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Master's Thesis of Science in Agriculture

**Development of transgenic rice plants
producing human basic fibroblast
growth factor, bFGF,
under drought conditions**

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ABF3	ABA Binding Factor 3
ABRE	ABA-responsive elements
Bar	Bialaphos-resistance gene
bFGF	Basic fibroblast growth factor
BLAST	Basic Local Alignment Search Tool
FST	Flanking sequence tag
LB	Left border
LEA	Late embryogenesis abundant
Nos	Nopaline synthase gene
NT	Non-transgenic
PC	Positive control
PinII	Potato proteinase inhibitor II
qRT-PCR	Quantitative real-time PCR
RB	Right border
Rbc	Ribulose-1, 5–bisphosphate carboxylase-oxygenase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGE	Transient gene expression
Tub	Tubulin

WAP	Weeks after pollination
Wsi18	Water-stress inducible protein 18
35S	Cauliflower mosaic virus 35S promoter

ABSTRACT

A member of the *FGF* gene family, the basic fibroblast growth factor (*bFGF*), is commonly used in clinical applications. It modulates wound healing, tissue repair and stimulates differentiation of fibroblasts. *FGFs* have been expressed in *E. coli*, insect cells and mammalian cells. Although expression systems have been reported, limitations such as low productivity and high cost exist. Plant molecular farming provides an alternative expression system for producing such recombinant proteins. Recombinant proteins from plants are safer, cost efficient and easier to scale up for mass production. In this study, the *bFGF* gene with myc-tag at its N- and C- terminal was transformed into rice protoplasts and its expression was detected using an anti-myc antibody. Single copy and intergenic transgenic rice plants were produced to be used for bFGF production. Sixty seven single copy lines were obtained from a total 186 transgenic lines and analyzed for T-DNA integration through flanking PCR. A total of 20 single copy, intergenic transgenic lines were finally obtained. Expression of bFGF proteins were confirmed in seeds of one multi copy T₀ line. In leaves of transgenic rice plants, *Wsi18:bFGF* transcripts as well as proteins were detected following drought-stress treatment. Collectively, these results proved that bFGF protein can be expressed in rice providing a great potential for molecular farming in plants.

Keyword: Basic fibroblast growth factor, bFGF, Transgenic rice plant,
Stress inducible, *Wsi18* promoter, Recombinant protein, Plant
molecular farming

Student Number: 2014-26006

INTRODUCTION

The basic fibroblast growth factor (bFGF) is a member of the heparin binding polypeptide family with a needed for therapeutic utilities. It is a single subunit protein molecular weight of 17 kDa (Wang et al. 2015). bFGF modulates growth and differentiation of epithelial cells and stromal bone marrow cells in mice (Pitaru et al. 1993; Lavandero et al.1998; Coumoul and Deng 2003). bFGF is also known to cure asthma by inducing and proliferating airway smooth muscle cells (Bossé and Rola-Pleszczynski 2008). It is also widely used for treating burn wounds, skin hardness, redness and elevation (Akita et al. 2005).

Because of its medical importance, genetic engineering for recombinant bFGF production is actively developed to obtain sufficient amounts. It has been expressed in *E. coli*, insects as well as in mammalian cells. Though several expression systems have been achieved, limitations such as low productivity and high cost still exist (Fischer. 2004). To address these limitations, plant-based production through plant molecular farming (PMF) offers an expression system capable of producing recombinant proteins in a much larger scale but with minimal cost (Xie et al. 2008). PMF have been used for producing pharmaceutical proteins, recombinant industrial enzymes and other secondary metabolites. PMF also reduces pathogen contamination in mammals with high scalability and protein yields (Stoger et al. 2005; Lau and Sun 2009). PMF is thus an ideal platform for producing pharmaceutical and industrial proteins (Sparrow et al. 2007).

The main commercial crop maize has high protein in seed and well developed for molecular works. However, the main disadvantage is cross-pollinating plant and leads contamination of environment. Wheat has low transformation efficiency and lack of efficient tools for genetic manipulations. Among other plants, rice has high grain yield, well established transformation protocols and molecular tools. It can also be easily scaled up and has an ideal trait for PMF. Other advantage of rice is being a self-pollinating plant which lowers the rate of unintended gene flow (Lau and Sun 2009). Also the protein content in rice bran can reached up to 17.2 % of bran composition (Saunders 1985).

In this study, water-stress inducible promoter *Wsi18* was used to drive the expression of *bFGF* transcripts. *Wsi18* promoter was selected to limit the expression of bFGF during the maturation stage of plants, where water availability in soil is purposely devoid. The activity of *Wsi18* promoter in transgenic rice plants have been identified to be active in drought-stress treated calli, leaves, root, flowers and whole grain (Yi et al. 2011). Here, It was observed that *Wsi18* promoter up-regulates mRNA and protein expression of *bFGF* in transgenic rice plants upon drought treatments. In addition, expression of the bFGF protein was induced in protoplasts by adding the *35S:ABF3*, which mimicked a water-stressed condition. In conclusion, the results indicate that ectopic expression of bFGF protein in rice is feasible and has a great potential for molecular farming in plants.

MATERIALS AND METHODS

1. Construction of myc-tagged *bFGF* vector

The Dongjin (*Oryza Sativa* L.) plants were prepared and the genomic DNA was extracted from the leaves with the DNAzol (Molecular Research Center, USA). The *Wsi18* promoter region (1.8 kb) (GQ903792) was amplified by forward primer (5'-AAGCTTATCGATCATATTTGACTCTT-3') and reverse primer (5'-GTTTCAGTTCGTGTGTTGGTAAGCTT-3'). PCR amplified fragments were ligated into pGEM-T Easy vector (Promega, USA) using T4 DNA ligase (New England Biolabs, USA). Cloned DNA fragments were sequenced and the cloning vector pSB11 was excised using *HindIII*. PCR products were linked to corresponding site of expression cassette. The expression vector contains specific *attR1* and *attR2* recombination site and 3' region of potato proteinase inhibitor II (*Pin II*) terminator. DNA sequences coding for human *bFGF* gene (Genbank accession No. NM 002006) was optimized with a rice codon bias using rice-preferred genetic codons. The PCR product was inserted between *BamHI* and *NotI* sites of myc-tagged entry vectors. N-terminal myc-tagged entry vector pE3n and C-terminal myc-tagged pE3c (Dubin et al. 2008) contains *attL1* and *attL2*. N- and C-terminal myc-tagged *bFGF* genes were replaced by LR reaction (Invitrogen, Canada).

2. Plant materials for protoplast isolation

Dongjin (*Oryza sativa* L.) seeds were sterilized with 70% EtOH for 5 min followed by 2% sodium hypochloride (NaOCl) for 30 min and washed with sterilized distilled water at least five times. Twenty five seeds were placed on MS medium with 1.5% sucrose, and grown in an incubator maintained at 28°C under dark for 9 days and light for 1 day.

3. Protoplast isolation

Protoplast isolation from rice seedlings was followed according to the method of Bergmann technique (Bergmann 1960) with some modifications of Deka and Sen (1976). Bundles of the plants (about 30 seedlings) from sheath of 200-250 rice seedlings were cut together into 0.5 mm with razor blade. The strips were transferred to 0.6 M mannitol for plasmolysis treatment and incubated for 10 min in dark. The mannitol solution was discarded and incubated in digestion solution (1.5% Cellulase R-10 (Yakult, Japan), 0.75% Macerozyme R-10 (Yakult, Japan), 0.5 M Mannitol, 10 mM MES at pH 5.7, 10 mM CaCl₂, 5 mM β-mercaptoethanol and 0.1% BSA) at 25°C with gentle shaking. The cell wall from protoplasts were treated with digestion solution containing cellulase and macerozyme for cell wall degradation. The effect of the combinations of these enzyme mixtures enhances protoplast isolation (Hashiba and Yamada 1982). Vacuum infiltration for 5 min and incubated at 25°C with gentle shaking at 40 rpm for 4 h. The incubated solution was collected and filtered first through a 70-μm then through 40-μm nylon mesh (Falcon, USA). Protoplasts were collected

by centrifugation at 300 g for 8 min. The protoplasts were rinsed using W5 solution (125 mM CaCl₂, 2 mM MES, 5 mM KCl and 154 mM NaCl). Further centrifugation for 2 min at 300 g and diluted with MMG solution (4 mM MES, 0.5 M Mannitol and 15 mM MgCl). Protoplasts were counted in a hemocytometer (Marienfeld, Germany) and adjusted to a 2.5×10^6 cells/100 μ l.

4. Protoplast transfection

Each 10 μ g of plasmid DNA and ABF3, provided by Dr. Se-Jun Oh (Oh et al. 2005) was introduced to the protoplasts. It mixed with PEG solution (0.3 M Mannitol, 100 mM CaCl₂ and 40% PEG 4000) and incubated at room temperature for 15 min in dark. After incubation, W5 solution were added and centrifuged at 300 g for 5 min. The pelleted protoplasts were treated with WI solution (4 mM MES, 20 mM KCl, 0.5 M Mannitol). It were incubated in 6 well plate at room temperature overnight in the dark. Incubated solutions were collected in new tubes and centrifuged at 300 g for 3 min.

5. Protein extraction and immunoblot analysis

For protein extraction, tissues were homogenized with 60 μ l of 1x passive lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, 1% Triton X-100 and Protease inhibitor cocktail (Roche, USA)). The lysis were clarified by centrifugation and quantified by the Bradford solution (Bio-Rad, USA). The protein extracts were analyzed by SDS-PAGE following the

methods of Hoffmann and Hampp (1994). The protein samples, molecular weight marker (Bio-Rad, USA) and positive controls were separated by 12% acrylamide gel with 4% stacking gel using Tris-glycine buffer (192 mM Glycine, 10% Methanol, 25 mM Tris, 0.037% SDS). Then recombinant human FGF basic 146 aa (R&D systems, USA) was loaded as positive control for quantitation of bFGF levels. The protein was transferred to nitrocellulose membrane (Millipore, USA) by a semi dry blot. The blots were blocked overnight with blocking buffer (5 M NaCl, 1 M Tris-HCl (pH 7.5), 0.1% Tween 20, 10% BSA). The blots were then immuno-stained using diluted 1:5000 human FGF basic antibody (R&D systems, USA) for 1 h. Donkey anti-goat IgG-HRP secondary antibody 1:5000 dilution with blocking buffer was probed (Santa Cruz, Canada) as second antibody for 1 h. The membranes were captured using Fusion SL imaging system (Vilber Lourmat, France) and quantified using Multi gauge software version 2.1 (Fuji, Japan). Separated total proteins were stained by Coomassie blue (Sigma, USA) diluted in 45% methanol and 10% acetic acid for 45 min. The blot was destained in 5% methanol and 7% acetic acid.

6. Vector construction and transformation of rice

The *Wsi18* promoter was inserted into pGEM-T Easy vector (Promega, USA) and digested with *HindIII*. The enzyme digested *Wsi18* promoter was ligated into pPZP200 vector containing *PinII* terminator, *bar* gene as a selectable marker, fused with 35S promoter and *nos* terminator. Rice-preferred codon optimized human *bFGF* gene (Genbank accession No. NM

002006) was inserted between *XhoI* and *BamHI* sites. To generate transgenic rice plants, *Agrobacterium* LBA4404 was transformed with *Wsi18:bFGF* vector using freeze-thaw method. Rice transformations were followed through Hiei et al. (1994). Co-cultivated transformed calli were selected with phosphinothricin (4 mg/l) and cefotaxime (250 mg/l). The regenerated transgenic rice plants were transferred and grown in a greenhouse (28 °C, 12 h light/dark).

7. TaqMan PCR

Genomic DNAs of transgenic rice plants were extracted using DNeasy (Qiagen, Germany). The *bar* and *nos* gene selection of transgenic rice plants were conducted with primer and probe in Table 1. For endogenous control, rice *tubulin alpha-1 chain* (AK102560) were used and Os03643486 (Applied Biosystems, USA) was used for probe. Real-time PCR reactions were conducted using Stratagene Mx3000p real-time PCR machine (Stratagene, USA) with 95 °C for 10 min and 40 cycles for denaturation 95 °C for 30 sec, annealing and extension for 1 min at 60 °C. The copy numbers were analyzed by Mx3000P software version 2.02 (Stratagene, USA).

8. Flanking PCR

Around 20-60ng/μl of genomic DNA was digested with *Bfal* (New England Biolabs, USA) for 4 h. Flanking sequence tag was performed as described by Thole et al. (2009). Adapters (5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGGGCAGGT-3' and 5'-

TAACCTGCCCAA-3') were ligated to DNA fragments at 23°C for 3 d using T4 DNA ligase (New England Biolabs, USA). Nested PCR was conducted to amplify the flanking sequence. Adapter-ligated DNAs were incubated 2X EF-Taq DNA Premix (SolGent, Korea) for PCR. Specific primers were designed for amplifying flanking region (Table 3). 1st PCR was conducted with RB-R1 and Nos-Fa1 primers with an initial 5 min denaturation at 98°C, followed by 40 cycles for denaturation 98°C for 20 sec, annealing at 60°C for 20 sec and extension for 2 min at 72°C then a final 5 min at 72°C. A 7 µl of the first PCR product was used for the 2nd PCR template. The 2nd PCR was performed under the same conditions. The 2nd PCR product was loaded and separated in 1% agarose gel. The amplified fragments were extracted using the ExpinTM Gel SV (Geneall, Korea) following the manufacturer's instructions. Extracted DNA were sent for sequencing by Cosmo Genetech (<http://www.cosmogenetech.com>). Data acquired were then used to determine positions of the T-DNA insertions using NCBI BLAST (<http://www.ncbi.nlm.nih.gov/blast>).

9. Drought-stress treatment and qRT-PCR

To confirm up-regulation of *bFGF* under drought conditions, leaves of transgenic rice plants were air dried at 28-30°C for 0, 2, 6 and 12 h. The drought-stress treated leaves were ground in liquid nitrogen with a mortar and pestle. Total RNA isolation and cDNA synthesis was performed using TRIzol (Invitrogen, Canada) and cDNA synthesis kit (Fermentas, Canada) according to the instructions. The PCR primers in Table 1 were used to

amplify *bar* and *nos*. PCR reactions were conducted 95°C for 10 min and 40 cycles for denature for 30 sec at 95°C, annealing and extension for 1 min at 60°C by Stratagene Mx3000p real-time PCR machine (Stratagene, USA). It was measured three times with independently prepared total RNA. The results were analyzed by Mx3000P software version 2.02 (Stratagene, USA). Two independent multi copy T₀ transgenic lines were used for *bFGF* expression analysis.

RESULTS

1. Vector construction for protoplast assay and rice transformation

Transient gene expression (TGE) assay is an alternative system for studying gene functions (Dekeyser et al. 1990). It is simple, cost effective and time-saving (Šimková et al. 2003). Codon optimized *bFGF* gene were successfully cloned into N- and C-terminal myc-tagging vectors in pPZP200 (Hajdukiewicz et al. 1994) (Fig. 1a). BLAST results showed correct DNA sequence as well as in-frame myc-tag sequence (Fig. 1b, c). Amino acid sequences were underlined in Fig. 1d. The *myc* and *bFGF* sequences are underlined. To produce bFGF protein in transgenic rice plants, *Wsi18:bFGF* was constructed (Fig. 1e). It was introduced in plants through *Agrobacterium*-mediated transformation. Transgenic rice plants were selected by growing in MS medium containing 7 mg/l of phosphinothricin.

2. Transient expression analysis in rice protoplasts

For TGE assay, *Wsi18:myc:bFGF* and *Wsi18:bFGF:myc* constructs were transfected into rice protoplasts. Myc-tagged bFGF proteins were detected using an anti-myc antibody (Fig. 2). The N- and C-terminal myc-tagged gene encode 249 (28.02 kDa) and 246 (27.83 kDa) amino acid, respectively. Thus showing slightly different sizes as seen in blot. The ABF3

treatment resulted in relatively higher expression levels of bFGF in myc-tagged protoplasts than those of non-treated ones. The bFGF proteins detected were 0.13% and 0.29% of the total soluble proteins in *Wsi18:myc:bFGF* and *Wsi18:bFGF:myc*, respectively. Based on these results, induction of bFGF expression was observed in protoplasts by mimicking drought-stress through ABF3 treatment.

3. Selection of single copy and intergenic T-DNA insertion lines

To prevent any unwanted effects on human or environment, single copy and intact T-DNA lines must be selected. Copy numbers of transgenic rice plants were determined through TaqMan PCR assay (Fig. 3a, b). The *bar* and *nos* gene specific probes were used to detect amplification products. These probes are labeled with fluorescent dye FAM. Probes for *bar* and *nos* were detected by FAM dye. The values were normalized by endogenous reference gene *OsTub1* and measured by VIC dye. Copy numbers were determined by comparing the fluorescence with known single copy homozygote controls (Bang et al. 2015) (Table 2). A total of 186 independent transgenic lines were generated using *Agrobacterium* transformation. Among these, 36% (67 lines) were found to be single copy lines. Two, three and multi copy lines were 16.7% (31 lines), 10.2% (19 lines) and 18.8% (35 lines), respectively. Around 18.3% (34 lines) were found to be null or dead. Single copy lines were further analyzed for insertion site location through flanking sequence tag (Fig. 4). The flanking

regions were digested with *Bfal* and ligated with adapters. 1st and 2nd PCR steps were conducted and separated in agarose gel. PCR products were sequenced and determined the insertion sites of T-DNA using NCBI BLAST. Through this analysis, 20 single copy lines among the 67 single copy lines were intergenic intact T-DNA (Table 2). Collectively, these results showed that single copy plants can be obtained with a frequency of 36% (Table 4). However, selecting intergenic single copy lines reduces the frequency to 11%.

4. bFGF protein expression in rice seed

Since single copy T₀ lines were allotted for propagation, bFGF protein accumulation was analyzed in T₀ multi copy lines. To investigate induction of bFGF protein under dried conditions, immunoblot analysis in seeds from mature plants was carried out. Transgenic rice seeds aged 2 and 4 weeks after pollination (WAP) were collected. Five µg of total proteins were separated by SDS-PAGE and bFGF protein was detected by anti-bFGF antibody (Fig. 5). These observations indicate that using *Wsi18* promoter to drive *bFGF* expression resulted in bFGF protein accumulation during seed development stage.

5. Detection of *bFGF* transcripts in transgenic rice leaves

To examine *bFGF* transcripts in leaves of transgenic rice plants, qRT-PCR analysis was performed. Two single copy (lines 166 and 179) and two

multi copy lines (lines 172 and 180) were selected and exposed to drought-stress treated conditions. The transcript levels of *bFGF* were measured in time course manner (Fig. 6). A 5.5-fold increase in line 172 was detected in 6 h after treatment while line 180 showed 7.6-fold increase. Compared to multi copy lines, single copy lines showed lower transcript ranging from 0.8 to 3.5 for lines 166 and 179, respectively following 6 h of drought-stress treatment. These results demonstrate that *Wsi18* promoter is efficient in driving *bFGF* expression under water-limited condition.

6. bFGF protein expression in leaves of transgenic rice plants under drought conditions

To assess the accumulation of bFGF protein under drought conditions, western blot analysis were performed. Lines 172 and 180 which showed significantly high *bFGF* transcripts were selected. The results showed significant bFGF proteins accumulation which started after 6 h following drought-stress treatment (Fig. 7). Overall, bFGF protein accumulation in drying leaves was successfully achieved through the use of *Wsi18* promoter.

Figure 1. Constructs for protoplast assay and rice transformation

Schematic representation of TGE assay and transformation vectors. The N- and C- terminal myc-tagged constructs for TGE assay (a). The constructs contain *Wsi18* promoter linked to *bFGF* and 3' *pinII*. 35S promoter and 3' *nos* flanked the *bar* coding region. BLAST alignments present corresponding the nucleotide sequences of (b, c) and amino acid sequence are underlined (e). Structure of rice transformation vector (e). *Wsi18*, water-stress inducible promoter; *bFGF*, basic fibroblast growth factor; *myc*, 6x myc epitope; 3' *PinII*, 3' region of the potato proteinase inhibitor II gene; 35S, cauliflower mosaic virus 35S promoter; *bar*, bialaphos-resistance gene; 3' *nos*, 3' region of the nopaline synthase gene; RB, right border; LB, left border.



(b) *Wsi18:myc:bFGF*

myc

Score	Expect	Identities	Gaps	Strand
859 bits(465)	0.0	465/465(100%)	0/465(0%)	Plus/Plus
Query 274	ATGGCTGCTGGATCTATTACTACCCTTCCAGCTTTGCCAGAGGATGGTGGATCTGGTGCT	333		
Sbjct 1	ATGGCTGCTGGATCTATTACTACCCTTCCAGCTTTGCCAGAGGATGGTGGATCTGGTGCT	60		
Query 334	TCCCCACCAGGACATTTCAAGGACCCAAAGAGGCTCTACTGCAAGAACGGTGGATTCTTC	393		
Sbjct 61	TCCCCACCAGGACATTTCAAGGACCCAAAGAGGCTCTACTGCAAGAACGGTGGATTCTTC	120		
Query 394	CTCAGGATTCACCCAGATGGAAGAGTGGATGGTGTGCGTGAAAAGTCCGATCCACACATT	453		
Sbjct 121	CTCAGGATTCACCCAGATGGAAGAGTGGATGGTGTGCGTGAAAAGTCCGATCCACACATT	180		
Query 454	AAGCTTCAGCTCCAGGCTGAAGAGAGGGGAGTTGTTTCTATTAAGGGCGTGTGCCCAAC	513		
Sbjct 181	AAGCTTCAGCTCCAGGCTGAAGAGAGGGGAGTTGTTTCTATTAAGGGCGTGTGCCCAAC	240		
Query 514	AGATACCTCGCCATGAAGGAAGATGGAAGGCTCCTCGCTTCTAAGTGCCTTACAGATGAG	573		
Sbjct 241	AGATACCTCGCCATGAAGGAAGATGGAAGGCTCCTCGCTTCTAAGTGCCTTACAGATGAG	300		
Query 574	TGCTTCTTCTTCGAACGTCTGGAATCCAACAACCTACAACACCTACAGTCCCGTAAGTAC	633		
Sbjct 301	TGCTTCTTCTTCGAACGTCTGGAATCCAACAACCTACAACACCTACAGTCCCGTAAGTAC	360		
Query 634	ACCTCTTGGTACGTGGCACTTAAGAGGACCGGACAGTACAAGCTTGGATCTAAGACTGGA	693		
Sbjct 361	ACCTCTTGGTACGTGGCACTTAAGAGGACCGGACAGTACAAGCTTGGATCTAAGACTGGA	420		
Query 694	CCAGGCCAGAAGGCTATTCTCTTCCTTCCAATGTCCGCCAAGTCC	738		
Sbjct 421	CCAGGCCAGAAGGCTATTCTCTTCCTTCCAATGTCCGCCAAGTCC	465		

bFGF

Score	Expect	Identities	Gaps	Strand
477 bits(258)	1e-139	258/258(100%)	0/258(0%)	Plus/Plus
Query 1	ATGGAGCAAAAGCTCATTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTCT	60		
Sbjct 1	ATGGAGCAAAAGCTCATTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTCT	60		
Query 61	GAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTCTGAAGAGGACTTGAATGAAATG	120		
Sbjct 61	GAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTCTGAAGAGGACTTGAATGAAATG	120		
Query 121	GAGCAAAAGCTCATTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTCTGAA	180		
Sbjct 121	GAGCAAAAGCTCATTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTCTGAA	180		
Query 181	GAGGACTTGAATGAAATGGAGAGCTTGGGCGACCTCACCATGGAGCAAAAGCTCATTCT	240		
Sbjct 181	GAGGACTTGAATGAAATGGAGAGCTTGGGCGACCTCACCATGGAGCAAAAGCTCATTCT	240		
Query 241	GAAGAGGACTTGAATTCC	258		
Sbjct 241	GAAGAGGACTTGAATTCC	258		

(c) *Wsi18:bFGF:myc*

bFGF

Score	Expect	Identities	Gaps	Strand
859 bits(465)	0.0	465/465(100%)	0/465(0%)	Plus/Plus
Query 1	ATGGCTGCTGGATCTATTACTACCCCTCCAGCTTTGCCAGAGGATGGTGGATCTGGTGCT	60		
Sbjct 1	ATGGCTGCTGGATCTATTACTACCCCTCCAGCTTTGCCAGAGGATGGTGGATCTGGTGCT	60		
Query 61	TTCCCACCAGGACATTTCAAGGACCCAAAGAGGCTCTACTGCAAGAACGGTGGATTCTTC	120		
Sbjct 61	TTCCCACCAGGACATTTCAAGGACCCAAAGAGGCTCTACTGCAAGAACGGTGGATTCTTC	120		
Query 121	CTCAGGATTCACCCAGATGGAAGAGTGGATGGTGTGCGTGAAAAGTCCGATCCACACATT	180		
Sbjct 121	CTCAGGATTCACCCAGATGGAAGAGTGGATGGTGTGCGTGAAAAGTCCGATCCACACATT	180		
Query 181	AAGCTTCAGCTCCAGGCTGAAGAGAGGGGAGTTGTTTCTATTAAGGGCGTGTGCGCCAAC	240		
Sbjct 181	AAGCTTCAGCTCCAGGCTGAAGAGAGGGGAGTTGTTTCTATTAAGGGCGTGTGCGCCAAC	240		
Query 241	AGATACCTCGCCATGAAGGAAGATGGAAGGCTCCTCGCTTCTAAGTGCCTTACAGATGAG	300		
Sbjct 241	AGATACCTCGCCATGAAGGAAGATGGAAGGCTCCTCGCTTCTAAGTGCCTTACAGATGAG	300		
Query 301	TGCTTCTTCTCGAACGTCTGGAATCCAACAACACTACAACACCTACAGGTCCCCTAAGTAC	360		
Sbjct 301	TGCTTCTTCTCGAACGTCTGGAATCCAACAACACTACAACACCTACAGGTCCCCTAAGTAC	360		
Query 361	ACCTCTTGGTACGTGGCACTTAAGAGGACCGGACAGTACAAGCTTGGATCTAAGACTGGA	420		
Sbjct 361	ACCTCTTGGTACGTGGCACTTAAGAGGACCGGACAGTACAAGCTTGGATCTAAGACTGGA	420		
Query 421	CCAGGCCAGAAGGCTATTCTTCTTCCCTCCAATGTCCGCCAAGTCC	465		
Sbjct 421	CCAGGCCAGAAGGCTATTCTTCTTCCCTCCAATGTCCGCCAAGTCC	465		

myc

Score	Expect	Identities	Gaps	Strand
477 bits(258)	1e-139	258/258(100%)	0/258(0%)	Plus/Plus
Query 1	ATGGAGCAAAGCTCATTCTGAAGAGGACTTGAATGAAATGGAGCAAAGCTCATTCT	60		
Sbjct 1	ATGGAGCAAAGCTCATTCTGAAGAGGACTTGAATGAAATGGAGCAAAGCTCATTCT	60		
Query 61	GAAGAGGACTTGAATGAAATGGAGCAAAGCTCATTCTGAAGAGGACTTGAATGAAATG	120		
Sbjct 61	GAAGAGGACTTGAATGAAATGGAGCAAAGCTCATTCTGAAGAGGACTTGAATGAAATG	120		
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Sbjct 121	GAGCAAAGCTCATTCTGAAGAGGACTTGAATGAAATGGAGCAAAGCTCATTCTGAA	180		
Query 181	GAGGACTTGAATGAAATGGAGAGCTTGGGCGACCTCACCATGGAGCAAAGCTCATTCT	240		
Sbjct 181	GAGGACTTGAATGAAATGGAGAGCTTGGGCGACCTCACCATGGAGCAAAGCTCATTCT	240		
Query 241	GAAGAGGACTTGAATTCC	258		
Sbjct 241	GAAGAGGACTTGAATTCC	258		

(d)

Wsi18:myc:bFGF

MEQKLISEEDLNEMEQKLISEEDLNEMEQKLISEEDLNEMEQKLISEEDLNEMEQKLISEEDLNEMESLGLDT

myc

MEQKLISEEDLNSGSPGSMAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVR

bFGF

EKSDPHIKLQLQAEERGVVSIKVCANRYLAMKEDGRLLASKCVTDECFERLESNNYNTYRSRKYTSWY

VALKRTGQYKLGSKTGPGQKAILFLPMSAKSAAA

Wsi18:bFGF:myc

MAAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVRKSDPHIKLQLQAEERGVV

bFGF

SIKVCANRYLAMKEDGRLLASKCVTDECFERLESNNYNTYRSRKYTSWYVALKRTGQYKLGSKTGPGQ

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myc

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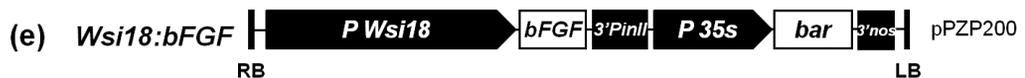


Figure 2. Analysis of bFGF protein expression levels in rice protoplasts

Induction of bFGF protein levels with ABF3 transcription factor in rice protoplasts. Myc-tagged *bFGF* under control of *Wsi18* promoter was introduced into protoplasts. Total proteins were extracted from the protoplasts were separated via SDS-PAGE. The signals were detected by anti-myc antibody. Accumulation of bFGF protein in rice protoplasts treated with ABF3. Negative control is non-transfected protoplasts, while 10 ng of positive control indicates recombinant human FGF. *Wsi18*, water-stress inducible promoter; *bFGF*, basic fibroblast growth factor; *myc*, 6x myc epitope; *3' PinII*, 3' region of the potato proteinase inhibitor II gene; M, protein marker; PC, positive control used by poly tag myc-GFP; NT, non-transfected protoplasts.

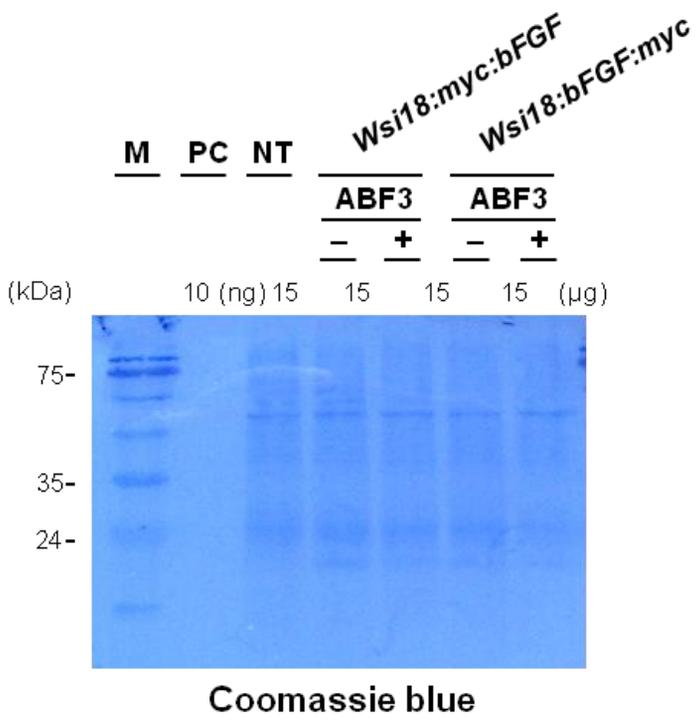
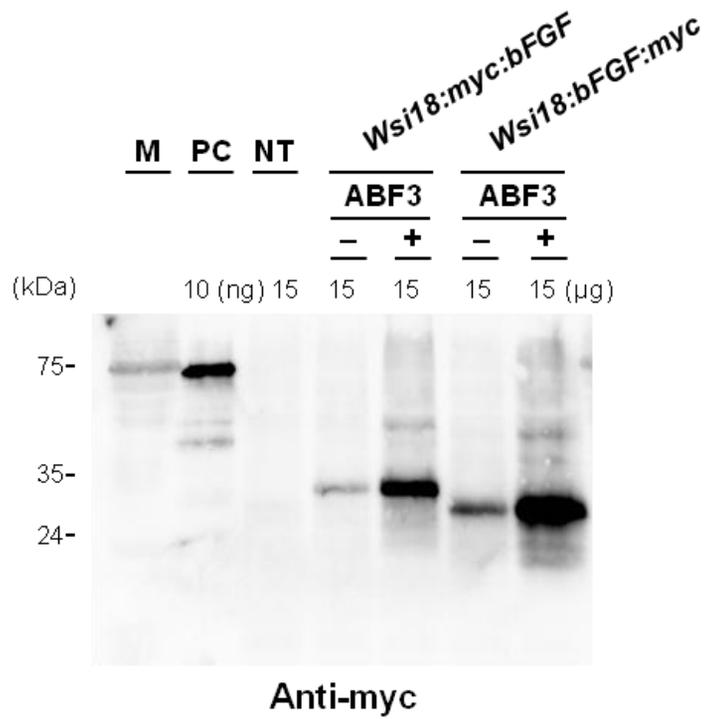
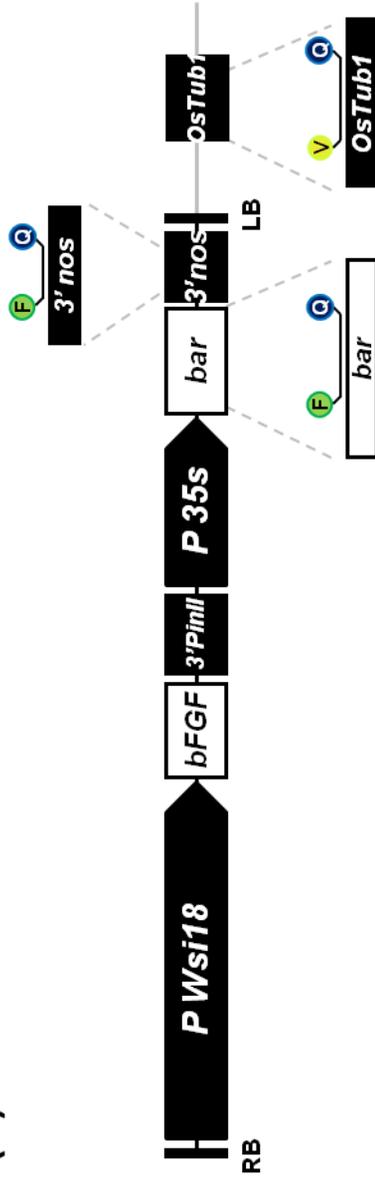


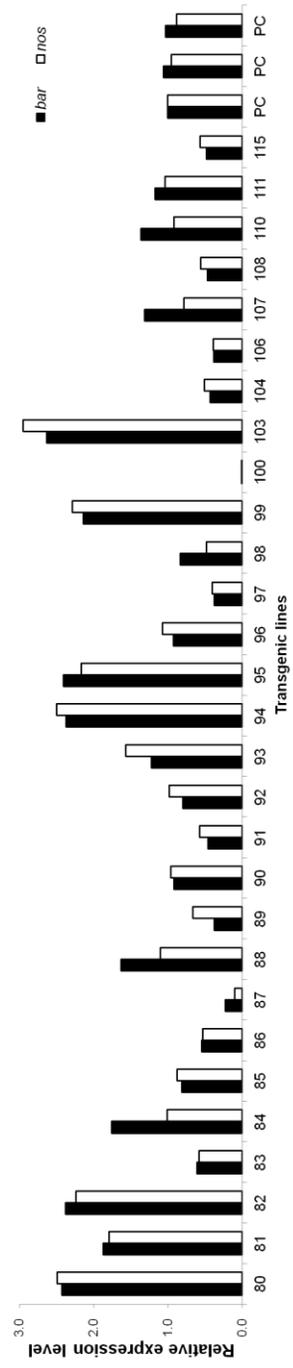
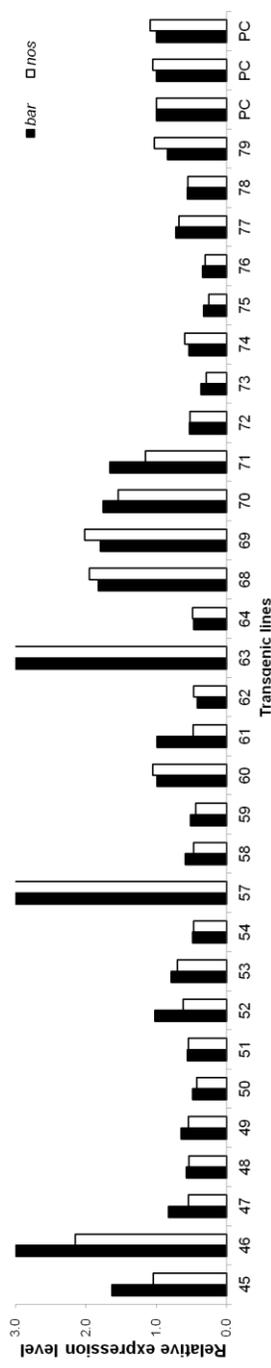
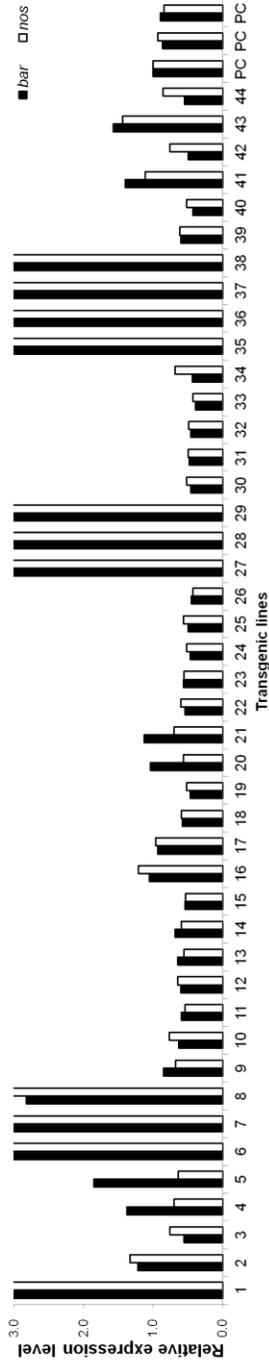
Figure 3. Transgene copy numbers of *Wsi18:bFGF*

Expression levels of *bar* and *nos* genes in *Wsi18:bFGF* transgenic rice plants. The *bar* and *nos* genes were inserted in transgenic clone vectors (a). Copy numbers were determined using TaqMan PCR by *bar* and *nos* probes. *OsTub1* was used as an endogenous reference gene. Copy numbers were calculated by comparing to known single copy control lines (b). Single copy homozygote rice plant was used as PC. F, FAM dye; V, VIC dye; PC, positive control.

(a)



(b)



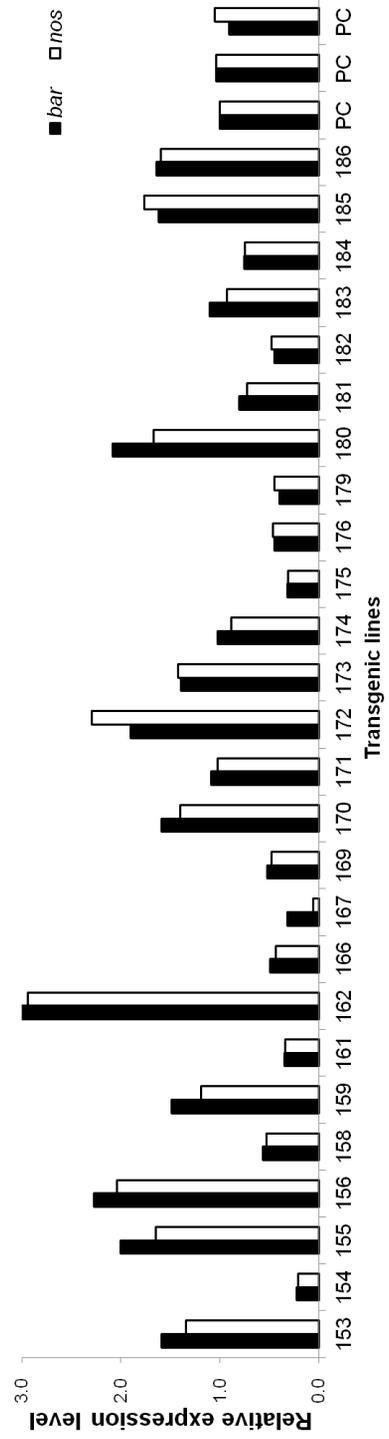
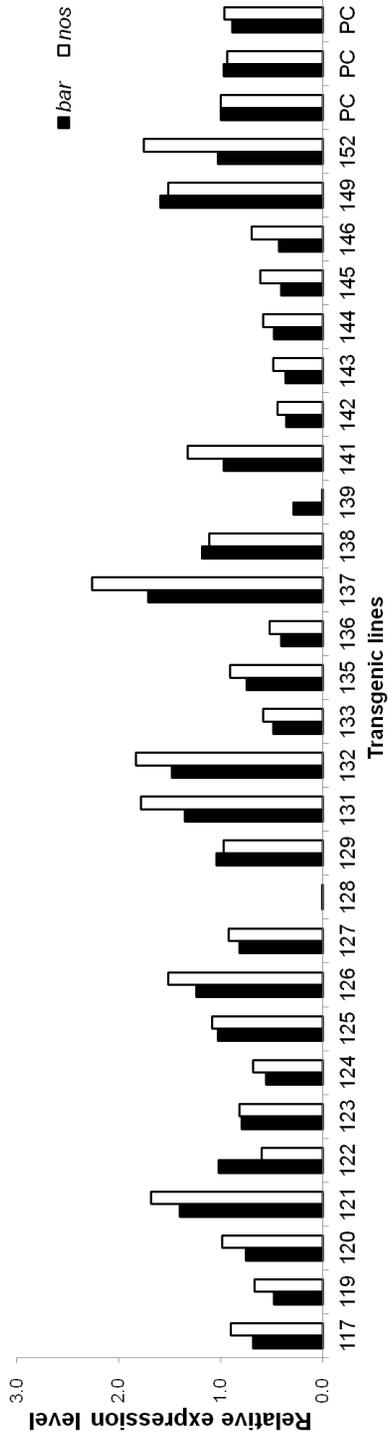


Figure 4. T-DNA insertion site confirmation through flanking sequence tag

Flanking sequence tag analysis of *Wsi18:bFGF* transgenic rice plants. The flanking regions were digested with *Bfal* (a). The digested fragments were ligated with *Bfal* adapters (b). 1st and 2nd PCR were conducted with specific primers (b). 2nd PCR products separated by gel electrophoresis (c). Schematic of T-DNA insertion site in rice genome (d). M, DNA molecular marker.

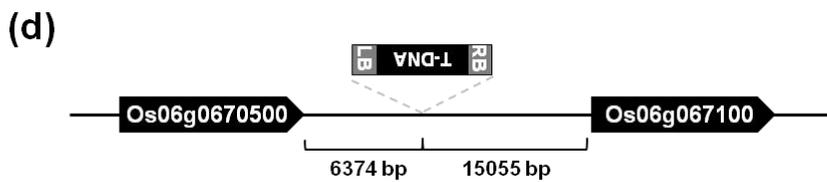
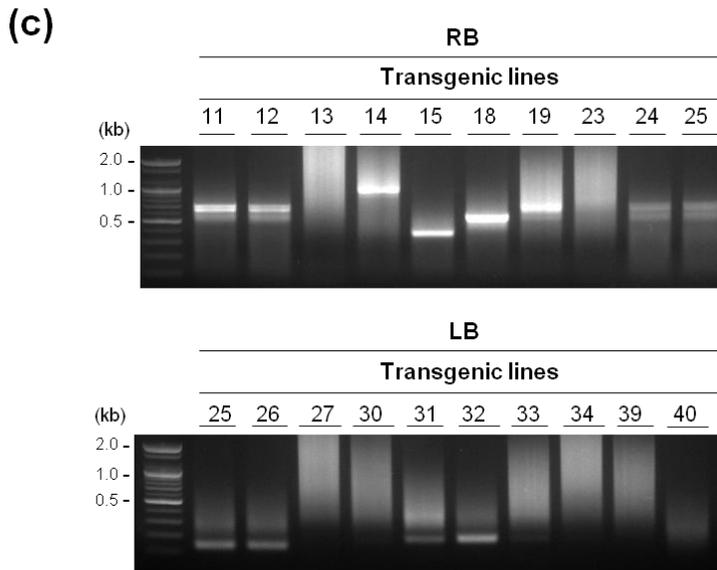
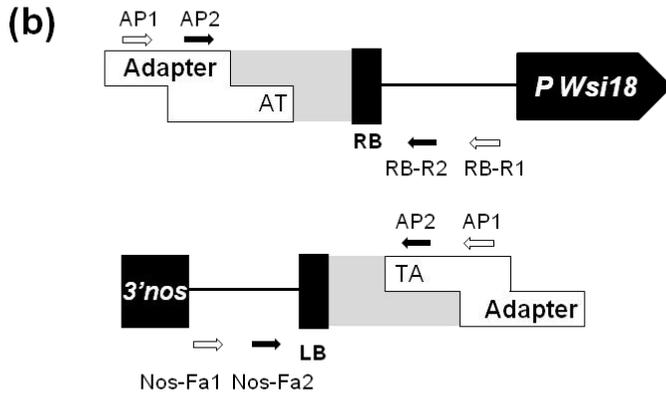
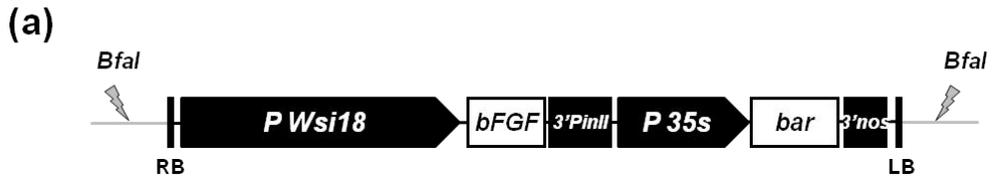
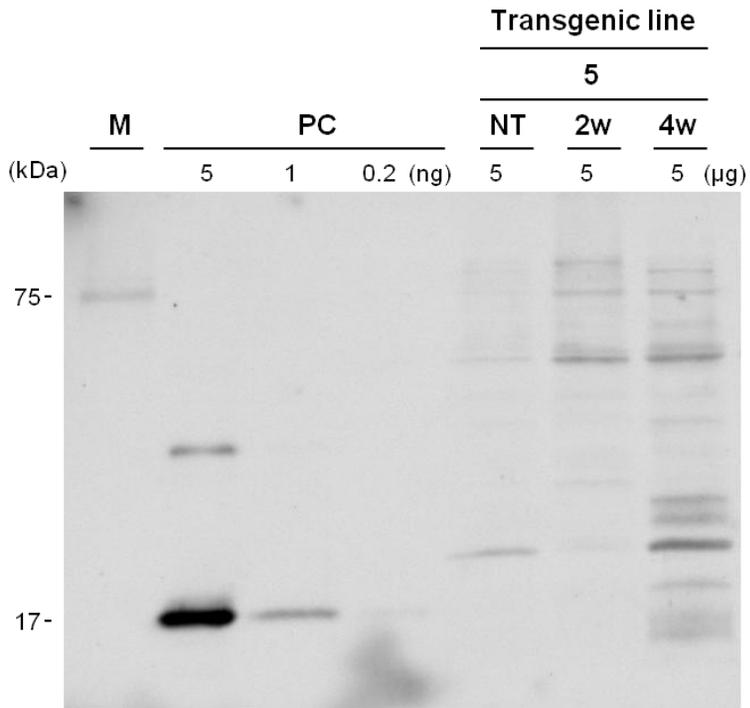
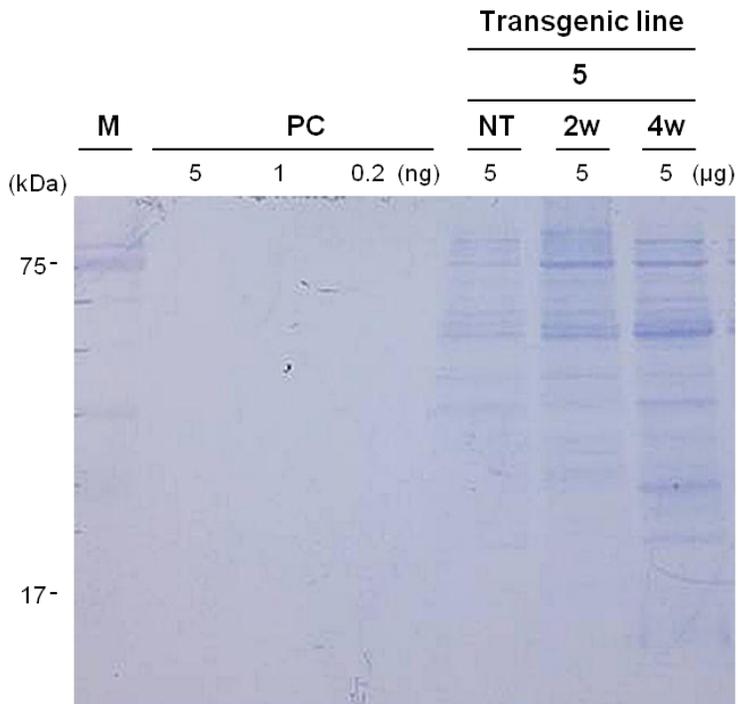


Figure 5. Immunoblot analysis of drought-stress treated transgenic rice seeds

Seeds from multi copy lines were matured for 2 and 4 WAP. 5 µg of total proteins from seeds were separated by SDS-PAGE. The protein bFGFs were detected at 17 kDa using anti-bFGF antibody. bFGF protein in 4 week was induced by drought conditions. Total proteins were stained with Coomassie blue. M, protein marker; PC, recombinant human FGF basic 146 aa; NT, non-transgenic rice plant.



Anti-FGF



Coomassie blue

Figure 6. Relative quantification of *bFGF* mRNA under water-stressed conditions

Total leaf RNA from two transgenic rice plants exposed to drought-stress for 0, 2, 6 and 12 h were extracted. The relative expression levels of mRNA were analyzed by qRT-PCR. Relative expression levels were calculated as the mean of the expression levels relative to 0 h (marked with asterisk). The error bars indicate standard deviations (n =3).

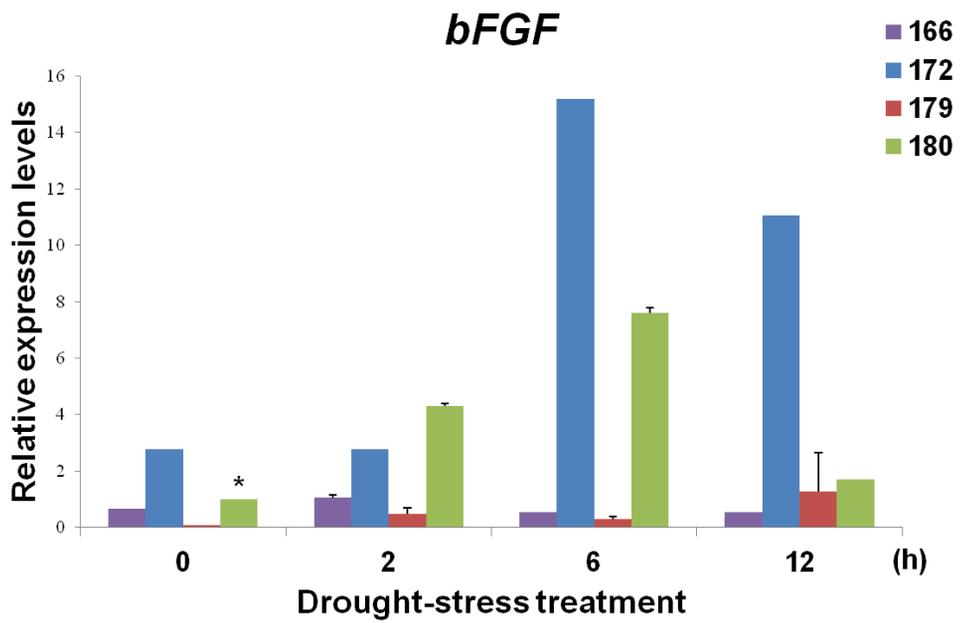


Figure 7. Immunoblot analysis of drought-stress treated transgenic rice leaves

Accumulation of bFGF proteins in response to drought. Two transgenic lines expressing bFGF were exposed to drought for 0, 2, 6 and 12 h. Total proteins were extracted and used 10 µg of total proteins. The results were separated using by SDS-PAGE and immunoblotted using anti-bFGF antibody. The separated proteins were visualized by Coomassie blue staining. PC, recombinant human FGF basic 146 aa; NT, non-transgenic rice plant.

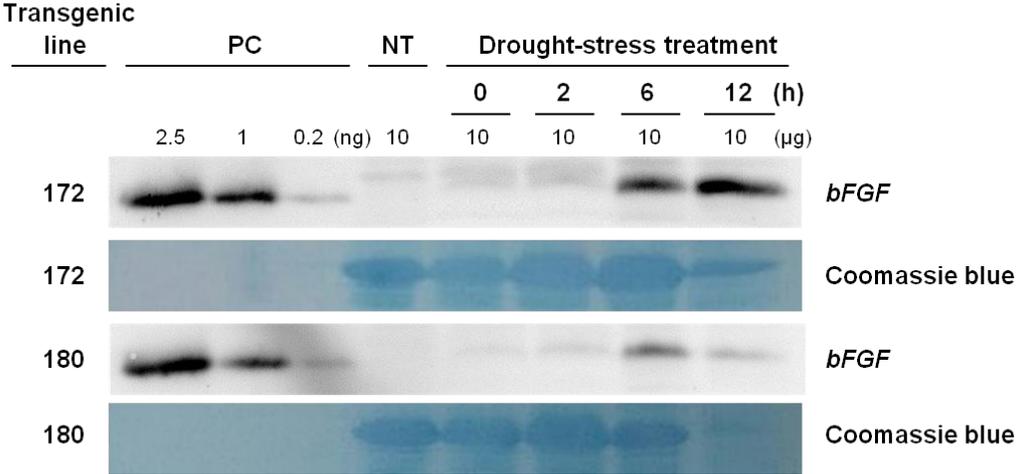


Table 1. Primers and probes used for TaqMan PCR and qRT-PCR

Primer sequence

	Forward	Reverse
Bar	5'-GAGACAAGCACGGTCAACTTC-3'	5'-CGAGGTCGTCCTCCGAC-3'
Nos	5'-GCATGACGTTATTTATGAGATGGGTTTT-3'	5'-TGGCGCTATATTTTGTCTATCG-3'
TaqMan PCR		
Bar-probe	FAM-TCCCTGCGGTTCCCTGC-NFQ	
Nos-probe	FAM-TAGAGTCCCGCAATTAT-NFQ	
bFGF	5'-TCCAGCTTTGCCAGAGGATG-3'	5'-TGGATCGGACTTTTCACGCA-3'
qRT-PCR		
Dip1	5'-GAGCTTGTCACCGGCATGGA-3'	5'-AGCTGGAGCTGGAGCTGGAT-3'
RbcS	5'-GGCAGGTACTGGACCATGTG-3'	5'-TTGTCGAAGCCCGATGATACG-3'

Table 2. Frequency of transgene copy numbers

Copy number	No. of lines	Percentage (%)
Single copy	67	36.0
2 copies	31	16.7
3 copies	19	10.2
More than 3 copies	35	18.8
Null	5	2.7
Death	29	15.6
Total	186	100.0

Table 3. Primers for flanking sequence tag

Primer sequence		
	AP1	5'-GGATCCTAATACGACTCACTATAGGGC-3'
1st PCR	RB-R1	5'-TTAGCTTGAGCTTGGATCAGATTGTCG-3'
	Nos-Fa1	5'-CGGGAATTCAATTCGGCGTTAATTCAG-3'
	AP2	5'-TATAGGGCTCGAGCGGC-3'
2nd PCR	RB-R2	5'-TCAGATTGTCGTTTCCCGCC -3'
	Nos-Fa2	5'-TTCAGTACATTA AAAACGTCCGC-3'

Table 4. Integrated T-DNA of transgenic rice plants

Integrated T-DNA	No. of lines	Percentage (%)
Intergenic	20	29.85
Intragenic	20	29.85
Not determined	27	40.30
Total	67	100.00

DISCUSSION

bFGF regulates cells during embryogenesis and tissue differentiation and tumor genesis. It has ability to repairs tissues applied for wound healing. The leading platform for PMF, rice have high grain yield, produces protein content in seeds and is easy to scale up (Lau and Sun 2009). Also, self-pollinating plants lower risk of pollen mediated gene flow.

For production of commercial recombinant protein, the choice of promoter is as essential as the gene of interest. In this study, *Wsi18:bFGF* transgenic rice plants for production of bFGF protein were constructed. *Wsi18* promoter, which is known to be activated under drought conditions, was used to drive the *bFGF* expression under drying or water-limiting stage. Studies show that drought conditions elevate ABA level and induce *LEA* genes in rice (Oh et al. 2005). *Wsi18* is a member of the *LEA* family which is known to contain ABREs and is activated by the ABF3 transcription factor. Other studies suggest that *Wsi18* promoter expressed *GFP* in drought-stress treated calli, leaves, root, and flowers and especially whole grains (Yi et al. 2011). For these reasons, *Wsi18* promoter was used to express bFGF protein in specific stages such as drought conditions or maturation stage when water contents in the field are decreased.

Prior to transgenic rice plants productions, the stability of bFGF protein expression in rice protoplasts was evaluated. The N- and C-terminal myc-tagged *Wsi18:bFGF* were significantly up-regulated by ABF3 transcription factor as shown in Fig. 2. The TGE assay has been proven to be a versatile

tool for characterization and functional analysis of genes (Chen et al. 2006; Yoo et al. 2007; Zhang et al. 2011). In this study, the ectopic expression of the bFGF protein in transgenic rice plants and production of bFGF protein is feasible.

The biosafety risk assessment is required to commercialize a GM crop to reduce or completely remove risks on humans as well as on environment (Lee et al. 2009). For these reasons, single copy with intact T-DNA must be selected. Reports have shown that two or more T-DNA insertions can result in post-transcriptional gene silencing (PTGS) (Tang et al. 2007; Hernandez-Garcia and Finer 2014). For identification of integration of the T-DNA in transgenic lines, Southern blot is time consuming, difficult and inefficient in large scales. Recently, TaqMan PCR has been shown to be reliable, fast, easy and could be performed on large number of lines (Shepherd et al. 2009). In this study, 36% of single copy lines were obtained from a total of 186 lines produced 16.7% and 10.2% were 2 and 3 copies, respectively. Through flanking sequence tag, T-DNA integration analysis was also performed. Out of the 36% single copy lines, 29.85% were found to be intergenic lines. This result was half of what was reported by other groups which acquired 62% intergenic insertion from single copy lines (Lim et al. 2016).

Since the single copy lines were at their early stage, multi copy lines were selected to detect expression and accumulation of bFGF proteins. *Wsi18* promoter driven bFGF mRNA and proteins were expressed in rice leaves as well as in seeds. The bFGF protein levels in leaves were induced in a time dependent manner. Though bFGF proteins at 12 h in 172 and 180

lines were not equally loaded, accumulation of bFGF proteins was still observed compared to 0 h. Result from other group claimed that using an endosperm specific promoter *Gt13a* can yield up to 8.33 ± 0.46 mg/kg (An et al. 2013). In this study however, there were no sufficient amounts of grain in T_0 plants since single copies and intergenic lines were propagated in the field.

In summary, we demonstrated that the *Wsi18* promoter is useful to drive the expression of *bFGF* under specific growth conditions, providing a good expression system for PMF. Further works are needed to produce a commercial-ready GM crop. These include a large production of transgenic rice plants and their agronomic traits evaluation in the field and bFGF protein quantification in selected elite lines.

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섬유아세포 성장인자 bFGF 생산용

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FGF gene family 인 Basic fibroblast growth factor (bFGF) 는 상처 치유, 조직 수복, fibroblast 의 분화 촉진 등의 조절에 관여하는 단백질로 의약분야에 널리 이용되어왔다. 그 동안 bFGF 는 *E. coli*, 곤충 세포, mammalian 세포 등에서 재조합 되어왔으나, 생산성이 낮고 고비용이 발생하는 등의 단점이 있다. 반면에 식물에서 재조합 단백질을 얻는 방식은 기존 방식보다 안전하고 비용적인 면에서도 효율적이며 대량 생산이 가능하다는 장점이 있다. 이러한 이유로 plant molecular farming (PMF)은 재조합 단백질 생산을 위한 새로운 방식으로 떠오르고 있다. 본 연구에서는 *bFGF* 의 N-, C-terminal 에 myc 을 tagging 하였고, rice protoplast 에서 안정된 bFGF 단백질 발현을 Myc antibody 로 확인하였다. 벼에서의 안정된 발현을 확인한 후, bFGF

생산용 single copy, intergenic 벼를 제작하였다. 제작된 186 개의 형질전환 체에서 67 개의 single copy 라인을 얻었으며, Flanking PCR 을 이용한 T-DNA integration 분석을 통해 최종적으로 20 개의 single, intergenic 라인을 획득하였다. 종자의 성숙 과정 중 수분 감소에 따른 *Wsi18* 프로모터의 유도 발현으로 목표 단백질의 발현 증가를 관찰하였다. 또한 건조된 벼의 잎에서 bFGF 의 mRNA 와 단백질의 유도 발현을 확인하였다. 위 결과 건조 처리 시간에 따른 증가 패턴을 관찰할 수 있었다. 본 연구결과들을 토대로 bFGF 단백질이 벼에서 발현 가능하다는 것을 증명하였으며, PMF 소재 가능성을 확인할 수 있었다.

주요어: Basic fibroblast growth factor, bFGF, Transgenic rice plant, Stress inducible, *Wsi18* promoter, Recombinant protein, Plant molecular farming

학 번: 2014-26006