

Induction of Male-Sterility by Controlling of Gibberellin Biosynthesis in Rice (*Oryza sativa*)

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ABSTRACT Gibberellins (GAs) are important hormones at every plant developmental stage including vegetative and reproductive. It is reported in many plant species that many dwarfism and male sterile mutants have defective GA biosynthesis genes. RNA interference (RNAi) technology can reduce expression of specific genes at the transcription level. Also, promoters can control the spatial and temporal expression of genes. Specific promoters containing the RNAi vector can control specific genes' spatial and temporal expression at the transcriptional level. In this study, at first, five anther specific promoters were chosen and verified by examining their tissue specific expression using promoter::GUS transgenic analysis. RNAi vectors, which contain five anther specific promoters and one constitutive promoter fused to GA biosynthesis related genes, were constructed for controlling of GA biosynthesis in stem and floral tissue. *OsAct1* promoter containing *OsGA20ox1* and *OsGA3ox2* RNAi T1 plants showed reduced gene expression and short plant height phenotype compare to wild type plant. *OsGA20ox* and *OsGA3ox* RNAi transformants also showed reduced fertility or sterile phenotype.

Keywords Dwarfism, Gibberellic acid, RNA interference, Pollen specific promoter, Male sterility

INTRODUCTION

The developmental stage of male reproductive organ in flowering plants begins with the establishment of the stamen (Ma 2005). The stamen consists of two distinct structures, the anther and filament. The anther is composed of highly specialized tissues for pollen generation while the filament provides support for the anther. Two sequential steps compose the male gametophytic development, microsporogenesis and microgametogenesis. Itoh *et al.* (2005) classified anther development into 8 stages. Initially, the anther conformation changes from ovoidal into four-cornered shape and the archesporial cells (ACs) initiate at the hypodermal layer of the anther. The ACs differentiates into primary sporogenous cells (PSCs) and primary parietal

cells (PPCs). After several rounds of division, the anther wall layer is formed. PPCs will continue to divide periclinally to generate the endothecium, a middle layer and a tapetum layer and will expand anticlinally. After the anther wall is completed, the PMCs undergo meiosis to produce haploid gametes. The microspore increases in size and undergoes an asymmetric cell division (pollen mitosis I) which produces two cells, the vegetative cell and generative cell. The generative cell undergoes symmetric cell division to produce two sperm cells before pollen tube germination. Finally, the tricellular pollen produced during male gametogenesis contains two sperm cells and one vegetative cell that are required for double fertilization of the egg cell and polar nuclei.

The flower structure of rice is relatively different from

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other cereal crops, like wheat and maize. The rice inflorescence is characterized by the presence of one spikelet that contains one floret enclosed by a pair of empty glumes (Zhang and Wilson 2009). Additionally, the rice floret is bisexual and the floral organs are asymmetrically arranged comprising of one lemma and one palea (first outer whorl), two lodicules (second whorl), six stamens (third whorl) and one pistil (fourth innermost whorl) (Yamaguchi *et al.* 2006; Hirano *et al.* 2008; Zhang and Wilson 2009).

Microarray analyses of the four stages (pre-meiotic, meiotic, single celled anther and trinucleate pollen) of anther development identified 1,000 genes specifically expressed in anther (Deveshwar *et al.* 2011). In a similar study by Fujita *et al.* (2010), the stages of rice reproductive development were dissected and gene expression levels were determined using microarray technology. The 2,593 probe sets were identified on developing anther, whereas only a few were detected in other reproductive stages like fertilization and embryonic stages. Therefore, proper understanding and knowledge of reproductive development processes are very important in the field of plant breeding, especially for hybrid seed production.

Generally, a gene's spatial and temporal expression pattern is determined by its own promoter. Use of an appropriate tissue specific promoter is a critical factor to control gene expression at specific stage and organ-specific manners. In rice, many kinds of pollen/anther/tapetum-specific genes with their promoters have been reported using traditional approaches and recently have been identified via transcriptome study through microarray analysis (Endo *et al.* 2004; Suwabe *et al.* 2008; Tang *et al.* 2010). Most of the genes that are expressed at early stage of microspore development are usually tapetum-specific since their cells usually play roles in leading the initial development of microspore. However, binucleate pollen stage at the beginning of tapetum cell degradation stage, gene expression patterns are rapidly changed. Expressions of tapetum specific genes disappeared and pollen specific genes begin to emerge at that point. The tapetum specific gene *Osc4* is expressed at the early uninucleate microspore stage for a short time (Tsuchiya *et al.* 1992; Tsuchiya *et al.* 1994). *Osg6b* show strong expression at uninucleate microspore stage and it is gradually reduced to trinucleate pollen stage

(Tsuchiya *et al.* 1992; Tsuchiya *et al.* 1994). In cases of pollen-specific genes, *OsSCP1* and *OsSCP2* belonging to a multi-gene family, *OsSCP*, are expressed at the late stage of pollen development from tricellular pollen stage to pollen tube germination (Park *et al.* 2006). *OsIPA* was identified as an indica rice derived pollen specific gene, is expressed at the late pollen development stage like *OsSCP* family (Gupta *et al.* 2007).

Gibberellins (GAs) diterpenoids that are based on a tetracyclic ent-gibberellane skeleton act as a growth regulator during plant development. Generally, GAs have an active role in plant vegetative development and also control the development of reproductive organs such as anther, tapetum and pollen. Generally, low GA contents can cause the delay or elimination of anthesis. Some studies showed that GA deficient or signaling mutant can induce male-sterile rice (Sakamoto *et al.* 2001; Kaneko *et al.* 2003). Also in *Arabidopsis*, GA-deficient mutant showed male-sterile phenotype with normal female organs (Koornneef and van der Veen 1980). *GAMYB* is widely known as GA signaling factor and a knock out mutant of rice *OsGAMYB* showed abnormal development of floral organs, shrunken and whitened anther, and delayed heading time in rice (Gubler *et al.* 1995; Kaneko *et al.* 2003). Additionally, GA-insensitive and defective mutants showed low fertilization by pollen viability and pollen tube growth (Chhun *et al.* 2007). Most GA-related studies in rice have been focused on plant vegetative aspects such as stem growth. Many research groups also reported that GA-related mutants can be cause of severe male-sterility in rice (Sakamoto *et al.* 2001; Oikawa *et al.* 2004; Sakamoto *et al.* 2004; Qiao *et al.* 2007). Interestingly, the most famous GA biosynthesis-related gene, knock-out mutant *sd1* and *d18*, did not show any fertility changes (Sakamoto *et al.* 2004). Therefore, *sd1* has been used as a critical gene for semi-dwarf rice breeding. This result, nevertheless, also provides another opportunity to study rice flower development with GA biosynthesis related genes - *OsGA20ox* gene including *sd1*, *GA20ox2* with four iso-genes, *OsGA3ox* gene including *d18* and *GA3ox2* with 2 iso-genes. It means that except *sd1* and *d18*, other genes have possibility of controlling flower development.

In this study we constructed six vectors containing five anther/pollen specific promoters and one constitutive promoter fused with GUS::GFP to verify the tissue specific expression of promoters. In addition, twenty kinds of RNAi vector were constructed to induce a male-sterile rice, which harbored five anther/pollen specific promoters of *Osc4*, *Osg6b* (Tsuchiya *et al.* 1994), *OsSCP1*, *OsSCP2* (Park *et al.* 2006) and *OsIPA* (Gupta *et al.* 2007), one constitutive promoter of *OsAct1*, and four GA biosynthesis related genes such as *OsGA20ox1*, *OsGA20ox3*, *OsGA3ox1* and *OsGA3ox2*; were constructed to induce a male-sterile rice. As a result, each GA-RNAi T₀ lines showed lower fertility than control T₀ lines but had similar grain numbers. Several GA-RNAi progenies showed short culm length trait as expected.

MATERIALS AND METHODS

Cloning of rice promoters

The promoter sequence information of six rice genes, *Osc4* (1,638-bp promoter), *Osg6b* (1,671-bp promoter), *OsSCP1* (2,033-bp promoter), *OsSCP2* (952-bp promoter), *OsIPA* (1,887-bp promoter) and *OsAct1* (1435-bp promoter) were obtained through reference papers (McElroy *et al.* 1990; Tsuchiya *et al.* 1994; Park *et al.* 2006; Gupta *et al.* 2007). The japonica rice cv. Dongjin genomic DNA was used as template for PCR reaction (Table 1). The condition for PCR reaction was 30 cycles of 94°C 30 seconds, 55°C

30 seconds, and 72°C 2 minutes, using Pfu-X DNA polymerase (Solgent, Korea). PCR product of *Osc4* promoter (hereafter pro) and *Osg6b* pro were cloned into pGEMT-easy vector (Promega, USA) and PCR product of *OsSCP1* pro, *OsSCP2* pro, *OsIPA* pro and *OsAct1* pro were cloned into T-Blunt vector (Solgent, Korea).

Construction of promoter-GUS::GFP fused vector

For the LR cloning, promoters cloned into pGEMT-easy and T-Blunt vector were used as a template DNA for PCR amplification. The 1st PCR reaction condition was 10 cycles of 94°C 30 seconds, 55°C 30 seconds, and 72°C 3 minutes using Pfu-X DNA polymerase (Solgent, Korea). The 2nd PCR was performed using an Attb primer pair and the PCR reaction condition was 5 cycles of 94°C 30 seconds, 45°C 30 seconds, and 72°C 1 minute and 20 cycles of 94°C 30 seconds, 55°C 30 seconds, and 72°C 3 minutes. PCR products were cloned into a pDONR201 entry vector using BP clonase II mix. The promoter inserted entry vectors were combined with pKGWFS7 destination vector for promoter analysis using LR clonase II mix.

Cloning of RNAi trigger region

The japonica rice cv. Dongjin DNA was used for PCR reaction. RNAi trigger regions from four rice genes - *OsGA3ox1*, *OsGA3ox2*, *OsGA20ox1* and *OsGA20ox3* - were PCR amplified using gene specific primers (Table 2). The PCR reaction conditions for *OsGA3ox1* and *OsGA3ox2* were 30 cycles of 94°C 30 seconds, 55°C 30

Table 1. Primer sequences for rice promoter cloning.

Promoter	Primer sequences (5'→3')	Size (bp)
<i>Osc4</i> pro	F: AAGCTTCAGACCGCTGGTCAGTGGA R: CTCGAGCTCCTGTGCTTCTTCAGAC	1,638
<i>Osg6b</i> pro	F: AAGCTTTTGTTAAACCCCTCAGGT R: CTCGAGCTTGAGCTAGCTTAATTAG	1,671
<i>OsSCP1</i> pro	F: AAAACTCGAGTCTGTTGTTCTACCCAATATTGTTAC R: TTTTATTAATTAGCACCGTTTCTATTGAGGGTAAC	2,033
<i>OsSCP2</i> pro	F: TAGCACCGTTTATGCTGAGCAGAATTGGTG R: ACTGGGTCGACTTGGGCCGGGAGGAGAGA	952
<i>OsIPA</i> pro	F: AAAAAAGCTTGTGTCGACCTCGGCATCCTCGGCG R: TTTTCTCGAGGTCGCTTTTATTTGGCTTGGTGTGTTT	1,887
<i>OsAct1</i> pro	F: AAAAAAGCTTTAGCTAGCATACTCGAGGTCATTCATATGC R: TTTTATTAATGATATCCTCGGCGTCAGCCATC	1,435

Table 2. Primer sequences for the cloning of RNAi trigger fragments.

Genes	Primer sequences (5'→3')	Size (bp)
<i>OsGA20ox1</i>	F: AAAAAGCAGGCTGCTGTCGTTCCGGTACTCATCGG R: AGAAAGCTGGGTTGCGGTACCTGGCGTTGGAGAG	457
<i>OsGA20ox3</i>	F: AAAAAGCAGGCTCAGGAGTACTGCGACGTGATGACG R: AGAAAGCTGGGTGGGTTGAGGAAGAAGGTGAGCGAC	422
<i>OsGA3ox1</i>	F: GGATCCCTAGGTGTCCAGATCCA R: CTCGAGGAAGTAGGGCATGGAG	272
<i>OsGA3ox2</i>	F: GGATCCGTCGCACTTGAAGAACC R: CTCGAGGGAGGAAGGGGAGAAGG	409

seconds, and 72°C 1 minute using Pfu-X DNA polymerase (Solgent, Korea). PCR products of *OsGA3ox1* and *OsGA3ox2* were double digested with *HindIII/XhoI* and cloned into pENTR donor vector. *OsGA20ox1* and *OsGA20ox3* were cloned into pDONR201 by using BP reaction. The 1st PCR reaction condition of *OsGA20ox1* and *OsGA20ox3* was 94°C 30 seconds, 55°C 30 seconds, and 72°C 1 minute and then the 2nd PCR was performed using an Attb primer pair. The 2nd PCR reaction condition was 5 cycles of 94°C 30 seconds, 45°C 30 seconds, and 72°C 1 minute and 20 cycles of 94°C 30 seconds, 55°C 30 seconds, and 72°C 1 minute. PCR products were cloned into pDONR201 vector using BP clonase II mix (Invitrogen, USA).

Promoters inserted RNAi destination vectors

Promoters (*Osc4* pro, *Osg6b* pro, *OsSCP2* pro, *OsIPA* pro and *OsAct1* pro) that cloned into T-Blunt vector were digested with *HindIII/XhoI*. The digested fragments were then cloned into promoter insertion sites in pK7gwiwGL RNAi vector (<http://www.psb.ugent.be/gateway>), which was digested with same restriction enzymes. *OsSCP1* promoter was cloned into pK7gwiwGL RNAi vector by double-digestion with *XhoI/AseI*. The resulting pK7gwiwGL RNAi destination vectors containing each promoter were combined with donor vector (pENTR; *OsGA3ox1*, *OsGA3ox2*; pDONR201; *OsGA20ox1*, *OsGA20ox3*) using LR clonase II (Invitrogen, USA).

Agrobacterium mediated rice transformation

The japonica rice cv. Dongjin was used as the material for transformation. Transgenic rice plants were generated

using *Agrobacterium*-mediated transformation of rice calli, as described by Toki *et al.* (2006). The transgenic rice plants were cultivated in the GMO experimental field of Kyungpook National University (Gunwi, Gyeongsangbuk-do province).

DNA extraction and T-DNA confirm PCR

Rice genomic DNA was extracted using Cetyltrimethylammonium bromide (CTAB) DNA isolation method as described by Ronald and Chen (1999). The presence of T-DNA in genome of transgenic plants was confirmed by PCR amplification using Attb primers. PCR condition was 30 cycles of 94°C 30 seconds, 45°C 30 seconds, and 72°C 1 minute.

Histochemical GUS assay

Anther from T₀ transgenic plants were collected at 1-2 weeks before heading and used for histochemical β -glucuronidase (GUS) assay. The GUS staining solution was 100mM sodium phosphate buffer, pH 7.0, 20% methanol, 0.1% triton X-100, 0.5mM K₃F₂(CN)₆ and 1mM X-GlcA (5-bromo-4-chloro-3-indoxyl- β -D-glucuronic acid). Five spikelets were incubated overnight at 37°C and then chlorophyll was removed by washing with 70% ethanol.

RNA extraction and semi-quantitative RT-PCR

Plant total RNA was extracted using Qiazol RNA extraction buffer (Qiagen, USA). RNA was extracted from 4-week old T₁ plants from each semi-dwarf line. Two micrograms of total RNA were used to generate first-stand cDNA through RT-PCR by using Diastar-RTase kit (Solgent, Korea) with oligo(dT)₁₈ primer (Genotech,

Korea). Primer sequences for RT-PCR analysis of *OsGA20ox1* and *OsGA3ox2* genes were designed based on information provided by Sakamoto *et al.* (2004). The total volume of single reaction was 20 μ L containing 1 μ L cDNA, 1X PCR buffer, 0.2 mM dNTPs, 0.2 μ M primer and 1 unit of Diastar-Taq DNA polymerase (Solgent, Korea). The reaction condition was 35 cycles of 94°C 30 seconds, 45°C 30 seconds, and 72°C 1 minute. Relative quantity of RT-PCR product was calculated with the used of Image Lab software (Bio-Rad Laboratories).

Fertility analysis

To estimate spikelet fertility, five panicles were selected randomly from each T_0 plant at maturity and their grain numbers were counted. Sunken grains in the distilled water were counted as fertile; seed and floated grains were counted as sterile. The fertility rate (FR) describes the fraction of grains with successfully seed sets to the total number of grains from 5 panicles of T_0 plant; Thus, $FR(\%) = (\text{fertile grain}/\text{total grain}) \times 100$.

Hydroponic culture of rice seedling

Rice seeds were soaked in seed disinfectant for a day and soaked seeds were incubated at 28°C in dark for 24 hours after removing the disinfectant. Germinated seeds were sowed on sterilized sand and were grown for a week under a condition of 32°C, 14-hour light and 28°C, 10-hour dark. Twenty well-grown, same size rice seedlings were planted on rice hydroponic solution (Yoshida *et al.* 1976). Rice seedlings were grown for 4 weeks at a culture condition of 32°C, 14-hour light and 28°C, 10-hour dark in growth chamber. Hydroponic solution was changed once a week. Within twenty seedlings, three were collected for RNA extraction and the others were used for GA contents analysis.

RESULTS

Construction of promoters-GUS::GFP fused vector for the analysis of anther/pollen specific promoters

To verify the tissue-specificity of promoters, pKGWFS7 promoter analysis vector was used. pKGWFS7 is the

promoterless-vector system fused GUS/GFP reporter genes containing attR1 and attR2 sites at the promoter region for LR cloning. Six promoters; *Osc4* pro, *Osg6b* pro, *OsSCP1* pro, *OsSCP2* pro, *OsIPA* pro and *OsAct1* pro, which were cloned to the pDONR201 were inserted into pKGWFS7 by LR cloning. Previously, it has been reported that, *Osc4* pro and *Osg6b* pro were characterized as tapetum-specific promoters, and *OsSCP1* pro, *OsSCP2* pro and *OsIPA* pro were pollen-specific promoters (Tsuchiya *et al.* 1994; Park *et al.* 2006; Gupta *et al.* 2007). Also, *OsAct1* pro is widely used constitutive promoter as a control. Consistent with previous reports, all analyzed T_0 transgenic plants exhibited strong GUS activity at the anther except *OsSCP2* pro. As we expected, *OsAct1* pro induced strong expression at whole spikelet tissue (Fig. 1).

RNAi vector construction for the suppression of GA biosynthesis

In the GA biosynthesis pathway, many different kinds of enzymes are involved. Among these enzymes, GA 20-oxidase and GA 3-oxidase are known key enzymes which catalyze the inactive GAs to active GAs. There are four GA 20-oxidase genes; (*OsGA20ox1*, *OsGA20ox2*, *OsGA20ox3*, and *OsGA20ox4*) and two GA 3-oxidase genes (*OsGA3ox1* and *OsGA3ox2*) in rice. All *OsGA20ox* genes are expressed in panicles at different levels. *OsGA20ox1*, *OsGA20ox2*,

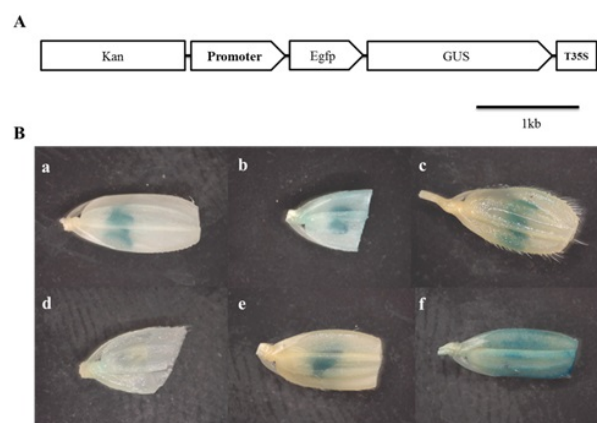


Fig. 1. Histochemical GUS analysis of rice flower. (A) Structure of pKFWFS7 destination vector carrying promoter-GUS::GFP gene fusion. (B) Results of GUS analysis. (a) *Osc4* pro, (b) *Osg6b* pro, (c) *OsSCP1* pro, (d) *OsSCP2* pro, (e) *OsIPA* pro, (f) *OsAct1* pro.

and *OsGA20ox4* are expressed in all vegetative organs at different levels but *OsGA20ox3* is only expressed at panicle. Like *OsGA20ox*, *OsGA3ox2* is expressed in all organs but *OsGA3ox1* is only expressed at panicle (Itoh *et al.* 2001; Kaneko *et al.* 2003; Sakamoto *et al.* 2004; Hirano *et al.* 2008). With this result, *OsGA20ox3* and *OsGA3ox1* are expected to be the floral tissue specific genes. The *sd1* (*OsGA20ox2*) and *d18* (*OsGA3ox2*) are also widely known dwarf mutants caused by GA deficiency (Itoh *et al.* 2001; Spielmeier *et al.* 2002). For controlling of GA biosynthesis at the anther/pollen development stage in rice, two *OsGA20ox* iso-genes (*OsGA20ox1* and *OsGA20ox3*) and two *OsGA3ox* iso-genes (*OsGA3ox1* and *OsGA3ox2*) were

chosen in order to construct the RNAi vector. Also, promoters of previously reported five anther/tapetum/pollen-specific genes (*Osc4*, *Osg6b*, *OsSCP1*, *OsSCP2*, *OsIPA*) and one constitutive expressed gene *OsAct1* were used to control tissue specific expression. Donor vector containing PCR amplified RNAi trigger fragments *OsGA20ox1* (457 bp), *OsGA20ox3* (422 bp), *OsGA3ox1* (272 bp) and *OsGA3ox2* (409 bp) were combined with pK7gwiwGL, and the resulting twenty RNAi destination vectors were constructed to generate rice transgenic plants (Fig. 2).

Generation of semi-dwarf mutant rice via transformation using RNAi vector

GA plays an important role in plant stem growth. Especially two GA biosynthesis-related genes, *OsGA20ox2* and *OsGA3ox1*, are known to be the major gene in rice vegetative growth. Two rice mutants, *sd1* and *d18*, which have mutations in these, were widely known to exhibit unique mutant phenotype of dwarfism due to a defective GA-biosynthesis pathway. It has also been reported that *OsGA20ox1* was involved in rice stem growth through GA processes. Some studies showed the possibility of semi-dwarfism induced through biotechnological approach via controlling of *OsGA20ox2* or *OsGA3ox2* (Itoh *et al.* 2002; Qiao *et al.* 2007).

To examine whether RNAi constructs of *OsGA20ox1* and *OsGA3ox2* affected GA biosynthesis in rice, transgenic lines were generated and analyzed on plant height and gene expression. If the RNAi vectors used in this study worked properly, we expected that resulting transgenic plants would be semi-dwarf as previously reported (Itoh *et al.* 2002; Oikawa *et al.* 2004; Qiao *et al.* 2007). As we expected, transgenic lines of *OsAct1* pro-*OsGA20ox1* and *OsAct1* pro-*OsGA3ox2* RNAi in T₁ generation, showed semi-dwarf phenotype but not in *OsGA20ox3* RNAi transgenics. T₁ transgenic progeny plants generated by RNAi vector of *OsAct1* pro-

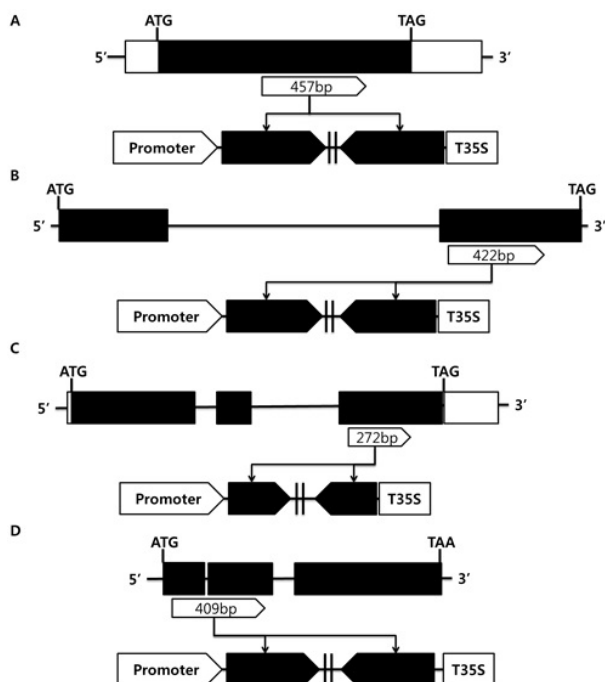


Fig. 2. Structure of *OsGA20ox* and *OsGA3ox* genes and their RNAi trigger regions used for construction of RNAi vectors. (A) *OsGA20ox1* (457 bp), (B) *OsGA20ox3* (422 bp), (C) *OsGA3ox1* (272 bp), (D) *OsGA3ox2* (409 bp).

Table 3. Summary of plant heights of semi-dwarf lines.

Line	Dongjin	20ox1-1	20ox1-2	20ox1-3	3ox2-1	3ox2-2	3ox2-3	3ox2-4
Plant height (cm)	100.8±8.3 ^{a,z)}	79.2±9.9 ^{bc}	85.6±5.0 ^b	74.3±5.9 ^c	75.3±3.4 ^{bc}	82.4±4.7 ^{bc}	84.1±5.3 ^b	80.9±10.0 ^{bc}

^{z)}Values with the same letter in a column are not significantly different at 5% probability level as determined by DMRT.

OsGA20ox1 gene exhibited approximately 20% shorter plant height which ranged from 74.3 ± 5.9 - 85.6 ± 5.0 cm compared to cv. Dongjin wildtype plant with average height of 100.8 ± 8.3 cm. T₁ transgenics from *OsAct1* pro-*OsGA3ox2* RNAi vector also showed similar pattern in height with the ranges of 75.3 ± 3.4 - 84.1 ± 5.3 cm (Table 3). Other transgenics containing specific promoter had not shown decreased height phenotype but some lines showed reduced fertility (data not shown).

Hence, it suggested that expression of these genes, *OsGA20ox1* and *OsGA3ox2*, were required for rice stem growth, strongly indicated that they played a role in GA biosynthesis pathway in rice. Furthermore, these results provide the possibility that these RNAi constructs can be applied to control GA biosynthesis in specific organs such as anther or pollen for the induction of male sterility.

Analysis of gene expression

Many GA-related studies previously reported the expression of *OsGA20ox1* and *OsGA3ox2* in all tissues and all stages of rice growth period (Kaneko *et al.* 2003; Sakamoto *et al.* 2004; Hirano *et al.* 2008). *OsGA3ox2* is an especially well-known major *GA3ox* gene in rice vegetative growth. Although *OsGA20ox2*, among *GA20ox* 4 isogenes, has been known as a major *GA20ox* in rice vegetative growth and *OsGA20ox1* has weaker expression than *OsGA20ox2*, *OsGA20ox1* also has the significant effect on rice stem growth (Oikawa *et al.* 2004).

To investigate the down-regulation of target gene expression of *OsAct1* pro-*OsGA20ox1* and *OsAct1* pro-*OsGA3ox2* RNAi transgenic plants, RT-PCR was performed using total RNA extracted from 4-week old rice stem tissues. Consistent with the results on plant heights, 4-week old seedlings also showed semi-dwarf phenotype (data not

shown). We confirmed that all plants contained T-DNA copies in their genome. The expression of the target gene in all *OsAct1* pro-*OsGA20ox1* RNAi lines tested was relatively weaker compared to wildtype (Fig. 3). However, in case of *OsGA3ox2* RNAi lines, expression levels were variable among transgenic lines (Fig. 4). Since the expression of *OsGA3ox2* in wild type plants was significantly weak, it's difficult to evaluate.

Among the transgenic lines generated from *OsGA3ox2*, we observed the typical semi-dwarf phenotype in two lines with similar expression levels compared to wildtype. Even though the increased expression level does not explain the observed phenotype on seedling height, these results suggested that RNAi constructs, to a certain extent, influenced the expression level of *OsGA3ox2*.

Generation of sterile rice regulation of *OsGA20ox* and *OsGA3ox* expression.

Our experimental goal is to generate rice transgenic lines which are male sterile induced by inhibition of GA biosynthesis in anther or pollen, and can be reversible to fertile plants by exogenous GA treatment. Therefore, it is essential to use anther/pollen specific promoters that can direct gene expression strictly at anther or pollen tissues during male gametophytic development in rice.

Total, twenty kinds of RNAi constructs containing six promoters combined with four RNAi trigger fragment of genes involving in rice GA biosynthesis pathway (*OsGA20ox1*, *OsGA20ox3*, *OsGA3ox1*, and *OsGA3ox2*) were constructed and introduced into rice. To compare with RNAi transformants, each promoter was also fused with GUS::GFP gene from pKGWFS7 vector and was introduced into rice to generate T₀ transgenic lines which were used as controls for the fertility evaluation.

T₀ plants transformed with RNAi vectors exhibited on

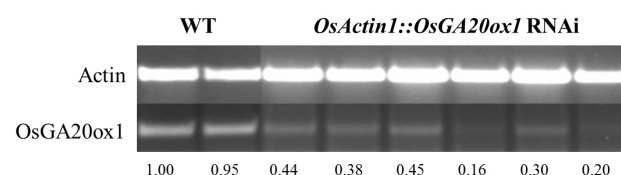


Fig. 3. RT-PCR result of *OsAct1* pro-*OsGA20ox1* semi-dwarf mutant lines. The numbers below gel photo are relative brightness value of the PCR band.

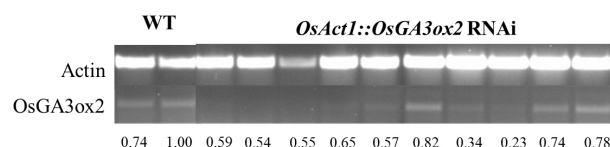


Fig. 4. RT-PCR result of *OsAct1* pro-*OsGA3ox2* semi-dwarf mutant lines. The numbers below gel photo are relative brightness value of the PCR band.

average lower fertility than T₀ plants from control vectors carrying the anther specific promoters fused with GUS::GFP genes. Every T₀ line showed lower fertility than that of wildtype rice. The average fertility rate (AFR) of T₀ line was 71% on *Osc4* pro-GUS::GFP, 57% on *OsIPA* pro-GUS::GFP, 58% on *OsSCP1* pro-GUS::GFP and 62% on *OsAct1* pro-GUS::GFP. Furthermore, severe sterile plants, less than 10% in AFR, were not observed in the control T₀ line. On the other hand, the highest AFR among RNAi lines containing genes involved in GA biosynthesis pathway was approximately 52% in T₀ line of *OsIPA* pro-*OsGA20ox3* in which complete-sterile plant was not found. While, the lowest AFR was 21% in *OsIPA* pro-*OsGA3ox2* in which 22 sterile plants were observed with the frequency of 42.5% (22/38). Among *OsGA20ox1* RNAi lines, a line carrying *OsSCP2* promoter showed AFR of 22%, but the other three lines showed 41%–49% AFR. It was slightly higher than other RNAi lines. In *OsGA20ox3* RNAi lines, every line showed similar AFR 44%–52%. It was the highest AFR value among RNAi transgenic lines. Among *OsGA3ox1* RNAi lines, those carrying *OsSCP1*, *OsIPA* promoters, showed 26% and 28% AFR respectively, and other lines exhibited AFR range of 32%–45%. It was the lowest AFR value among RNAi transgenic lines. In *OsGA3ox2* RNAi lines, lines controlled by *OsSCP1*, *OsIPA* promoter showed 24% and 21% AFR, and other lines showed AFR range of 37%–51% (Table 4).

In conclusion, AFRs in RNAi T₀ lines of *GA20ox1*, *GA20ox3*, *GA3ox1*, and *GA3ox2* were 37%, 49%, 32% and 36% respectively. Severe sterile plants have been found in most populations of T₀ RNAi plants except for that of

OsIPA pro-*GA20ox3* T₀ plants. Hence, our data of fertility analysis showed that all AFRs were similar, except for the *GA20ox3* T₀ RNAi line. Although T₀ lines of *GA20ox3* exhibited the highest AFR, it was lower than any of T₀ the control lines.

Among the six promoters, more effective promoters were those of *OsSCP2*, *OsSCP1* and *OsIPA*. In *OsGA3ox* RNAi lines, *OsSCP1* and *OsIPA* promoter showed more effective than other promoters. In *OsGA20ox* RNAi lines, unlike with *OsGA3ox*, *OsSCP2* promoter had stronger effect than other promoters. Although fertility of RNAi T₀ transgenic plants was slightly lower than transgenic control lines, numbers of glumous flowers in transgenic plants were almost similar between RNAi and control transgenic plants.

Fertility of transgenic lines

In our study, the accurate assessment of a fertility-related phenotype that occurs frequently in transgenic plants caused by somaclonal variation during the tissue-culture process was the important point. They usually showed reduced-fertility or even sterility because T-DNA insertions can generate the defects in anther development due to chromosome re-arrangements, and also because *Agrobacterium*-mediated transformation tends to decrease fertility frequency (Xing and Zachgo 2007). For instance, Hsing *et al.* (2007) have produced approximately 55,000 T₀ rice transgenic lines by using *Agrobacterium*-mediated transformation, and 8% of them are sterile. Although our population was smaller than of previous experiment, no severe sterile phenotype was found on control transgenic plants.

Table 4. Average fertility (%) of T₀ RNAi lines from RNAi vectors of genes involving in GA biosynthesis.

Promoters	Genes					Total
	<i>GUS</i>	<i>GA20ox1</i>	<i>GA20ox3</i>	<i>GA3ox1</i>	<i>GA3ox2</i>	
<i>OsAct1</i> pro	62	39	50	26	48	58 ^{a,z)}
<i>Osc4</i> pro	71	-	-	45	40	56 ^a
<i>Osg6b</i> pro	-	-	-	32	52	58 ^a
<i>OsSCP1</i> pro	58	41	50	26	24	46 ^b
<i>OsSCP2</i> pro	-	22	44	33	37	37 ^c
<i>OsIPA</i> pro	57	49	52	28	21	41 ^{bc}
Total	61 ^a	37 ^c	49 ^b	32 ^c	36 ^c	

^{z)}Values with the same letter in a column are not significantly different at 5% probability level as determined by DMRT.

Progeny analysis of RNAi lines with low fertility

We also planted progenies of RNAi lines, including severe sterile line and normal fertility line. Through several generations (T_1 - T_3), RNAi transgenic lines showed segregated fertility or restore fertility. It was equally *OsGA20ox* and *OsGA3ox* lines. Undoubtedly, progenies of lower FR lines showed lower FR than higher FR lines progenies. However, in most cases transgenic lines fertility was restored. Even though their T_0 line showed severe sterile phenotype (less than 5% FR) their progenies showed restored fertility up to 80%; it is almost same with wildtype. In some cases, sterile phenotype was also transferred to T_3 progeny but it also showed restored fertility up to 30%. Some transgenic lines lose their T-DNA through several generations and because of that their fertility was restored. Also, some lines might have their trait by somaclonal variation during rice tissue culture process; it might have disappeared through several generations.

DISCUSSION

Effect of GA in rice flower development

Many experiments have shown that the shortage of endogenous GA content causes the male-sterile phenotype. Since it has been known that GA's major function in plants is controlling stem growth and breaking dormancy, researchers have been focused on plant height especially on dwarf phenotype. Also, GA-related dwarf mutants had been used for critical genetic source of modern rice breeding. In spite of the following facts, some studies suggest that GA also controls fertility and flower development. The goal of this study is to produce a reversible male-sterility by controlling the hormones, specifically GA for the production of F_1 hybrid rice. Traditionally in F_1 hybrid production, the three-parent system requires an MS line, maintainer line and restorer line. However, this system is not an effective way of producing F_1 hybrid seed because of its cost-ineffectiveness. By using the reversible male-sterile line, the 2-parent system, which requires an MS line and a restorer line, can be employed. The future goal of this study is to focus on

examination of fertility recovery by exogenous GA treatment.

Among GA-biosynthesis genes, *OsGA20ox3* and *OsGA3ox1* predominantly express gene at floral tissues and *OsGA20ox1* and *OsGA3ox2* express at both vegetative tissues and floral tissues. For this reason, we focused on *OsGA20ox3* and *OsGA3ox1* as a candidate gene of GA biosynthesis regulative gene at floral tissues. Additionally, *OsGA20ox1* and *OsGA3ox2* were reported as genes that are concerned with plant height in rice and their expression was detected on flower tissues. In our study, the most effective gene that might control the fertility of rice flower was the *OsGA3ox1*. The second most effective gene was *OsGA3ox2*. The *OsGA20ox* group was less effective than of *OsGA3ox* group. It is thought that there are four kinds of genes in the *OsGA20ox* group and so each gene expresses complementarily during the flower development stage, even though *OsGA20ox3* is preferential gene. Actually, every *OsGA20ox* gene family and *OsGA3ox* gene family has expression at the flower, with different expression intensity. In *OsGA3ox* group, two genes exist and both have an important role in flower development stage. Among anther specific promoters used to induce male-sterility in this study, *OsSCP1* and *OsIPA* promoters were the most effective when used together with *OsGA3ox* gene family, while *OsSCP2* promoter was the most effective when used together with *OsGA20ox* gene family. Actually, *OsSCP* genes constitute a gene family and was reported to show same expression pattern. RNAi lines with promoters of *OsSCP1* and *OsSCP2* showed small different results, indicating that both promoters are effective for reducing expression levels of *OsGA20ox* and *OsGA3ox* genes.

Male-sterility is an essential component for the successful production of F_1 hybrid rice seed. Many crops have traditionally been developed through a three-line system: the cytoplasmic genic male-sterile (CGMS) line, maintainer line, and restorer line. However, these systems have disadvantages, such as a limitation on restorer germplasm, a complex procedure for parental seed production, and time-consuming breeding of CGMS (Virmani 1994). China and several other Asian countries scientists have recently found out a new technological approach to replace the traditional CGMS system and simplified two-line protocol was successfully established,

variety of germplasms are available as parents which maintainer lines did not need (Virmani 1994). The new hybrid production system is based on environment-sensitive a genetic male-sterile (EGMS) line which is controlled by environmental condition like photoperiod or temperature (Virmani 1994). One weakness point of EGMS lines is that environmental conditions must be constant. Therefore, unexpected environmental changes can cause of fertility restoration that lead to failure in hybrid seed production.

Recoverable male sterility by chemical treatment can be a useful and crucial tool for hybrid seed production. Most male-sterile mutants produce defective in pollen generation or showed abnormal developmental process in sporophytic tissue in the tapetum and filaments. Other developmental processes are related to changes in the levels of plant growth regulator (Huang *et al.* 2003). For instance, the need for gibberellins in formation of male gametophytes was supported by studies of defective mutants that can restore the male sterility through application of GAs (Jacobsen and Olszewski 1991). Although endogenous phytohormones modulation can cause male sterility in many higher plants, their fertility can be recovered with exogenous application of suitable hormones.

Here we report that sterility or reduced fertility in rice can be induced through the controlling of endogenous GA biosynthesis by using *OsGA20ox* and *OsGA3ox* RNAi vector. Therefore, these results suggest the useful biotechnological approaches to engineer male sterility for the widespread use of hybrid production in self-pollinated crops.

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REFERENCES

- Chhun T, Aya K, Asano K, Yamamoto E, Morinaka Y, Watanabe M, *et al.* 2007. Gibberellin regulates pollen viability and pollen tube growth in rice. *Plant Cell* 19: 3876–3888.
- Devshwar P, Bovill WD, Sharma R, Able JA, Kapoor S. 2011. Analysis of anther transcriptomes to identify genes contributing to meiosis and male gametophyte development in rice. *BMC Plant Biol.* 11: 78.
- Endo M, Tsufchya T, Saito H, Matsubara H, Hakozaiki H, Masuko H, *et al.* 2004. Identification and molecular characterization of novel anther-specific genes in *Oryza sativa* L. by using cDNA microarray. *Genes Genet. Syst.* 79: 213–226.
- Fujita M, Horiuchi Y, Ueda Y, Mizuta Y, Kuibo T, Yano K, *et al.* 2010. Rice expression atlas in reproductive development. *Plant Cell Physiol.* 51: 2060–2081.
- Gubler F, Kalla R, Roberts JK, Jacobsen JV. 1995. Gibberellin-regulated expression of a *myb* gene in barley aleurone cells: evidence for Myb transactivation of a high-pl α -amylase gene promoter. *Plant Cell* 7: 1879–1891.
- Gupta V, Khurana R, Tyagi AK. 2007. Promoters of two anther-specific genes confer organ-specific gene expression in a stage-specific manner in transgenic systems. *Plant Cell Rep.* 26: 1919–1931.
- Hirano K, Aya K, Hobo T, Sakakibara H, Kojima M, Shim RA, *et al.* 2008. Comprehensive transcriptome analysis of phytohormone biosynthesis and signaling genes in microspore/pollen and tapetum of rice. *Plant Cell Physiol.* 49: 1429–1450.
- Hsing YI, Chern CG, Fan MJ, Lu PC, Chen KT, Lo SF, *et al.* 2007. A rice gene activation/knock mutant resource for high throughput functional genomics. *Plant Mol. Biol.* 63: 351–364.
- Huang S, Cerny RE, Qi Y, Bhat D, Aydt CM, Hason DD, *et al.* 2003. Transgenic studies on the involvement of cytokinin and gibberellin in male development. *Plant Physiol.* 131: 1270–1282.
- Itoh H, Ueguchi-Tanaka M, Sentoku N, Kitano H, Matsuoka M, Kobayashi M. 2001. Cloning and functional analysis of gibberellin 3 β -hydroxylase genes that differently expressed during the growth of rice. *Proc. Natl. Acad. Sci. U.S.A.* 98: 8909–8914.
- Itoh H, Ueguchi-Tanaka M, Sakamoto T, Kayano T, Tanaka

- H, Ashikari M, *et al.* 2002. Modification of rice plant height by suppressing the height-controlling gene, *D18*, in rice. *Breed. Sci.* 52: 215-218.
- Itoh J, Nonomura K, Ikeda K, Yamaki S, Inukai Y, Yamagishi H, *et al.* 2005. Rice plant development: from zygote to spikelet. *Plant Cell Physiol.* 46: 23-47.
- Jacobsen SE, Olszewski NE. 1991. Characterization of the arrest in anther development associated with gibberellins deficiency of the *gib-1* mutant of tomato. *Plant Physiol.* 97: 409-414.
- Kaneko M, Inukai Y, Ieguchi-Tanaka M, Itoh H, Izawa T, Kobayashi Y, *et al.* 2003. Loss-of-function mutations of the rice *GAMYB* gene impair α -amylase expression in aleurone and flower development. *Plant Cell* 16: 33-44.
- Koornneef M, van der Veen JH. 1980. Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana* (L.) heynh. *Theor. Appl. Genet.* 58: 257-263.
- Ma H. 2005. Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. *Annu. Rev. Plant Biol.* 56: 393-434.
- McElroy D, Zhang W, Cao J, Wu R. 1990. Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* 2: 163-171.
- Oikawa T, Koshioka M, Kojima K, Yoshida H, Kawata M. 2004. A role of *OsGA20ox1*, encoding an isoform of gibberellin-20-oxidase, for regulation of plant stature in rice. *Plant Mol. Biol.* 55: 687-700.
- Park JL, Hakozaiki H, Endo M, Takada Y, Ito H, Uchida M, *et al.* 2006. Molecular characterization of mature pollen-specific genes encoding novel small cysteine-rich proteins in rice (*Oryza sativa* L.). *Plant Cell Rep.* 25: 466-474.
- Qiao F, Yang Q, Wang CH, Fan YL, Wu XF, Zhao KJ. 2007. Modification of plant height via RNAi suppression of *OsGA20ox2* gene in rice. *Euphytica* 158: 35-45.
- Ronald PC, Chen DH. 1999. A rapid DNA miniprep method suitable for AFLP and other PCR applications. *Plant Mol. Biol. Rep.* 17: 53-57.
- Sakamoto T, Kobayashi M, Itoh H, Tagiri A, Kayano T, Tanaka H, *et al.* 2001. Expression of a gibberellin 2-oxidase gene around the shoot apex is related to phase transition in rice. *Plant Physiol.* 125: 1508-1516.
- Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M, Ishiyama K, *et al.* 2004. An overview of gibberellin metabolism enzyme genes and their related mutants in rice. *Plant Physiol.* 134: 1642-1653.
- Spielmeyer W, Ellis MH, Chandler PM. 2002. Semidwarf (*sd-1*), “green revolution” rice, contains a defective gibberellin 20-oxidase gene. *Proc. Natl. Acad. Sci. U.S.A.* 99: 9043-9048.
- Suwabe K, Suzuki G, Takahashi H, Shiono K, Endo M, Yano K, *et al.* 2008. Separated transcriptomes of male gametophyte and tapetum in rice: validity of a laser microdissection (LM) microarray. *Plant Cell Physiol.* 49: 1407-1416.
- Tang X, Zhang ZY, Zhang WJ, Zhao XM, Li X, Zhang D, *et al.* 2010. Global gene profiling of laser-captured pollen mother cells indicates molecular pathways and gene subfamilies involved in rice meiosis. *Plant Physiol.* 154: 1855-1870.
- Toki S, Hara N, Ono K. 2006. Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice. *Plant J.* 47: 969-976.
- Yoshida S, Forno DA, Cock JH, Gomez KA. 1976. Laboratory manual for physiological studies of rice. International Rice Research Institute. Manila, Philippines. pp. 61-66.
- Tsuchiya T, Toriyama K, Nasrallah ME, Ejiri S. 1992. Isolation of genes abundantly expressed in rice anthers at the microspore stage. *Plant Mol. Biol.* 20: 1189-1193.
- Tsuchiya T, Toriyama K, Ejiri S, Hinata K. 1994. Molecular characterization of rice genes specifically expressed in the anther tapetum. *Plant Mol. Biol.* 26: 1737-1746.
- Virmani SS. 1994. Heterosis and hybrid rice breeding. Monograph on theoretical and applied genetics. Vol. 22. Springer-Verlag, Berlin Heidelberg.
- Yamaguchi T, Lee DY, Miyao A, Hirochika H, An G, Hirano H. 2006. Functional diversification of the two C-Class MADS Box genes *OSMADS3* and *OSMADS58* in *Oryza sativa*. *Plant Cell* 18: 15-28.
- Xing S, Zachgo S. 2007. Pollen lethality: a phenomenon in *Arabidopsis* RNA interference plants. *Plant Physiol.* 145: 330-333.
- Zhang DB, Wilson ZA. 2009. Stamen specification and anther development in rice. *Chinese Sci. Bull.* 54: 2342-2353.