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Alteration of rice growth and development via antisense expression of *OsGA20ox2* gene

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GA 20-oxidase is a key enzyme involved in gibberellin (GA) biosynthesis. To investigate the roles of *OsGA20ox2* in regulating plant growth and development, we used reverse genomic approach to generate dwarf transgenic rice plants with antisense technology. *OsGA20ox2* nucleic acid sequence includes three exons and two introns. We constructed three antisense vectors named pA1CK, pA2CK, pACK, respectively, major containing the first exon, the second exon, and the whole cDNA sequence of *OsGA20ox2* gene. Through *Agrobacterium*-mediated transformation of rice, six dwarf lines were obtained from antisense vector pACK. Three dwarf lines (5101 to 5103) in T_1 generation displayed severe dwarf in height (12.31 cm, 11.4% of wild type plants), fewer tiller numbers (4.87, 35.3% of wild type plants), smaller dark green leaves, shorter internode length (7.05 cm, 10.9% of wild type plants), and seed abortion compared to the wild type plants. Other three dwarf lines in T_2 generation (5104 to 5106) showed dwarf (56.18 to 60.64 cm, 52.0 to 56.1% of wild type plants), late flowering, few tiller numbers (11.60 to 12.60, 84.1 to 91.3% of wild type plants), short internode length (34.69 to 35.37 cm, 53.4 to 54.5% of wild type plants), 100-seed weight reduction (1.53 to 1.57 g, 74.9 to 75.8% of wild type plants), and reduced seeds reproductive. The results indicate that dwarf lines decreased expression of *OsGA20ox2* gene in stem and panicle but no change in root. These results show that the antisense *OsGA20ox2* gene restricted rice architecture, suggesting that *OsGA20ox2* was required for panicles and stem development. We further showed that the antisense dwarf lines could be restored to normal plant height by applying exogenous GA_3. Results also demonstrate that antisense expression of *OsGA20ox2* affected plant stature and development.

Key words: OsGA20ox2, antisense, dwarf, development.

INTRODUCTION

Loss-of-function mutations of the *OsGA20ox2* gene (sd1), known as the 'green revolution gene', cause semi-dwarfism, resulting in lodging resistance and increased grain yields as is exemplified in an *indica* cultivar IR8 (Sasaki et al., 2002). Up to now, molecular-engineering approaches to modulate plant height have been demonstrated: suppression gibberellin (GA) biosynthesis and catabolism gene. Coles et al. (1999) had successfully modified and plant development in *Arabidopsis* by antisense expression of *GA20ox* gene, which encodes GA 20-oxidase catalyzing in the GA biosynthetic pathways. Similarly, Carmen et al. (2007) suggested that *Carrizo citrange* plants have produced antisense *CcGA20ox1* under control of the 35S promoter to modify plant architecture, which showed shorter phenotypes, shorter thorns compared with wildtype plants (Figure 2).

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Abbreviations: PCR, Polymerase chain reaction; **CTAB,** cetyl trimethyl ammonium bromide; **GA,** gibberellin.

Table 1. Primers for amplified antisense DNA fragments of OsGA20ox2.

Primer	Sequence (5′→3′)	AB077025 (position)
CHK5 (BamH Ⅰ)	ATGGATCC CTCCCCATTGGCGCGAAGTC	540-559
CHK6 (*Kpn* Ⅰ)	TAGGTACCATGGTGGCCGAGCACCCCACG	1-20
CHK3 (*Bam*H Ⅰ)	CCGGATCCGAAGGTGTCGCCGATGTTGAT	856-876
CHK4 (*Kpn* Ⅰ)	TAGGTACCAAGCTCCCATGGAAGGAGACC	448-468
Q2(*Bam*H Ⅰ)	ATGGATCCTCAGCTGGCCGCCTCGACCTG	1150-1170
Q1 (*Kpn* Ⅰ)	TAGGTACCATGGTGGCCGAGCACCCCACG	1-20

**Bam*H Ⅰ, *Kpn* Ⅰ restriction sites were underlined.

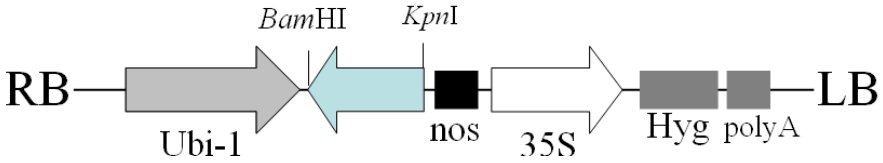

Figure 1. Construction of antisense vector. RB, right border; LB, left border; Ubi-1, the maize (*Zea mays*) ubiquitin promoter; NOS, NOS terminator; 35S, CAMV 35S promoter; Hyg, hygromycin phosphotransferase gene.

Here, we describe the production of transgenic dwarf rice, featuring antisense suppression of *OsGA20ox2* expression. The transgenic dwarf lines could be shown to exhibit a marked reduction of transcripts for *OsGA20ox2* in stem and panicle. Furthermore, the antisense technology developed in this study genetically modified rice growth and development with antisense vectors.

MATERIALS AND METHODS

Plant materials

The *japonica* rice line zhongzuo8923 and zhongzuo0201 and their transformants were used in this study. Rice seeds were immersed in water for two days, sown in pots and grown for one month in a greenhouse, and then transplanted to the field under the natural conditions in Beijing.

Isolation of three fragments of *OsGA20ox2* and antisense vectors construction

The fragments of 559, 408 and 1170 bp of *OsGA20ox2* were obtained from primers CHK5/CHK6, CHK3/CHK4 and Q1/Q2, respectively. These primers were designed based on the sequence of *OsGA20ox2* gene from rice with GenBank accession number AB077025 (Table 1). The 559 bp fragment includes the first exon (557 bp) and 2 bp of the first intron based on genomic DNA amplification, the 408 bp fragment includes part (107 bp) of the first exon and the whole second exon (321 bp) based on genomic DNA amplification, and the 1170 bp fragment was full cDNA of *OsGA20ox2* by reverse transcription polymerase chain reaction (PCR) method. The three amplified fragments were cloned into the pGEM®-T easy vector (Promega, USA) and sequenced using universal SP6 and T7 primers to conform their reality (Sangon, Shanghai). The intermediate vector pCK303 was derived from pCAMBIA1300 by introducing the maize (*Zea mays*) ubiquitin promoter and NOS terminator and was used for our vectors construction (Figure 1). The 559, 408, and 1170 bp fragments of *OsGA20ox2* gene, respectively, was inserted into the *Bam*H Ⅰ-*Kpn* Ⅰ site of pCK303 to construct antisense vector pA1CK, pA2CK, pACK (Figure1).

Agrobacterium-mediated transformation of rice

Mature seeds of rice were dehusked, soaked for 2 min in 70% ethanol and then sterilized by immersion in 0.1% (m/v) $HgCl_2$ for 15 min. Then they were washed five times in sterilized water. Rice embryonic calli were induced on scutella from mature seeds (He et al., 2010) and transformed with the *EHA105* strain of *Agrobacterium tumefaciens* carrying the desired binary antisense vectors. The details of the transformation procedures were as described by Lin and Zhang (2005). Transgenic plants were selected on the medium containing 50 mg /L hygromycin. Hygromycin-resistant T_0 plants were transplanted into soil and grown in greenhouse at 28°C. The T_1 and T_2 populations of transgenic plants were used for further analysis in experiments.

Southern-blot analysis

Genomic DNA was extracted from rice leaves by the cetyl trimethyl ammonium bromide (CTAB) method described by Murray and Thompson (1980). Genomic DNA was extracted from T_0-T_2 populations and subjected to polymerase chain reaction (PCR) analysis of the hygromycin phosphotransferase (*Hyg*) gene as described below. Ten (10) micrograms of genomic DNA was digested with *EcoR*I or *Hind*III and electrophoresed on a 0.8% (w/v) agarose gel and then transferred to a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech). A 770 bp fragment of hygromycin resistant gene, *Hyg* was amplified from pACK plasmid by PCR using the primers 5′-TCC ACT ATC GGC GAG TAC TTC T-3′ (forward), and 5′-AGA GCC TGA CCT ATT GCA TCT C-3′ (reverse), and was used as the DNA probe that was labelled with α-^{32}P-dCTP by the random priming method kit (Takara, Japan).

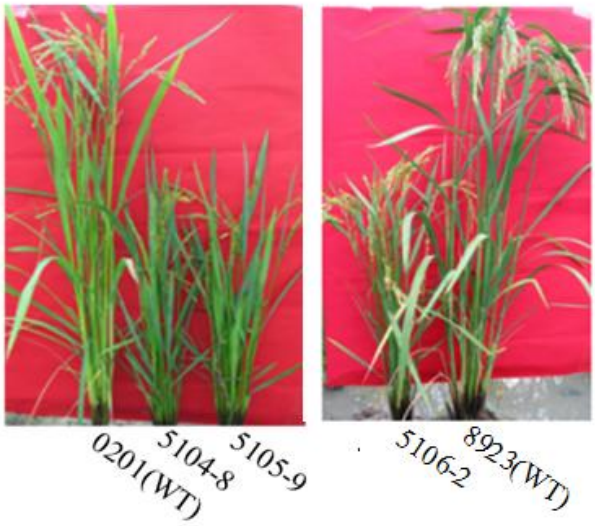

Figure 2. Phenotype of dwarf plants in T2 generation. (A) Phenotypic comparison of representative plants from T2 generation of 5104, 5105 and the wild-type zhongzuo0201 at the adult phase. (B) The height of dwarf lines 5106 in T2 generation and wild-type zhongzuo8923 at the adult phase.

Hybridization was performed in 5 × SSC, 5 × Denhardt´s solution, 0.6% (w/v) SDS, 10% (w/v) dextran sulfate, and 20 mg/L salmon sperm DNA at 65°C for 14 h. Filters were washed twice in 2 × SSC, 0.1% SDS at 65°C for 10 min, once in 1 × SSC, 0.1% SDS at 65°C for 10 min, and once in 0.5 × SSC, 0.1% SDS at 65°C for 10 min. Blots were exposed on a phosphorImager plate and signals were detected by the molecular imager® FX (BIO-RAD).

Extraction of total RNA and semi-quantitative RT-PCR analysis of *OsGA20ox2* transcripts

Total RNA was isolated from leaves in seedling stage, roots in mature stage, flowering panicles in flowering stage, and stems in tillering stage according to the protocol of the manufacturer (Micro-to-Midi Total RNA Purification System, Invitrogen, USA). Two micrograms of total RNA was subjected to reverse transcription with the oligo-(dT)18 primer by AMV First-Strand cDNA Systhesis Kit (Takara, Japan) in a 20 µl reaction volume. The RT-PCR reaction (50 µl) contained 2.0 µl of the cDNA solution, 1x PCR buffer, 0.2 µM dNTPs, 0.2 µM primer, and 1.0 unit of LA Taq polymerase (Takara, Japan).

The expression patterns of *OsGA20ox2* in antisense dwarf lines and wild type were analyzed using semi-quantitative RT-PCR. The *OsGA20ox2* primers were utilized with Z5: AAG CTC CCA TGG AAG GAG ACC, Z3: TCG CCG ATG TTG ATG ACC AT. For loading control and testing DNA contamination, *Actin* (AB047313.1) primers were used, AF: TCC TCC GTG GAG AAG AGC TA, AR: CTG GTA CCC TCA TCA GGC AT.

The expression of *OsGA20ox2* was carried out by RT-PCR under the conditions of up to 35 cycle of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min. Aliquots were sampled at appropriate cycles and, after fractionation on agarose gel electrophoresis, visualized with ethidium bromide staining. The amount of products was densitometrically estimated and the optimal PCR cycle for exponential phase of amplification was determined for each gene.

Treatment of rice plants with exogenous gibberellic acid (GA_3)

Rice seeds were surface-sterilized in 1% NaClO for 10 min, soaked for 2 min in 70% ethanol, and soaked in sterile distilled water at 4°C for 48 h. Then sterilized rice seeds were placed on a 1% (w/v) agar plate containing 30 µM GA_3. Seedlings were grown for 8 days under the conditions described above, and then the lengths of the second leaf sheaths were measured (Oikawa et al., 2004).

RESULTS

Dwarf, reduced internode length and short dark leaves

The antisense vectors pA1CK, pA2CK and pACK were used to transform the rice line zhongzuo8923 and zhongzuo0201. After *Agrobacterium*-mediated transformation, no dwarf transformant from antisense vectors pA1CK and pA2CK was obtained in T_0 generation. Six dwarf transformants named 5101-5106 from pACK were obtained in T_0 generation, and PCR analysis revealed that the *Hyg* gene presented in all the dwarf individuals but not in the tall individuals (data not shown). Fifteen (15) dwarf lines in T_1 generation confirmed the transgenic plants by PCR analysis of *Hyg* gene (data not shown). 5101 to 5103 dwarf individuals in T_1 generation displayed severely dwarf, which were 12.31 cm (11.4% of wild type plants) on average in height during mature period (Table 2). 5101-5103 in T_1 generation showed dark green and small leaf length (12.87 cm average, 33.7% of wild type plants), very few tillering number (4.87 average, 35.3% of wild type plants), late flowering (late 20 days), few seeds or no full seeds failure in germination and growth (Table 2). The internode length of 5101 to 5103 dwarf individuals was 7.05 cm and 10.9% of wild type plants. The panicle axis length of 5101 to 5103 dwarf individuals was 5.02 cm and 24.5% of wild type plants. The differences in height, internode length, panicle axis length, tiller number per panicle, leaf length between 5101 to 5103 dwarf individuals and the wild type plants were statistically significant ($P < 0.05$, Table 2).

The differences in height, internode length, panicle axis length, seeds per panicle, leaf length, 100-seed weight between 5104 to 5106 dwarf plants and wild type plants were statistically significant ($P < 0.05$, Table 2). The height of dwarf plants (56.18 to 60.64 cm) from 5104 to 5106 lines in T_2 generation were about 52.0 to 56.0% of wild type plants (Table 2, Figure 3). The internode length of dwarf plants (34.69 to 35.37 cm) were about 53.4 to 54.5% of wild type plants (Table 2). The panicle axis length of dwarf plants (11.17 to 12.80 cm) were about 53.0 to 62.5% of wild type plants (Table 2). The seeds per panicle of dwarf plants (56.18 to 62.19) were about 51.9 to 56.2% of wild type plants (Table 2). The leaf length of dwarf plants (34.73 to 35.40 cm) were about 90.0 to 92.5% of wild type plants (Table 2). The 100-seed weight of dwarf plants (1.53 to 1.57 g) were about 74.9 to 75.8% of wild type plants (Table 2). The tiller numbers

Table 2. Phenotypic characteristics of wild type and transgenic plants in T_1 or T_2 generation.

Item	Zhonguo0201	5101- 5103	5104	5105	5106	Zhongzuo8923
Plant height (cm)	108.07 ± 1.56^{a}	12.31±1.50^{d}	56.18±1.96^{c}	56.71±2.12^{c}	60.64±2.96^{b}	108.23 ± 2.56^{a}
Internode length (cm)	64.94 ±3.44 ^{a}	7.05 ±0.58^{c}	34.69 ±2.16^{b}	34.71 ±2.41^{b}	35.37 ±2.71^{b}	65.74 ±3.49 ^{a}
Panicle axis length (cm)	20.49 ± 2.04^{a}	5.02±1.39^{c}	12.80±2.63^{b}	11.17±2.44^{b}	11.79±2.44^{b}	22.23 ± 2.12^{a}
Seeds per panicle	108.18 ± 15.5^{a}		56.18±6.96^{c}	60.69±7.68^{b}	62.19±6.90^{b}	110.57 ± 17.3^{a}
Tiller numbers per plant	13.80 ± 1.42 ^{a}	4.87±1.36^{c}	12.60±1.14^{ab}	11.60±3. 03^{b}	12.13±1.26^{ab}	14.57 ± 2.14 ^{a}
Leaf length (cm)	38.27±2.29^{a}	12.87±1.36^{c}	35.33±1.36^{b}	35.40±1.58^{b}	34.73±3.38^{b}	38.57±2.11^{a}
100-seed weight (g)	2.07±0.21^{a}		1.55±0.25^{b}	1.57±0.19^{b}	1.53±0.26^{b}	2.04±0.19^{a}

Data were analyzed in mature stage. zhongzuo0201 and zhongzuo8923 were wild type plants. 5101-5105 were from zhongzuo0201, 5106 from zhongzuo8923. Results are shown from the 15 plants each dwarf lines and are represented as mean ± SE. 15 fully expanded leaves per plant in mature stage were analyzed. 5101- 5103 dwarf plants were analyzed in T_1 generation and 5104- 5106 dwarf plants were analyzed in T_2 generation. Variance analysis was used DPS 7.55. Different letters represent values that are significantly different ($P < 0.05$) between wild type and antisense plants.

Figure 3. Dwarf plants from 0201/pACK in T_2 generation in the field; A: 5104; B: 5105.

per plant of dwarf plants from 5105 and wild type plants were statistically significant ($P < 0.05$, Table 2), and were about 84.1% of wild type plants (Table 2). As a result, the dwarf lines exhibited semi-dwarf, late flowering for 10 days and reduced productivity.

Southern blot analysis of dwarf plants

Southern blotting was conducted to confirm the integration of the antisense vector structure into the rice genome. Based on the fact that the endogenous gene *OsGA20ox2* is homologous to the target sequence of antisense construct, the *Hyg* gene was used as the DNA probe for Southern blotting. Genomic DNA from the three dwarf lines 5104-8, 5105-9, and 5106-2 were digested with *Eco*RI and *Hind*III, and the DNA blots were probed by α-^{32}P-dCTP-labelled *Hyg* gene fragment. The wild type zhongzuo0201 was used as the negative control and no hybridization band was found (data not shown). The hybridization bands of the dwarf lines 5104-8, 5105-9, and 5106-2 are shown in Figure 4. For line 5106-2, only one strong hybridized band presented when the DNA was digested with *Eco*RI or *Hind*III. For 5104-8 and 5105-9, two hybridized bands were observed when the DNA was digested with *Hind*III and *Eco*RI. These results clearly indicate that the antisense vector pACK has been integrated into the genome of rice, and a single or two copy insertions were sufficient to generate dwarfism.

Semi-quantitative RT-PCR analysis of dwarf lines

The expression patterns of *OsGA20ox2* in dwarf lines and wild-type were analyzed using semi-quantitative RT-PCR. As shown in Figure 5, high level and even expression of *Actin* gene was detected in both the dwarf lines and the wild type by RT-PCR with 28 cycles. *OsGA20ox2* were expressed at similar level in mature leaves and roots between dwarf lines and the wild type. The transcript level of *OsGA20ox2* was dramatically decreased in stem and flower panicles of dwarf lines compared with that of wild type. The above results indicate that the expression of *OsGA20ox2* was suppressed in the dwarf plants.

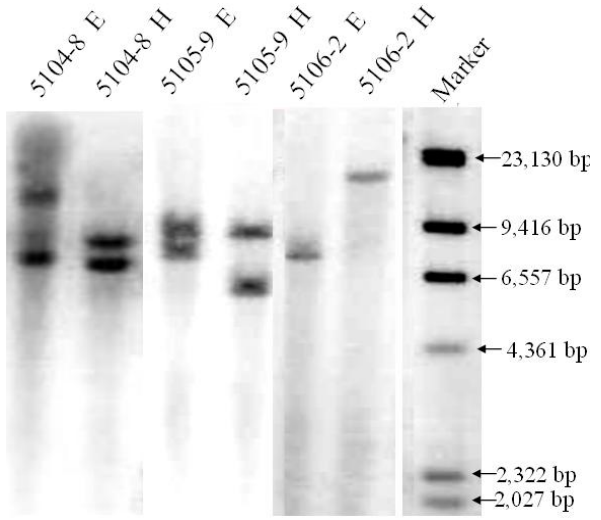

Figure 4. Southern blot analysis of transgenic lines in T_2 generation. Three dwarf plants 5104-8, 5105-9 from zhongzuo0201/pACK, and 5106-2 from zhongzuo8923/pACK. For each lane, 8 µg of rice genomic DNA were digested with *Eco*RI (E) or *Hind*III (H) and resolved on a 0.8% agarose gel. A 770-bp PCR fragment of *Hyg* gene was used as a DNA probe labeled with α-^{32}P-dCTP. Marker is λDNAHindIII digested.

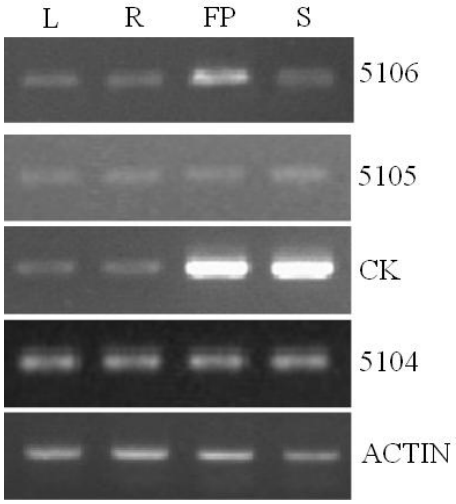

Figure 5. RT-PCR analyses of the expression of *OsGA20ox2* genes in dwarf lines. RNA was extracted from mature leaves (L), roots (R), flowering panicles (FP), and stems (S). 5104, 5105, 5106 lines all were dwarf plants. CK is wild type plant. Rice actin gene was used as a control. The cycle numbers of RT-PCR occurred 28, 35 tested for *Actin*, *OsGA20ox2*, respectively.

Effect of GA_3 on antisense dwarf lines

To further demonstrate that dwarf phenotype of antisense dwarf lines was caused by the decreased contents of biologically active GA, we applied 30 µM exogenous GA_3 to antisense dwarf plants and examined their GA response. Results show that antisense dwarf seedlings responded strongly to GA_3, and their second sheath length was recovered similar to that of the wild type at 30 µM GA_3 for eight days (Table 3). The differences in length of the second leaf sheath between the antisense dwarf plants and wild type plants untreated by exogenous GA_3 were statistically significant (Table 3, $P<0.05$). However, differences in length of the second leaf sheath between the antisense dwarf plants and wild type plants treated with exogenous GA_3 were not statistically significant (Table 3, $P>0.05$). Our results show that the growth of antisense dwarf seedlings was stimulated by the exogenous GA_3. These results further confirm that the endogenous contents of biologically active GAs in antisense dwarf lines were deficient caused by antisense suppression of the *OsGA20ox2* gene.

DISCUSSION

GA20-oxidase genes from many plants were cloned and their function and expression analysis studied (Xiao et al., 2006; Lee et al., 2007; Rieu et al., 2008; Zhao et al., 2010; Carzoli et al., 2008; Huerta et al., 2009; Rodríguez et al., 2011; Song et al., 2011). Coles et al. (1999) produced transgenic Arabidopsis expressing antisense copies of *AtGA20ox1*, *AtGA20ox2* and *AtGA20ox3* cDNAs. Plants expressing antisense copies of *AtGA20ox1* had short hypocotyls and reduced rates of stem elongation. Antisense expression of *AtGA20ox2* had no apparent effects in long days, but stem growth in one transgenic line grown in short days was reduced by 20%. Antisense copies of *AtGA20ox3* had no visible effect, except for one transgenic line that had short hypocotyls (Coles et al 1999). These results demonstrate that plant growth and development can be modified by manipulation of GA 20-oxidase expression in transgenic plants. In the investigation reported here, we examined the efficacy of employing the antisense strategy to knockdown the *OsGA20ox2* gene, which is involved in the biosynthesis of GA, a key factor of the stem elongation of rice. We have demonstrated that antisense method-mediated gene for OsGA20ox2 had successfully generated transgenic rice plants with dwarfism phenotype. Our results also reveal that the lower transcriptional level of *OsGA20ox2* in dwarf lines flowering panicles and stems disrupted the shoot elongation, delayed in flowering and reproductive development.

A ‘‘random antisense cDNA mutagenesis (RAM)’’ approach was utilized in higher plant (Jun et al., 2002). 1,000 transgenic plants of *Arabidopsis thaliana* L. expressing random antisense cDNA(s) were generated, and 37% of the mutations were likely due to antisense effects (Ji et al., 2002). In our experiments, the height of zhongzuo8923 and zhongzuo0201 were reduced by

Table 3. Effect of 30 μM GA_3 treatment on the second leaf sheathes.

2nd Leaf sheath length	Wild type	5104	5105	5106
Untreated with GA_3 (cm)	2.67±0.28[a]	1.95±0.23[b]	1.97±0.19[b]	2.00±0.19[b]
Treated with GA_3 (cm)	8.22 ±0.40[a]	8.03±0.30[a]	8.06±0.36[a]	8.08±0.34[a]

Results are shown from the 15 seedlings each dwarf lines and wild type, and are represented as mean ± SE. Statistical significant was determined by t-test. Different letters represent values that are significantly different ($P < 0.05$) between wild type and dwarf plants.

antisense method knockdown expression of *OsGA20ox2*. In T_0 generation, six dwarf transgenic lines from pACK were obtained, but no dwarf lines from pA1CK and pA2CK. The antisense effect generated by the different constructs suggests that there was a major factor of target antisense fragment. One explanation for the difference in height for different antisense vectors may be target length of encoding fragment in constructs, and the longer target length of encoding fragment (pACK, 1170 bp) was more efficient than the short length of encoding fragment (pA1CK, 559 bp; pA2CK, 408 bp) in dwarf rate. The antisense target encoding fragment was too short to induce antisense knockdown of target gene. Another explanation for our low dwarf rate may be connected with the T-DNA insertion to rice genome site. In T_2 generation, the dwarf degree (rate between average height of dwarf lines and that of wild-type plants) of 5104 to 5106 lines from pACK was 52 to 56%. However, in T_1 generation seeds from 5101-5103 lines were sterile and not further analyzed in this study. Moreover, it is necessary to study forwardly about relation between target fragment and the antisense effect.

Reverse genetics is a powerful tool for assessing gene function, and several alternative reverse genetic approaches to study gene function, such as RNA interference (RNAi) and antisense technology (Cheng et al., 2005; Moriguchi et al., 2011; Antonio et al., 2011). In RNAi technology, the introduction of double-stranded RNAs (dsRNAs) into cells inhibits the expression of the corresponding endogenous gene at transcriptional and posttranscriptional levels (Tang et al., 2003; Matthew, 2004; Travella et al., 2006). Consistent with earlier discoveries that the gene-silencing efficiency of antisense transgene is lower than that of RNAi transgene (Li et al., 2011); growth/developmental defects were observed in the *OsGA20ox2*- antisense transgenic lines.

In conclusion, it has been shown that it is possible to modify the architecture of rice plants by genetic manipulation of endogenous GA20ox expression in transgenic plants. Reduction of plant height by modifying the expression of genes of GA biosynthesis and metabolism is potentially of high agronomic interest.

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