Zhao X, Pan X, Xie Q, Zhu L. 2006. The rice *HIGH-TILLERING DWARF1* encoding an ortholog of Arabidopsis *MAX3* is required for negative regulation of the outgrowth of axillary buds. *Plant Journal* **48**: 687–698.

Supplementary Material

The following supplementary material is available for this article online:

Table S1 Endogenous hormones in the junction of root and shoot of *Ostil1* and the wild-type Nip

Table S2 Primers used for analysing the expression of known genes

Fig. S1 Phenotype of wild-type background *OsNAC2* RNA-interfering transgenic lines. (a) Phenotype of wild-type and transgenic lines. (b) Quantitative RT–PCR analysis of *OsNAC2* in the wild-type and RNA-interfering transgenic lines.

Fig. S2 Analysis of the relationship of *OsNAC2* with known genes. (a) Yeast two-hybrid analysis for the interaction of OsNAC2 and OsTB1. (1) AD-OsTB1 + BD; (2) AD-OsTB1 + BD-OsNAC2 (1–154 aa); (3) AD-OsTB1 + BD-OsNAC2 (148–343aa); (4) positive control, AD-SV40 + BD-P53. (b) Semi-quantitative RT–PCR analysis of known genes involved in shoot branching between the mutant and WT plant.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2007.02177.x
(This link will take you to the article abstract).

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the journal at *New Phytologist* Central Office.

