Elucidation of Gene Diversity Affecting Gibberellin Producibility in Fusarium fujikuroi

Elucidation of Gene Diversity Affecting Gibberellin Producibility in *Fusarium fujikuroi*

(*Fusarium fujikuroi* のジベレリン産生性に影響を与える遺伝子の多様性解明)

2020

The United Graduate School of Agricultural Science, Gifu University

Science of Biological Environment

(Gifu University)

BAO WANXUE

Elucidation of Gene Diversity Affecting Gibberellin Producibility in *Fusarium fujikuroi*

（*Fusarium fujikuroi* のジベレリン産生性に影響を与える遺伝子の多様性解明）

BAO WANXUE

CONTENTS

GENERAL INTRODUCTION

Rice *bakanae* is a fungal disease that results in 30% to 95% yield loss in the world (Gupta *et al* 2015). It was first identified in 1828 at Japan (Ito and Kimura 1931) and was designated as '*bakanae*' disease after 100 years (Seto 1928). Diseased plants often exhibit yellowish leaves and abnormal stem elongation, especially at the seedling stage (Gupta *et al* 2015). During the crop growth, diseased seedlings die because all organs are attacked (Gupta e*t al* 2015). *Bakanae* disease causes huge yield and economical loss in rice growing regions, such as Japan, Taiwan, Turkey and Philippines (Nelson *et al* 1993, Cumagun *et al* 2011).

Fusarium fujikuroi Nirenberg is the pathogen responsible for causing rice *bakanae* by producing gibberellins (GAs) (Zainudin *et al* 2008). *F. fujikuroi* is a member of the *Fusarium fujikuroi* species complex (FFSC), which includes more than 50 phylogenetically different species (O'Donnell *et al* 1998), comprising of 12 mating populations (MP-A to MP-L) (Lima *et al* 2012). According to the phylogenetic analyses, the FFSC was divided into the American, the African and the Asian clade (O'Donnell *et al* 1998). Recently, three clades were proved to be evolved from a common ancestor and the Asian clade was branched in the earliest time (Fourie *et al* 2013). The FFSC members are known to cause disease on various crops (Bacon *et al* 1996) but morphological similarities make species identification without molecular diagnosis difficult (Leslie and Summerell, 2006).

F. fujikuroi has been intensively studied for its producibility of a broad spectrum of secondary metabolites, such as mycotoxins, plant hormone and pigments. The mycotoxins induce food and feed contamination and further a health risk to human and animals. *F. fujikuroi* has been identified to produce fusarin (Barrero *et al* 1991), fusaric

acid (Bacon *et al* 1996), beauvericin (Logrieco *et al* 1998), moniliformin (Rabie *et al* 1982), fumonisin (FUM) (Abbas *et al* 1994) and so on. The pigments that *F. fujikuroi* produced include neurosporaxanthin (Prado-Cabrero *et al* 2009), bikaverin (Wiemann *et al* 2009) and fusarubin (Studt *et al* 2012b).

GA is a family of diterpenoids containing 136 chemical forms whereas the most bioactive forms as plant hormone are GA_1, GA_3, GA_4 and GA_7 (Sponsel and Hedden 2004). In most plants, final GA products are GA_1 and GA_4, whereas the main product is GA_3 in *F. fujikuroi* (Hedden *et al* 2001). GA_3 promotes seed germination (Finch-Savage and Leubner Metzger 2006), responses to abiotic stress (Cole-brook *et al* 2014), and enhances fruit growth (Li *et al* 2011) and flowering (Sharma and Singh 2009). GA production is not essential for *F. fujikuroi* survival on medium or in rice, but promotes invasion of *F. fujikuroi* into cells of rice root (Wiemann *et al* 2013).

CHAPTER I

GA gene complementation in F-group strains

Introduction

In *F. fujikuroi*, GA is biosynthesized by a gene cluster that consists of 7 adjacent genes in chromosome V, including the P450 mono oxygenase genes (*P450-1*, *P450*-2, *P450*-3 and *P450*-4), the *ent-copalyl* diphosphate /*ent*-kaurene diphosphate synthase gene (*CPS/KS*), the geranylgeranyl diphosphate synthase gene (*GGS2*) and the desaturase (*DES*) (Tudzynski and Höler 1998; Wiemann *et al* 2013) (Figure 1). Acetyl-CoA is converted to farnesyl diphosphate (FDP) by mevalonic acid pathway and GA biosynthesis starts from FDP (Figure 2). By catalyzation of GGS2, geranylgeranyl diphosphate (GGDP) generates (Tudzynski and Höler 1998). GGDP is transferred into the GA-specific intermediate *ent*-kaurene by CPS/KS (Tudzynski and Höler 1998). *Ent*-kaurene is converted into *ent*-kaurenoic acid by oxidization of P450-4 then into *ent*-7α-hydroxy-kaurenoic acid by oxidation of P450-1 (Tudzynski and Höler 1998). GA_{12} and GA_{14} are synthesized from *ent*-7α-hydroxy-kaurenoic acid by multifunction of P450-1 (Rojas *et al* 2001). P450-2 catalyzes the conversion from GA_{14} to GA_4 (Tudzynski *et al* 2002). GA_1 and GA_7 are generated from GA_4 by P450-3 and DES, respectively (Tudzynski *et al* 2002, Tudzynski *et al* 2003). The main product GA_3 is converted from GA_7 under catalyzation of P450-3 (Tudzynski *et al* 2003). GA biosynthetic pathway in *F. fujikuroi* is different from that in high order plants, suggesting independent evolution of GA production between *Fusarium* and plant (Bömke and Tudzynski 2009).

Nitrogen conditions have been shown to distinctly affect GA production in *F. fujikuroi*. GA yield is low under high nitrogen conditions and *vice versa* (Wiemann *et al* 2013). A GATA-type transcription factor (TF), AreA, which regulates the utilization of alternative nitrogen sources (nitrate, arginine, urea and allantoin) when optimized nitrogen sources (glutamine and ammonium) are absent (Marzluf 1997, Tudzynski

2014), and AreB whose function is unknown, are essential for expression of the GA gene cluster (Michielse *et al* 2014a). AreA, which directly binds to GATA motifs in promoters of the GA gene cluster, positively regulates GA gene expression except *P450-3* (Milan *et al* 2003). AreB regulates expression of GA genes positively and negatively at the meantime (Michielse *et al* 2014a). Another TF FfSge1, which is orthologue of Sge1 but without regulation of conidiogenesis as in *Fusarium oxysporum*, upregulates expression of the GA genes under limited nitrogen conditions (Michielse *et al* 2014b). On the contrast, the basic leucine zipper (bZIP) TF MeaB negatively regulates expression of the GA genes (Wagner *et al* 2010). The upregulation of the acetylation of histone H3 lysine 9 (H3K9Ac) and dimethylation of lysine 4 at histone 3 (H3K4me2) with expression of the GA genes was revealed in *F. fujikuroi* (Wiemann *et al* 2013). Recently, association of TFs and histone-modification in *F. fujikuroi* was proposed by Pfannmüller *et al* (2017) as "Deletion AreA and AreB results in reduced H3K9Ac levels at GA gene cluster, which is in line with an abolished biosynthesis of GA". The global regulator velvet protein, *FfVel1*, *FfVel2* and *FfLae1* are activators for GA biosynthesis (Wiemann *et al* 2010). The glutamine synthetase encoding gene *GLN1* has positive effect on GA biosynthesis (Teichert *et al* 2004). The NADPH cytochrome P450 reductase (CPR), an electron donor of P450 monooxygenase, is also essential for GA biosynthesis (Malonek *et al* 2004). In addition, GA biosynthesis is positively influenced by cAMP signaling in *F. fujikuroi*. *FfAc* and *FfPka2* have positive effect on GA biosynthesis (García-Martínez *et al* 2012, Studt *et al* 2012a).

Among the FFSC members, GA production has been considered to present only in *F. fujikuroi* (MP-C) for long time. However, the significant GA production was detected in other FFSC members. *F. proliferatum* (MP-D) strain KGL0401 was found to be a

GA producer. The GA producibility of KGL0401 was even higher than a wild type *F. fujikuroi* strain (Rim *et al* 2005). Orchid-associated *F. proliferatum* strain ET1 produced significant amount of GA_4 and GA_7 (Tsavkelova *et al* 2008). The *F. proliferatum* strains with GA producibility were endophytes in plant root rather than pathogens for maize (Troncosco *et al* 2010). Another phylogenetically closely related member in the Asian clade, the *F. sacchari* (MP-B) strains B-7610, B-12756 and B-1732 produced GA_3 although the production was lower than the *F. fujikuroi* strain (Kawaide 2006, Troncosco *et al* 2010). However, *F. konzum* (MP-I), which is classified in the American clade, was also identified to produce GA. *F. konzum* strain I-10653 synthesized GA_1 (Leslie *et al* 2004). Several years later, Troncoso *et al* (2010) found that *F. konzum* strains I-11616 and I-11893 produced a lot of GA_1 and a few GA_3.

Intriguingly, GA production has been proved to be diverse in *F. fujikuroi* in recent 30 years although *F. fujikuroi* is well known as a GA producer. In addition, negative tendency of GA producibility and FUM producibility has been observed in *F. fujikuroi* (Niehaus *et al* 2017, Suga *et al* 2019). Fifty-eight *F. fujikuroi* strains which were isolated from diseased rice seedlings produced GA with 1 to 74 μg/g, and FUM production were detected in 8 strains of them: mean concentration of FB_1 and FB_2 was 380 μg/g and 23 μg/g, respectively (Yoshizawa *et al* 1994). Ten *F. fujikuroi* strains were proved to produce GA_3 meanwhile traces of FUM. These strains induced yellowish and slender leaves in rice seedlings (Wulff *et al* 2010). According to the phylogenetic and pathogenicity analyses, Niehaus *et al* (2017) found that *F. fujikuroi* strain B14 induced stunting and early withering on rice seedlings rather than abnormal elongation, which is the typical symptom of *bakanae* disease. Meanwhile, B14 produced FUM at high level instead of GA. Suga *et al* (2019) designated Japanese *F. fujikuroi* strains as Gibberellin

(G)-group and Fumonisin (F)-group based on the results of phylogenetic analyses and FUM and GA production assay. G-group produces a large amount of GA but no FUM, whereas F-group produces a large amount of FUM but GA at very low or no level. G-group strains induce abnormal elongation on rice seedlings while F-group strains do not induce that symptom. According to Piombo *et al* (2020), GA production difference was detected among three *F. fujikuroi* strains. The strain with the highest GA_3 production induced rice seedlings abnormal elongation whereas the strain with the lowest production induced stunting. GA production of these strains were affected by temperature *in vivo*. In addition, FUM production was detected in the GA low-producer *in vitro*. Expression of *CPS/KS* were in accordance to GA production in these strains.

In previous research, GA production difference was detected between G- / *bakanae* group and F- / stunting group (Niehaus *et al* 2017, Suga *et al* 2019). While, the genetic causes of the difference remain unclear. In this CHAPTER, using G-group strain Gfc0801001 and F-group strain Gfc0825009, GA production difference was identified to be associated with the GA gene cluster.

Materials and methods

Fusarium fujikuroi strains

G-group strain Gfc0801001 isolated from rice and F-group strain Gfc0825009 isolated from maize were used (Suga *et al* 2019). We also used SL0271 strain which was isolated from tomatoes (Imazaki and Kadota, 2015). SL0271 was identified as being a member of the F-group based on the single nucleotide polymorphisms (SNPs), T618G in the translation elongation factor I α (Suga *et al* 2014) and C3250T in the RNA polymerase II-second-largest subunit (Suga *et al* 2019). SL0271 was also found to be a member of the F-group, following positive FUM production.

Culture conditions

For the GA production assay of crossing progenies or transformants and for reverse transcription polymerase chain reaction (RT-PCR) analysis, a mycelial plug was transferred into 40 ml of 10% (w/v) ICI medium (Imperial Chemical Industries Ltd. UK) containing 0.05% (w/v) ammonium nitrate as nitrogen source and grown for 7 d on a reciprocal shaker (123 rpm) at 25°C. The mycelial mat and culture liquid were separated with filter paper. The culture liquid was centrifuged at 14,000 rpm for 15 min at 25°C and supernatant was kept at -30°C until analysis.

For quantitative RT-PCR (RT-qPCR), a mycelial pellet was transferred into 40 ml of 100% (w/v) ICI medium containing 0.5% (w/v) ammonium nitrate as nitrogen source and grown for 3 d on a reciprocal shaker (123 rpm) at 25°C. The pre-induced GA mycelial mat was obtained by filtering half of the culture. The remaining culture was centrifuged at 4,500×g for 25 min, and the mycelial mat was transferred into 40 ml of 10% (w/v) ICI medium and then cultured for 7 d. The post-induced GA mycelial mat

and culture liquid were separated with filter paper. The mycelial mat was kept at -80℃ until RNA extraction. Culture filtrate was kept at -30℃ until GA production assay.

DNA extraction

Genomic DNA was extracted from a mycelial mat cultured in potato dextrose broth (PDB) medium for 3 d. After the extra liquid and the agar piece were removed, the mycelial mat was transferred onto a filter paper and air-dried naturally over 24 h. A small piece of dried mycelial mat (3 mm^2) and two short wires (1~1.5 cm) were vortexed for 20 min with max strength. The mycelial powder was suspended with potassium ethyl xanthogenate (PEX) solution (6.25 mM potassium ethyl xanthogenate, 100 mM Tris HCl, pH 7.5, 700 mM NaCl, and 10 mM EDTA, pH 8.0) then was incubated at 60°C for 30 min and centrifuged at 14,000 rpm for 5 min at 25°C. The supernatant was collected and mixed with ethanol (EtOH) by inversion then was centrifuged again. Collecting the precipitate then mixing with 600 μl 70% (v/v) EtOH and incubating for 1 min then removing the rest liquid. After drying under vacuum for 10 min, the genomic DNA was harvested. Genomic DNA was kept in 400 μl water at -30℃.

PCR and sequencing

The DP4, P1, P2G2 and CP3 regions in the GA gene cluster (Figure S1) were amplified by PCR using KOD-Plus-Neo DNA polymerase (Toyobo, Tokyo, Japan) and the iCycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following cycling parameters: 94°C for 2 min, 30 cycles of 98°C for 10 s, 60°C for 30 s, and 68°C for 10 min. The primers used for PCR and sequencing of the GA gene cluster are shown in Table S1 and Table S2. PCR products were mixed with 60% (v/v) polyethylene glycol (PEG) solution (30% polyethylene glycol 6000, 1.6 M NaCl) and inoculated at 37°C for

10 min. The suspension was centrifuged at 12,000×g for 10 min and DNA was precipitated by rinsing 70% (v/v) EtOH. The DNA was resuspended in water and used for sequencing. Nucleotide sequence was obtained by BigDye terminator V3.1 with cycle sequencing kits (Life Technologies, Carlsbad, CA, USA) and an ABI 3130 genetic analyzer (Life Technologies, Carlsbad, CA, USA). The bidirectional promoter region between *P450-1* and *P450-4* (Ca. 1kb) in the G- and F-group strains was similarly amplified by PCR with the use of primers P4-Fus1 and P1-Fus2 instead (Table S2). The following cycling parameters were used: 94°C for 2 min, 30 cycles of 98°C for 10 s, 55°C for 30 s, and 68°C for 10 min. Primers P4-Fus1 and P1-Fus2 were also used for sequencing. Sequence data was processed with Chromas Pro (Technelysium Pty., Tewantin, Queensland, Australia) and Genetyx (Genetyx, Tokyo, Japan). Sequences of the GA gene cluster of Gfc0801001 (accession number LC390205) and Gfc0825009 (accession number LC390342) are available in DDBJ/EMBL/GenBank database.

Integration of the TP4501 fragment (Ca. 4.3kb) in the transformants was detected by PCR using a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA), KOD Plus-Neo DNA polymerase (Toyobo) and the primer pair of HS755 (Table S1) / M13M4 (5'-GTTTTCCCAGTCACGACGTTGTAAA-3') with the following cycling parameters: 94°C for 2 min, 35 cycles of 98°C for 10 s, 60 °C for 30 s, and 68°C for 7 min.

RNA extraction

Total RNA was extracted from 300 mg mycelial mat by the Maxwell RSC and the Simply RNA Tissue kit (Promega, Madison, WI, USA). RNA was eluted in 50 µl water and kept at -80℃. The RNA concentration was determined to 20 ng/µl using NanoVue (GE Healthcare, Munich, Germany).

Construction of the transformation vector

A part of the GA gene cluster shown in Figure 1 in the GA gene cluster was amplified from Gfc0801001 by PCR using the primer pair shown in Table S1. PCR products treated with *Not* I (New England Biolabs, Ipswich, MA, USA) were inserted into the *Not* I site in pCB1004 (Caroll *et al* 1994), pDNAT1 (Kück and Hoff, 2006) or pBSNII99-3 (Sultana *et al* 2019) using DNA Ligation Kit Ver. 2 (Takara Bio Inc., Otsu, Japan) (Table S3). This type of plasmid construct provides a simple gene integration to the genome rather than homologous recombination by the fungal transformation.

Fungal transformation

The budding cell was prepared by mycelial mat culturing in the PDB medium for 48 h. The cells were obtained by centrifugation after filtration with Kim Wipes (Kimberly-Clark, Tokyo, Japan), then suspended in PDB medium at 5.0×10^5/ml. The suspension was shaking incubated on 90 rpm at 25℃ over 7 h until the length of the budding filament reached 2~3 times the length than before. The budding filaments were collected by filtration under vacuum and washed with 1.2 M NaCl. To construct the protoplast, the budding filaments were suspended in an enzyme solution containing 20 mg/ml of lysing enzyme (Sigma), 10 mg/ml of Cellulase Onozuka RS (Yakult, Tokyo, Japan), 10 mg/ml of Zymolyase 20T (Seikagaku Kogyo, Tokyo, Japan), 10 mg/ml of b-Glucuronidase type H-1 (Sigma), and a small amount of chitinase (Sigma) in 1.2 M NaCl, then stirred gently at 30℃ for 4~6 h. The protoplast was collected by filtration washed with SE (1 M sorbitol, 50 mM EDTA, pH 8.0) and centrifugated at 2000 rpm for 10 min, then filtration was washed with STC (1 M sorbitol, 25 mM Tris-HCl, pH 7.0, 25 mM $CaCl_2$). The final concentration was adjusted to $5\sim10\times10^5$/100 μl. Transformation was incubated under illumination with one percent water agar (w/v)

containing 250 μg/ml hygromycin B (Wako, Osaka, Japan), 800 μg/ml nourseothricin (Axxora, San Diego, CA, USA), or 450 μg/ml geneticin (G418 disulfate salt, Sigma, St Louis, Mo, USA) to select transformants containing the plasmid of interest.

RT-PCR

RT-PCR was performed with PrimeScript™ One Step RT-PCR (Takara Bio Inc., Otsu, Japan) with the primer pairs shown in Table S4 according to the manufacturer's instructions. A reaction mixture containing enzyme mix, buffer, 1.6 ng/μl RNA and 0.4 μM each primer was prepared. RT-PCR was performed in a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following cycling parameters: 50°C for 30 min and 94°C for 2 min, 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min. The amplified product was detected by 1% (w/v) agarose gel electrophoresis.

RT-qPCR

Reverse transcription was performed using the Superscript VILO cDNA synthesis kit (Invitrogen Carlsbad, CA, USA) according to the manufacturer's instructions. A reaction mixture containing Enzyme Mix and 1 ng/μl RNA was incubated in 25°C for 10 min, followed by 42°C for 60 min and 85°C for 5 min. Two μl of the solution was added to a 25 μl PCR mixture of SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) containing 2 μM each primer (Table S5). PCR amplification was performed in triplicate on a StepOne Plus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) under the conditions: 40 cycles each of 95°C for 15 s and 63°C for 60 s. Specific amplification was confirmed by a single peak of the melt (dissociation) curve analysis. The ΔΔCt method was used for quantification. The ΔCt value was obtained by subtracting the Ct value of *Histone H3* from that of the interest gene. The

ΔΔCt value was obtained by subtracting the ΔCt mean value of GA pre-induction culture of Gfc0801001 from that in each respective sample. RNA from three independent cultures for each strain or transformant were used and their mean $2^{-\Delta\Delta Ct}$ values were obtained.

Gibberellin analysis

Thin-layer chromatography (TLC) was performed by culture liquid of 7 d culture in 10% ICI medium (w/v). Here, five-hundred ng GA_3 standard (Wako Pure Chemicals Ind., Ltd., Osaka, Japan) was spotted as a reference and 20 μl culture liquid was spotted on the TLC. Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was performed to quantify GA_1, GA_3, GA_4 and GA_7 in the liquid culture according to Suga *et al* (2019). Working solutions containing GA_1, GA_3, GA_4, and GA_7 at concentrations of 0.5~5.0 mg/l (GA_3/GA_7: 0.5, 1.0, 2.0, 5.0 mg/l; GA_1/GA_4: 0.5, 1.0, 1.5, 2.0 mg/l; in four bottles) were used to generate a calibration curve. The limit of quantification (LOQ) was the lowest concentration (0.5 mg/l) on the calibration curves. In case a peak smaller than the LOQ was detected in the sample by UPLC-MS/MS, we determined it as trace after confirmation of specific MS/MS spectra. In case that the concentration of the sample exceeded the range given by the calibration curve, four times and 10 times diluted sample were prepared and reanalyzed. Mean values of three independent cultures for each strain or transformant were obtained. When trace amounts were detected in at least 1 of 3 replicative cultures, this was determined as a trace.

Pathogenicity test

The dwarf rice cultivar, Tanginbozu, was used to perform the pathogenicity test. This cultivar is known to be sensitive to GA. The rice seeds were disinfected by

immersing in 60°C water for 10 min, then cooled with running water rapidly before pregermination. The strains were cultured on potato dextrose agar (PDA) medium for 7 d at 25°C and spore suspension at concentration of 1×10^5/ml. The seeds were immersed into the spore suspension and incubated at 30°C in the darkness for 48 h. Ten germinant seeds were washed by and sank into sterile water to remove the redundant mycelium. Then the seeds were sown in pots (5.5 cm×7 cm) containing 50 ml wet sterile nutritional soil. The pots were placed in the plastic case (13 cm×21 cm×11 cm) and covered a piece of plastic wrap. Incubating at 30°C under continuous illumination for 4 d, the plastic wrap was removed and the seedlings were watered. The incubation continued at 23°C a 16 h light / 8 h dark cycle. The height of three highest seedlings in each pot was measured after incubating for 3 d. The disease level was evaluated as the ratio of mean height of three tallest individual seedlings with inoculation to that of healthy seedlings. The pathogenicity tests were performed in three pots (replications) of each strain or transformant. The statistical significance (p-value <0.01) of pathogenicity in transformants and the donor strain Gfc0801001 versus the original strain (Gfc0825009 or SL0271) was calculated using the ANOVA-Dunnett Multiple Comparisons Test in Microsoft Excel.

Results

Linkage analysis between gibberellin producibility and SNP markers

Linkage analysis was performed to determine whether the cause of GA low production in F-group strain Gfc0825009 is present in the GA gene cluster. Forty-two progenies between G-group strain Gfc0801001 and Gfc0825009 (Sultana *et al* 2019) were used. These progenies were cultured in 10% (w/v) ICI medium for 7 d and GA_3 in the culture liquid was detected by TLC. GA_3 spots were detected in Gfc0801001 and some progenies, but not in Gfc0825009 and other progenies. SNP and Mating type (MAT) data of progenies from the study by Sulnata *et al* (2019) were used for linkage analysis with GA_3 production. Among the five SNP markers, the P4504_C842T marker is present in *P450-4* that is located in the GA gene cluster. The CPR_C1152A marker is present in *CPR* which is involved in GA production (Malonek *et al* 2004) whereas is not located in the GA gene cluster. GA_3 production was completely linked with the P450-4_C842T marker (Table S6). These results suggest that the cause of GA low production in Gfc0825009 is present in the GA gene cluster.

Sequence comparison

The nucleotide and amino acid sequences of the genes in the GA gene cluster were compared between G-group strain Gfc0801001 and F-group strain Gfc0825009 (Table 1) and we found that all the differences were substitutions. A homology > 99.3% was observed in nucleotide and amino acid sequence of genes with the exception of *CPS/KS*. *CPS/KS* had the lowest homology (98.4%) and 15 amino acid substitutions were detected. GA_3 production has been detected in Gfc0825009 despite the fact that a GA_3 spot could not be detected by TLC (Suga *et al* 2019). Gene constitution in the GA gene cluster is retained in Gfc0825009 and is consistent with its GA_3 production.

RT-PCR

Expression of genes in the GA gene cluster was investigated in Gfc0801001 and Gfc0825009 by RT-PCR (Figure 3). DNA with expected size was amplified in all genes though amplification of *DES* in Gfc0825009 was not obvious as that in Gfc0801001 (Figure 3). The result suggests that all GA biosynthetic genes can express in F-group strain Gfc0825009.

Gibberellin production increase by gene complementation

In order to find the cause of low GA production in Gfc0825009, gene complementation in the GA gene cluster were performed in Gfc0825009 using G-group strain Gfc0801001 as a gene donor. At first, the GA gene cluster was divided into two regions; DP2 including *DES*, *P450-4*, *P450-1*, and *P450-2* and GP3 including *GGS2*, *CPS/KS* and *P450-3* (Figure 1). We assumed that either DP2 or GP3 from Gfc0801001 increases GA production in Gfc0825009. However, transformants with increased GA production were detected in both DP2 and GP3 (Figure 1, Table S7). We identified positive detection of a GA_3 spot on TLC as GA production increase in transformant because the original strains did not display GA_3 spot on TLC (Figure S2). The GA_3 spot on TLC was positive in 14 of 32 DP2 transformants, while one of 32 was positive in GP3 transformants. Therefore, we focused on the genes in DP2 though the result suggests that the genes in GP3 also contribute to the increase in GA production.

Transformants of each *DES*, *P450-4*, *P450-1* or *P450-2* were further created and the increase in GA production was detected in several transformants of each gene (Figure 1, Table S7). These results suggest that each gene in the DP2 region of G-group Gfc0801001 could increase GA_3 production in F-group Gfc0825009. The amino acid sequence of *P450-1* is identical between Gfc0801001 and Gfc0825009 (Table 1) and

therefore, we hypothesized that reintegration of *P450-1* from Gfc0825009 to itself may increase GA production. As we expected, GA production increase was observed in the self-transformants (FfGibselfTP4501 in Figure 1 and Table S7). These results suggest that transfer of a single gene in the GA gene cluster can increase GA production in Gfc0825009. For further studies, we defined transformant with positive GA_3 spot on TLC as GA recovery transformant and transformant without the spot as GA nonrecovery transformant. We selected two representative GA recovery and one nonrecovery transformants to assay GA production and gene expression though only one transformant with GA recovery was available for *P450-2* (FfGibTP4502(#49)). The GAs concentrations were investigated by UPLC-MS/MS. In consideration of gene expression studies, the representative transformants were firstly cultured in 100% (w/v) ICI medium as GA pre-induction and then in 10% ICI (w/v) medium as GA post-induction. GA_1, GA_3, GA_4 and GA_7 in liquid culture of GA post-induction were quantified by UPLC-MS/MS. We identified obvious increase of GA_3 and slight increase of GA_7, GA_4 and GA_1 in the GA recovery transformants compared to the original strains (Table 2). Among the single gene transformants, comparatively high GA_3 production was observed in *P450-4* transformants (Table 2). GAs production in the GA nonrecovery transformants were not altered from the original strains (Table 2). Expression of *P450-1*, *P450-4* and *P450-2* in the transformants were assayed by RT-qPCR (Table 2). In the case of GA recovery transformants of *P450-1* (FfGibTP4501(#8) and (#10)), expression of *P450-1*, *P450-2* and *P450-4* increased following GA post-induction, although these levels were lower than G-group Gfc0801001 (Table 2). The increase in *P450-1* expression was also observed in GA nonrecovery transformant (FfGibTP4501(#4)). However, simultaneous expression

increases of *P450-2* and *P450-4* were not detected in it. In GA nonrecovery transformants FfGibselfTP4501(#1) and FfGibTP4504(#31), the expression of all *P450-1*, *P450-2* and *P450-4* slightly increased (Table 2). Increases in the expression of the transferred gene and other genes were observed in the GA recovery transformants of self *P450-1*, *DES*, *P450-4*, and *P450-2* though that of the transferred gene could not be confirmed for *DES* transformants (Table 2). In the case of the GA nonrecovery transformants, the expression of the transferred gene did not always increase and the expression of other genes did not increase either (Table 2). In order to clarify whether the simultaneous increase in the gene expressions of GA recovery transformants is unique to Gfc0825009, *P450-1* and *P450-4* transformants were created with another F-group strain, SL0271. Similar results were observed (Table 2). We assumed that the failure to increase GA production in the GA nonrecovery transformants may attribute to partial integration of the transferred gene. Therefore, full-length integration of the transferred gene in *P450-1* transformants was confirmed by PCR (Figure S3). DNA with expected size was amplified in GA recovery transformants whereas was not amplified in GA nonrecovery transformants except for FfGibTP4501(#4) (Table 2, Figure S3). DNA with expected length was amplified and an increase of *P450-1* expression was observed in FfGibTP4501(#4); however, the expression of *P450-4* and *P450-2*, and the production of GA_3 did not increase. Integration of the transferred gene into the GA gene cluster may be responsible for the expression increase of GA genes. Therefore, the lengths of the GA gene cluster in the GA recovery transformants FfGibTP4501(#10) and FfGibselfTP4501(#20) were investigated by PCR. We found that the length did not alter in the transformants (Figure S4). Therefore, the integrated region of transferred gene is outside of the GA gene cluster in these transformants. In

order to understand the reasons behind the low expression of *P450-1* and *P450-4* in F-group strains, the nucleotide sequences of the bidirectional promoter region between *P450-4* and *P450-1* in F-group strains Gfc0825009 and SL0271 were compared with G-group strain Gfc0801001 (Figure S5). We detected four substitutions in Gfc0825009 and two substitutions in SL0271. None of the substitutions were shared in these strains.

Pathogenicity to rice seedling

In order to assess the effect of increases in GA production in the transformants, the pathogenicity of GP3, *DES*, *P450-1*, self-*P450-1* and *P450-2* transformants were investigated (Table 3). G-group strain Gfc0801001 (gene donor) was 1.3 times the height of rice seedlings compared to non-inoculated seedlings whereas the original F-group strains Gfc0825009 and SL0271 was 0.9 and 1.0 times the height, respectively (Figure 4, Table 3). Regardless of GA recovery and nonrecovery transformants, significant differences in plant height were not detected in inoculated seedlings, including FfGibGP3(#12) (Table 3) which produced GA_3 with an approximate half concentration of G-group strain Gfc0801001 by culturing *in vitro* (Table 2).

Discussion

In this CHAPTER, transferring each of *P450-1*, *P450-4*, *P450-2*, or *DES* from a G-group strain increased GA production in two F-group strains. We also observed a simultaneous increase in *P450-1*, *P450-4*, and *P450-2* expression in the GA recovery transformants.

GA production in *F. fujikuroi* has been intensively studied. However, mechanisms behind the differences in GA production among strains are not fully understood. In this CHAPTER, we found that the GA production difference between G-group Gfc0801001 and F-group Gfc0825009 is due to the GA gene cluster.

In *F. fujikuroi*, G-group strains produce large amounts of GA and produce no FUM. Whereas F-group strains produce large amounts of FUM and little or no GA (Suga *et al* 2019). The level of gene expression in the GA gene cluster has been shown to be involved in GA production difference between G- and F-group strains (Table 2). Nucleotide substitutions in the bidirectional promoter of *P450-1* and *P450-4* might be responsible for the low expression of these genes in F-group strains although the substitutions were not located in the GATA motifs (Figure S5). However, substitutions in GATA motifs were detected in GA non-produced *F. proliferatum* strain D02945 compared to the IMI58289, a GA producer of *F. fujikuroi* (Malonek *et al* 2005b). The inactivity of *CPS/KS* and *GGS2* has been detected in the *F. proliferatum* strain D00502 (Malonek *et al* 2005c). While, in the case of F-group Gfc0825009 and SL0271, these genes have proved to be functional because GA_3 production was detected in these strains and their transformants (Table 2).

In pathogenicity tests, Gfc0801001 induced abnormal elongation of rice seedlings while Gfc0825009 and SL0271 induced little to no stunting (Figure 4). Pathogenicity

did not significantly alter in GA recovery transformants (Table 3). This may be due to the production of FUM and fusaric acid which have a stunting effect on rice seedlings in the transformants (Niehaus *et al* 2017). When stunting type strain B14 of *F. fujikuroi*, which produces a significant amount of FUM, was inoculated to rice seedlings together with exogenous GA_3, no elongation of rice seedlings was observed (Niehaus *et al* 2017). Based on further study, Niehaus *et al* (2017) concluded that "Conversion of B14 into a *bakanae* type pathotype by addition of GAs is only possible after deleting the key genes for the production of fumonisins and fusaric acid". If FUM production is deleted in the GA recovery transformants, the elongation symptom might be more obvious. In addition, symptom of inoculated rice seedlings was affected by temperature (Piombo *et al* 2020). The infected seedlings were mixed with elongated and stunting although they were infected by *F. fujikuroi* strain with high or low GA production at a time. When temperature increased, seedlings infected with the GA producer showed elongation obviously. Installing the eligible temperature of pathogenicity test might be emerging conspicuous symptom.

In this CHAPTER, GA production also increased in one GP3 transformant (Figure 1, Table S7). According to Piombo *et al* (2020), low expression of *CPS/KS* was detected in the GA nonproducer of *F. fujikuroi*. This suggests that a single gene complementation with *GGS2*, *CPS/KS* or *P450-3* may also increase GA production by simultaneous increase expression of other genes in the GA gene cluster.

Results of RT-qPCR showed that expression of *P450-1*, *P450-4*, and *P450-2* are low in both F-group strains Gfc0825009 and SL0271 after GA post-induction compared to G-group strain Gfc0801001 (Table 2). This suggests that low GA production in F-group strains is due to low expression of GA genes.

Partial integration of TP450-1 did not enhance expression of other genes (Table 2, Figure S3) although full length integration and an increase in *P450-1* expression was observed in the GA nonrecovery transformant FfGibTP4501(#4). The lack of increase in GA production of the transformants may attribute to the lack of a simultaneous increase in expression of other genes in the GA gene cluster. Further comparative analyses of FfGibTP4501(#4) and GA recovery transformants would contribute to our understanding of the regulatory mechanisms of GA biosynthesis.

Malonek *et al* (2005b) suggested that the correct integration of a GA gene cluster is important for its transcription rate. In this CHAPTER, we confirmed that the transferred gene was not integrated into the GA gene cluster in two GA recovery transformants (Figure S4). This suggests that gene integration in the GA gene cluster is not necessary to increase the expression of other genes in the cluster.

Transfer of the GA gene might activate regulators of the GA gene cluster, such as histone modification. Wiemann *et al* (2013) indicated that H3K9ac and H3K4me2 occurred primarily under nitrogen-limiting conditions. Alteration of H3K9ac and H3K4me2 in the GA gene cluster might occur in the GA recovery transformants. Park *et al* (2015) demonstrated that zearalenone (ZEA) itself induces expression of *ZEB2* that is a TF for ZEA biosynthetic genes at the early stage of ZEA production in *F. graminearum*. A single gene transfer may cause increase of chemical intermediate for GA_3 production and it may induce simultaneous gene expression in the GA gene cluster.

Figure and table

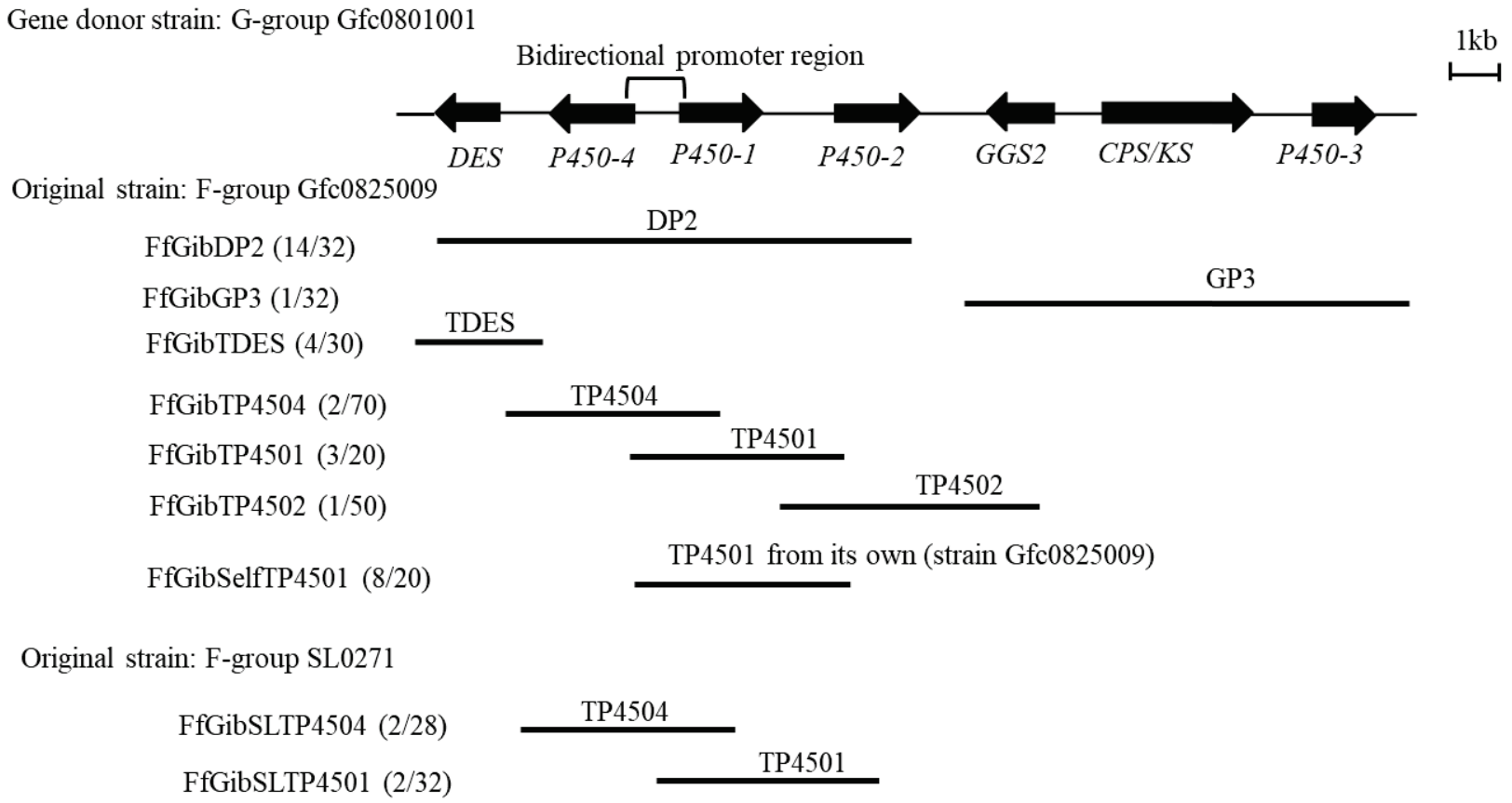

Figure 1. The GA biosynthetic gene cluster and result of gene complementation.

The GA gene cluster for transformation was provided by G-group Gfc0801001 as donor strain. Transformants were created by using F-group Gfc0825009 (FfGibT-) and SL0271 (FfGibSLT-) as original strains. Region transferred from Gfc0801001 to the F-group strains was indicated by horizontal line. GA recovery transformant was determined by positive detection of GA_3 spot on TLC. The number in bracket means GA recovery transformants / tested transformants.

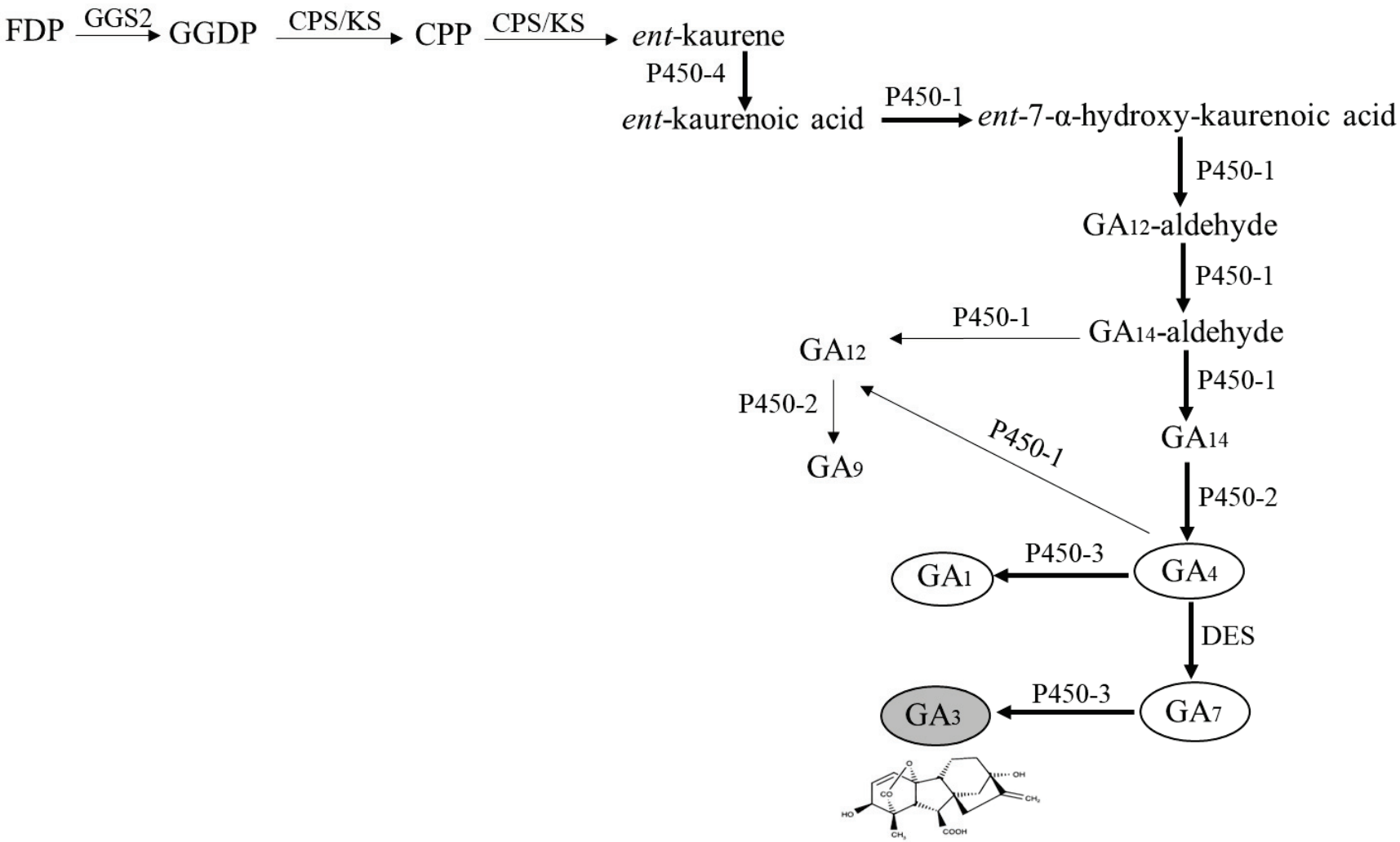

Figure 2. GA biosynthetic pathway in *F. fujikuroi.*

The pathway shows genes enzymes and products. The main pathway is shown by bold arrows. FDP, GGDP and CPP means farnesyl diphosphate, geranylgeranyl diphosphate and ent-copalyl diphosphate, respectively. The final products are indicated by ellipses. The chemical structure of GA3 was indicated at the bottom.

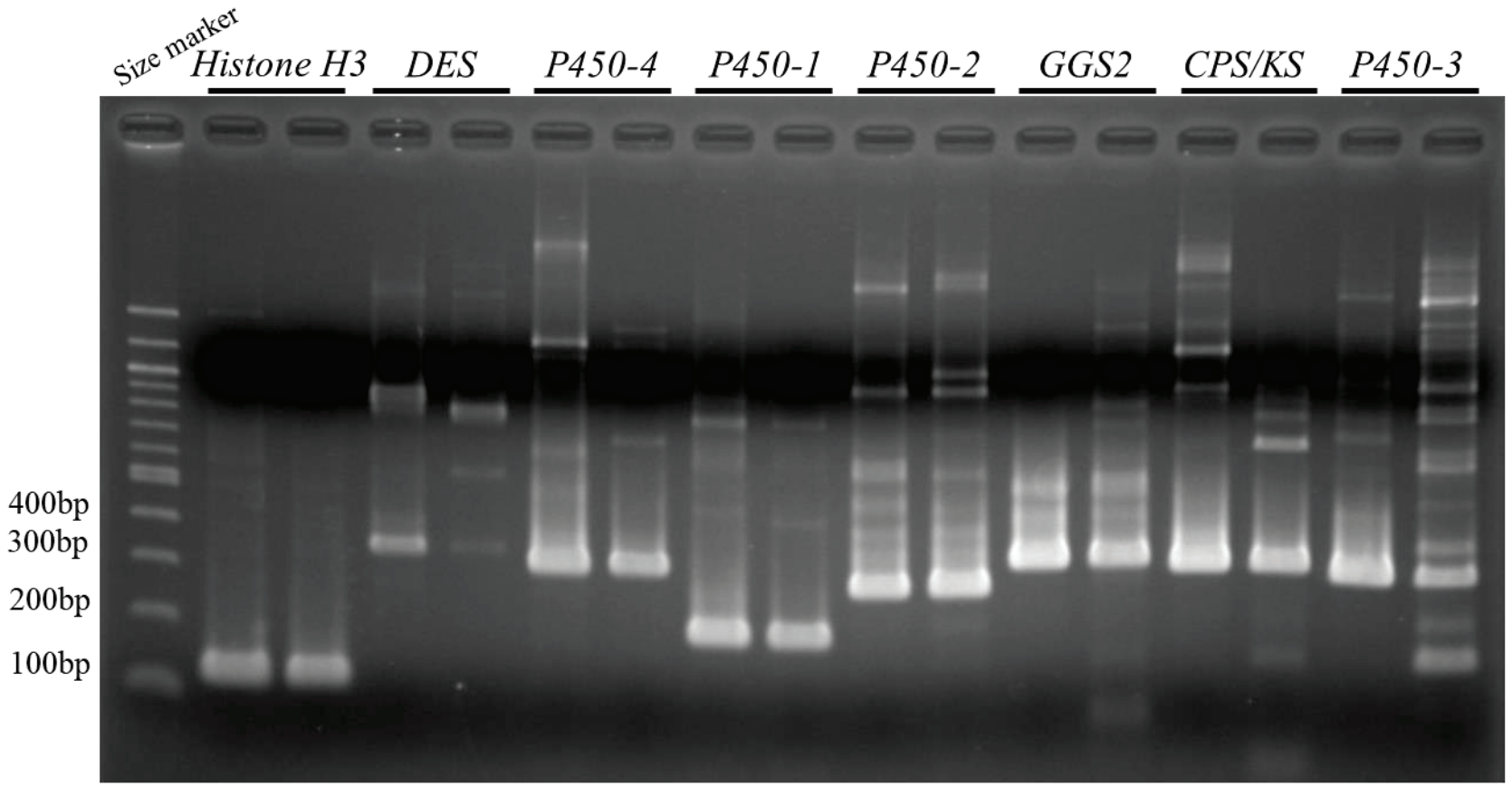

Figure 3. RT-PCR of the GA biosynthetic genes.

RT-PCR product was subjected to 1% (w/v) agarose gel electrophoresis. The left and the right lane in each gene belongs to Gfc0801001 and Gfc0825009, respectively. Histone H3 was used as the positive control. The size of the expected amplification is found in Table S4.

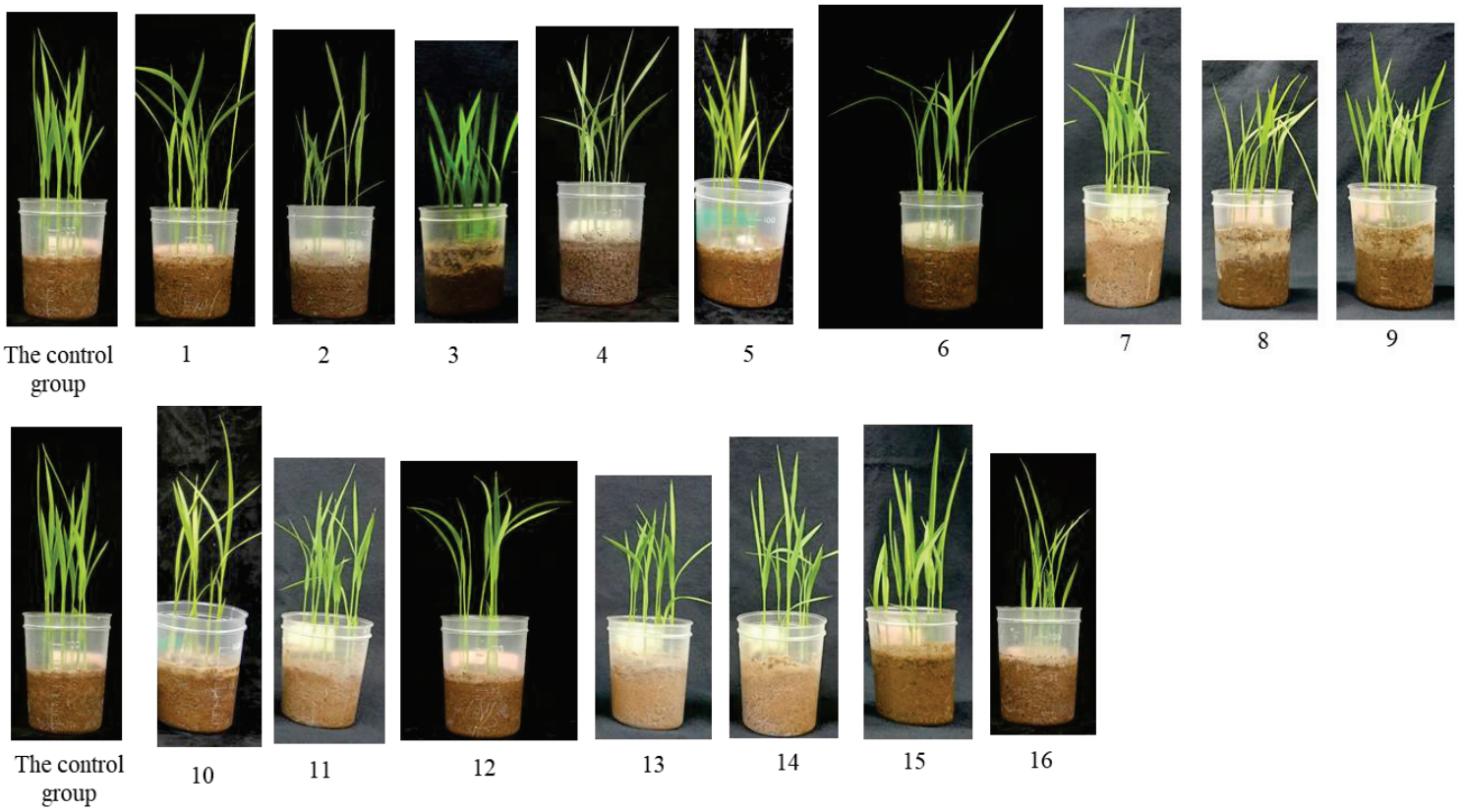

Figure 4. The symptom of infected rice seedlings

Water was used as control. Rice seedlings were inoculated with G-group strain Gfc0801001 (No.1); F-group strains Gfc0825009 (No.2) and SL0271 (No.3); GA recovery transformants: FfGibGP3(#12) (No.4), FfGibTDES(#12) (No.5), FfGibTP4501(#8) (No.6) and (#10) (No.7), FfGibSLTP4501(#19) (No.8) and (#20) (No.9), FfGibselfTP4501(#6) (No.10) and (#20) (No.11), FfGibTP4502(#49) (No.12); and GA nonrecovery transformants: FfGibTP4501(#4) (No.13), FfGibselfTP4501(#1) (No.14), FfGibSLTP4501(#3) (No.15) and FfGibTP4502(#5) (No.16).

Table 1. Sequence homology of the GA gene cluster[a)].

Gene	Gene ID[b)]	Function[c)]	Homology (%)		Length of amino acid[d)]	The number of different amino acid[e)]
			Nucleic acid	Amino acid		
DES	FFUJ_14331	desaturase	99.4	99.4	342	2 substitutions
P450–4	FFUJ_14332[f)]	P450 monooxygenase	99.6	99.8	525	1 substitution
P450–1	FFUJ_14333[f)]	P450 monooxygenase	99.5	100.0	526	Nothing
P450–2	FFUJ_14334	P450 monooxygenase	99.5	99.3	481	3 substitutions
GGS2	FFUJ_14335	geranylgeranyl diphosphate synthase	99.3	99.4	393	2 substitutions
CPS/KS	FFUJ_14336	*ent-copalyl* diphosphate synthase/*ent*-kaurene diphosphate synthase	98.4	98.4	952	15 substitutions
P450–3	FFUJ_14337	P450 monooxygenase	99.4	99.4	527	3 substitutions

a) The homology between the G-group strain Gfc0801001 and the F-group strain Gfc0825009.

b) Gene ID of whole genome sequence of strain IMI58289 (Wiemann *et al* 2013).

c) Functions were refered to Studt and Tudynski (2014).

d) Value of Gfc0801001.

e) Sequence of Gfc0825009 compared to that of Gfc0801001.

f) Coding region was modified from that of IMI58289 (Wiemann *et al* 2013) in the database.

Table 2. Gibberellin production and gene expression of transformant.

Original strain	Transformant[a]	GA production[b]	Gibberellin (mg/l)[c]				Comparative expression level[d]						Full length integration PCR
							P450-4		*P450-1*		*P450-2*		
			GA_3	GA_7	GA_4	GA_1	pre-inductio	post-induction	pre-induction	post-induction	pre-induction	post-induction	
Gfc0825009	FfGibDP2(#8)	recovery	41.3(±0.2)	3.3(±0.5)	3.9(±0.1)	0.9(±0.1)							
	FfGibGP3(#12)	recovery	40.2(±2.8)	trace	14.8(±0.4)	5.4(±0.1)							
	FfGibTDES(#12)	recovery	3.2(±0.6)	0.6(±0.0)	0.6(±0.0)	trace	0*	3.9(±0.2)	0*	6.0(±0.2)	0*	1.1(±0.5)	
	FfGibTDES(#21)	recovery	14.2(±6.1)	2.4(±0.1)	2.4(±0.4)	0.8(±0.0)	0.0(±0.0)	4.6(±1.5)	0.0(±0.0)	11.0(±3.9)	0.0(±0.0)	11.1(±2.1)	
	FfGibTDES(#7)	nonrecovery	trace	trace	trace	trace	0.0(±0.0)	0.2(±0.1)	0.0(±0.0)	0.3(±0.1)	0.0(±0.0)	0.2(±0.1)	
	FfGibTP4504(#1)	recovery	32.9(±2.2)	3.0(±0.4)	2.7(±1.0)	1.1(±0.4)	3.0(±1.2)	16.0(±1.8)	0.1(±0.0)	10.9(±1.7)	0.1(±0.0)	5.0(±0.4)	
	FfGibTP4504(#54)	recovery	45.8(±1.2)	2.3(±0.1)	5.4(±3.2)	1.5(±0.6)	3.7(±0.9)	17.3(±3.8)	0.1(±0.0)	16.2(±2.6)	0.0(±0.0)	5.0(±0.8)	
	FfGibTP4504(#31)	nonrecovery	trace	trace	trace	trace	0.0(±0.0)	1.8(±1.3)	0.1(±0.0)	2.6(±2.0)	0.0(±0.0)	1.8(±1.1)	
	FfGibTP4501(#8)	recovery	5.5(±1.2)	0.6(±0.1)	1.2(±0.1)	0.5(±0.0)	0.1(±0.1)	6.3(±1.5)	0.8(±0.3)	53.1(±8.6)	0.0(±0.0)	2.9(±0.6)	Positive
	FfGibTP4501(#10)	recovery	2.5(±0.2)	0.6(±0.0)	0.6(±0.1)	trace	0.4(±0.0)	4.0(±1.2)	0.8(±0.1)	38.3(±8.7)	0.0(±0.0)	0.9(±0.0)	Positive
	FfGibTP4501(#4)	nonrecovery	ND	ND	trace	ND	0*	0*	0.4(±0.1)	54.8(±6.6)	0*	0.0(±0.0)	Positive
	FfGibSelfTP4501(#6)	recovery	2.1(±0.5)	ND	1.1(±0.2)	ND	0.0(±0.0)	2.7(±1.0)	0.1(±0.0)	15.9(±0.5)	0.0(±0.0)	2.6(±0.1)	Positive
	FfGibSelfTP4501(#20)	recovery	8.8(±0.3)	1.6(±0.1)	3.5(±0.6)	0.6(±0.0)	0*	3.4(±0.6)	0.1(±0.0)	8.8(±2.4)	0*	13.4(±0.3)	Positive
	FfGibSelfTP4501(#1)	nonrecovery	trace	trace	trace	ND	0.0(±0.0)	0.3(±0.1)	0.4(±0.2)	3.4(±1.1)	0*	1.1(±0.1)	Negative
	FfGibTP4502(#49)	recovery	27.4(±4.4)	1.7(±0.4)	4.2(±0.7)	2.4(±0.6)	1.7(±0.2)	5.9(±1.6)	1.0(±0.1)	5.9(±2.1)	0.8(±0.3)	3.0(±0.7)	
	FfGibTP4502(#5)	nonrecovery	trace	trace	trace	trace	0*	0.0(±0.0)	0*	0.8(±0.7)	0*	0.1(±0.1)	
SL0271	FfGibSLTP4504(#2)	recovery	>50.0	4.4(±0.2)	1.8(±0.1)	1.7(±0.1)	0.5(±0.2)	4.6(±2.6)	1.0(±0.5)	12.2(±4.7)	0.6(±0.0)	9.3(±0.8)	
	FfGibSLTP4504(#25)	recovery	>50.0	3.1(±0.3)	1.5(±0.2)	1.8(±0.1)	0.3(±0.1)	6.0(±3.6)	0.5(±0.2)	14.6(±4.9)	0.4(±0.0)	8.5(±0.3)	
	FfGibSLTP4504(#1)	nonrecovery	trace	ND	trace	ND	0.1(±0.0)	0.4(±0.2)	0*	0.1(±0.0)	0*	0.0(±0.0)	
	FfGibSLTP4501(#19)	recovery	14.1(±2.8)	0.9(±0.1)	1.5(±0.1)	1.0(±0.1)	0*	1.1(±0.0)	0.2(±0.0)	9.7(±4.6)	0.0(±0.0)	2.5(±0.4)	Positive
	FfGibSLTP4501(#20)	recovery	3.2(±0.6)	0.3(±0.0)	0.6(±0.0)	0.3(±0.1)	0*	13.6(±6.7)	0*	31.9(±22.4)	0.0(±0.0)	1.2(±0.1)	Positive
	FfGibSLTP4501(#3)	nonrecovery	trace	trace	trace	ND	0*	0*	1.1(±0.1)	6.8(±1.4)	0.0(±0.0)	0.0(±0.0)	Negative
	Gfc0801001 (Gene donor)		>50.0	6.2(±0.7)	1.9(±0.2)	1.4(±0.1)	1.0**(±0.0)	13.5(±0.4)	1.0**(±0.1)	21.6(±1.3)	1.0**(±0.1)	15.8(±1.0)	
	Gfc0825009 (Original strain)		trace	trace	trace	ND	0*	0.1(±0.1)	0.0(±0.0)	0.3(±0.2)	0*	0.2(±0.0)	
	SL0271 (Original strain)		ND	ND	trace	ND	0.0(±0.0)	0.0(±0.0)	0.0(±0.1)	0.0(±0.0)	0*	0*	

a) (#numer) means individual transformant.

b) GA_3 spot was present (recovery) or absent (nonrecovery) by TLC.

c) Mean of three replicates. Standard deviation was indicated in parenthesis. In case of lower concentration than the limit of quantification, the peak with specific MS/MS spectrum is present (trace) or absent (ND).

d) * means that all three replicates were less than detectable level. ** is used as a standard expression level.

Table 3. Pathogenicity of strains and transformants

Strains	Type	Pathogenicity[a)]
FfGibGP3(#12)	GA recovery transformants	1.0(±0.0)
FfGibTP4501(#8)		1.1(±0.1)
FfGibTP4501(#10)		0.9(±0.1)
FfGibTP4501(#4)		0.9(±0.1)
FfGibSelfTP4501(#6)		1.1(±0.0)
FfGibSelfTP4501(#20)		1.0(±0.1)
FfGibSelfTP4501(#1)		0.8(±0.0)
FfGibSLTP4501(#19)	GA non-recovery transformants	1.0(±0.0)
FfGibSLTP4501(#20)		1.0(±0.1)
FfGibSLTP4501(#3)		1.0(±0.0)
Gfc0801001	Gene donor	1.3(±0.1)*
Gfc0825009	Original Strain	0.9(±0.0)
SL0271		1.0(±0.0)

a) Mean of three replicates. Standard deviation was indicated in parenthesis. * indicates significant difference to both of original strains (Gfc0825009 and SL0271) by Dunnett test ($P<0.01$).

CHAPTER II

Bidirectional promoter region of *P450-4* and *P450-1*

Introduction

F. proliferatum, the closest relative species to *F. fujikuroi*, had been thought to be incapable of GA production for long time even though an entire GA gene cluster was detected in its genome (Malonek *et al* 2005a). According to Malonek *et al* (2005b), the lack of GA production in *F. proliferatum* strain D02945 is due to 60% lower expression of *P450-1* and a nonfunctional protein encoded by *P450-4*. Nucleotide sequences of the bidirectional promoter region between *P450-4* and *P450-1* showed 79% homology between D02945 and *F. fujikuroi* strain IMI58289 that is a GA producer (Malonek *et al* 2005b). Two double GATA motifs in this bidirectional promoter region were revealed to be essential for transcription of *P450-4* for interaction with the GATA TF AreA (Mihlan *et al* 2003). Mutations in the GATA motifs, especially in the double GATA motifs were identified to be associated to low expression of these GA genes in D02945 (Malonek *et al* 2005b).

In CHPATER I, compared to the G-group strain Gfc0801001, the disparate nucleotide substitutions were detected in bidirectional promoter region between *P450-4* and *P450-1* in F-group strains Gfc0825009 and SL0271 (Figure S5). Unlike condition in *F. proliferatum* strain D02945, nucleotide substitutions in Gfc0825009 and SL0271 were not located in GATA motifs (Figure S5). Combined with the result that low- or non-expression of *P450-4* and *P450-1* in Gfc0825009 and SL0271 (Table 2), additional ten strains of G-group and F-group in *F. fujikuroi* were used to investigate if the expression difference of *P450-4* and *P450-1* is general between G-group and F-group strains and genetic cause of the difference.

Materials and methods

Fungal strains

Strains randomly selected from Suga *et al* (2019) were used (Table 4); eleven and thirteen strains belong to G- and F-group, respectively.

Culture conditions

Culturing for RT-qPCR and GA production assay was performed with three replicates as the descriptions in CHAPTER I.

DNA and RNA extraction

The DNA and RNA extraction were performed as the descriptions in CHAPTER I.

Sequencing

The bidirectional promoter region between *P450-4* and *P450-1* was amplified by PCR with KOD-Plus-Neo DNA polymerase (Toyobo, Tokyo, Japan) and primers P4-Fus1 and P1-Fus2 (Table S2) according to the description in CHAPTER I. The parameters were as following: 94℃ for 2 min, 35 cycles of 98℃ for 10 s, 55℃ for 30 s, and 68℃ for 10 min. PCR products were directly sequenced by P4-Fus1 and P1-Fus2 according to the description in CHAPTER I. Sequence data was processed with Chromas Pro (Techelysium Pty., Tewantin, Queensland, Australia) and Genetyx (Genetyx, Tokyo, Japan).

RT-qPCR

RT-qPCR for *P450-4* and *P450-1* were performed according to the description in CHAPTER I. Superscript VILO cDNA synthesis kit (Invitrogen Carlsbad, CA, USA) and SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) were used. PCR amplification was performed in technical triplicates on a StepOne Plus Real-time PCR System (Life Technologies, Carlsbad, CA, USA). The ΔΔCt method was used for

quantification. The ΔCt value was obtained by subtracting the Ct value of *Histone H3* from that of *P450-1* or *P450-4*. The ΔΔCt value was obtained by subtracting the ΔCt mean value of GA pre-induction of Gfc0801001 (one of the three biological replicates) from that in each respective sample. RNA from three independent cultures (biological replicates) for each strain was used and their mean $2^{-\Delta\Delta Ct}$ values were obtained.

Gibberellin analysis

TLC and UPLC-MS/MS were used to assay GA production of G- and F-group strains, performing as the descriptions in CHAPTER I.

Results

Sequence of the bidirectional promoter region of *P450-4* and *P450-1*

In CHAPTER I, two F-group strains (Gfc0825009 and SL0271) were revealed to have nucleotide substitutions in the bidirectional promoter region between *P450-4* and *P450-1* to that in G-group strain Gfc0801001 (Figure S5). In order to further compare the promoter region, sequences in additional 10 G-group strains and 11 F-group strains were obtained. Ten G-group strains showed identical sequence with Gfc0801001 and it was defined as G1 type. Whereas, 7 types of sequence (F1~F7) were detected in 13 F-group strains including previously sequenced Gfc0825009 and SL0271 (Table 4, Figure 5). It was detected that one substitution in F3 and F4, two substitutions in F2 and F5, four substitutions in F1 and F7, and eight substitutions in F6 compared to G1. Some substitutions were shared with several types; A297G in F1 and F6, C363T in F2, F3 and F7, A387G in F2 and F5, G474T, T515G and C820A in F1 and F7 (Figure 5). None of the substitutions was present in GATA motifs shown in Malonek *et al* (2005b) (Figure 5).

Gene expression and GA production

To investigate if the substitutions in the bidirectional promoter associate with gene expression of *P450-4* and *P450-1*, we selected 3 G-group strains and one strain of each 7 F-type, respectively (Table 4) to assay GA production and gene expression. The concentration of GA_1, GA_3, GA_4 and GA_7 in culture filtrate of GA post-induction was confirmed by UPLC-MS/MS. In G-group strains, amount of GA_3, GA_4, and GA_7 reached an obvious level in post-induction culture though GA_1 was lower than other GAs (Table 5). In F-group strains, GAs amounts were too low to be quantified (Table 5). Comparative expression levels of *P450-4* and *P450-1* in these G- and F-group

strains were investigated by RT-qPCR based on expression level of GA pre-induction culture in G-group strain Gfc0801001 as standard (Table 5). Expression increase of *P450-4* and *P450-1* was detected in all G-group strains after GA post-induction (Table 5). While, expression of *P450-4* and *P450-1* in all F-group strains were under detectable level or at very low level even in post-induction culture (Table 5).

Discussion

In this CHAPTER, various nucleotide substitutions compared to G-group strains were revealed in the bidirectional promoter region between *P450-4* and *P450-1* in F-group strains. In G-group strains, nucleotide sequence of this region was identical. In addition, GA production and high expression of *P450-4* and *P450-1* in GA post-induction culture were detected in G-group strains. Whereas GA production and expression of *P450-4* and *P450-1* was very low or even not able to be detected in GA post-induction culture in F-group strains (Table 5).

In GA biosynthetic pathway of *F. fujikuroi*, P450-4 and P450-1 oxidize *ent*-kaurene (Tudzynski and Höler 1998). P450-1 also catalyzes production of GA_{14} (Rojas *et al* 2001). GA_{14} is the precursor of GA_4 (Figure 2). In CHAPTER I, it was detected that transferring of each *P450-1*, *P450-4*, *P450-2* or *DES* from G-group strain Gfc0801001 could increase GA production of F-group strain Gfc0825009. In this CHAPTER, it was identified that GA production and expression of *P450-4* and *P450-1* were at very low or undetectable level in GA post-induction culture of all F-group strains (Table 5). These suggest that GA low or non-production of F-group might be attributed to low or no expression of *P450-4* and *P450-1*.

The GATA type TF, AreA was proved to upregulate expression of the GA genes in *F. fujikuroi* (Marzluf 1997, Tudzynski *et al* 2014). AreA directly binds to the promoter regions among the GA genes (Mihlan *et al* 2003). Two double GATA motifs (246th and 649th in Figure 5) which are essential to expression of *P450-4* and *P450-1* are present (Mihlan *et al* 2003) in the bidirectional promoter region. Malonek *et al* (2005b) identified the mutations occurred in one of the double GATA motifs (649th in Figure 5) and also in other GATA motifs (110th, 124th, 146th, and 500th in Figure 5) in GA non-

produced *F. proliferatum* strain D02945. In this CHAPTER, none of the substitutions in the bidirectional promoter region were shared in all F-group strains and all GATA motifs were retained not only in G-group strains, but also in F-group strains (Figure 5). The results suggest that one specific mutation in the bidirectional promoter did not bring low or no expression of *P450-4* and *P450-1* in F-group strains.

Another possibility of different expression of *P450-1* and *P450-4* between G- and F-group may be attributed to histone modification. Histone regulates gene expression by changing structure of chromatin (Wiemann *et al* 2013). It was reported that H3K9Ac and H3K4me2 upregulate expression of GA biosynthetic genes in *F. fujikuroi* (Wiemann *et al* 2013). In this CHAPTER, only one substitution was detected in F3 and F4 type sequence, respectively (Figure 5). However, expression of *P450-4* and *P450-1* was very low in the strains with F3 and F4 type sequence compared to G-group strains (Table 5). Difference of H3K9Ac and H3K4me2 may be associated to expression difference of *P450-4* and *P450-1* between G- and F-group.

Suga *et al* (2019) conducted phylogenetic analyses on 95 *F. fujikuroi* strains. The results showed F-group is closer to *F. proliferatum*, which is generally GA low or nonproducer. F-group has higher gene diversity than G-group (Suga *et al* 2019) as various substitutions in the bidirectional promoter region were observed only among F-group strains (Figure 5). These results suggest that population carrying high expression type sequence established as G-group rather than that specific mutation in the bidirectional promoter region caused low expression of *P450-1* and *P450-4* in F-group.

The production of secondary metabolites may affect evolution of the fungi (Fox and Howlett 2008). In previous study, it was proved that a large amount of GA is only

produced in G-group strains (Suga *et al* 2019). While, FUM production was detected only in F-group strains (Suga *et al* 2019). FUM has antibiotic effect which is confirmed *in vitro* (Keyser *et al* 1999). The antibiotic effect of FUM producers in *F. fujikuroi* might be beneficial to compete with other microorganisms in nature. Whereas GA is not necessary for growth of *F. fujikuroi* itself but contributes to effective invasion into the rice root cells (Wiemann *et al* 2013). GA and FUM production difference are probably involved in subdivision of *F. fujikuroi* to G- and F-group.

The GA gene cluster is present or partially present in the FFSC members (Malonek *et al* 2005a). GA non-production of the FFSC members with the whole GA gene cluster is responsible to non-functional GA genes. Horizontal gene transfer (HGT) is considered as main pattern in evolution (Rosewich and Kistler 2000) and distribution of the FFSC strains. In addition, distribution of GA producibility in the FFSC is also affected by differential inheritance from a common ancestor or comprehensive effects with HGT (Malonek *et al* 2005a). The result in CHAPTER I showed that the GA gene cluster is entirely present in F-group strain Gfc0825009. In this CHAPTER, we found the diversity of the bidirectional promoter region of *P450-4* and *P450-1* in F-group.

Further studies of GA production difference between G- and F-group could reveal regulatory mechanisms of the GA gene cluster in *F. fujikuroi*.

Figure and table

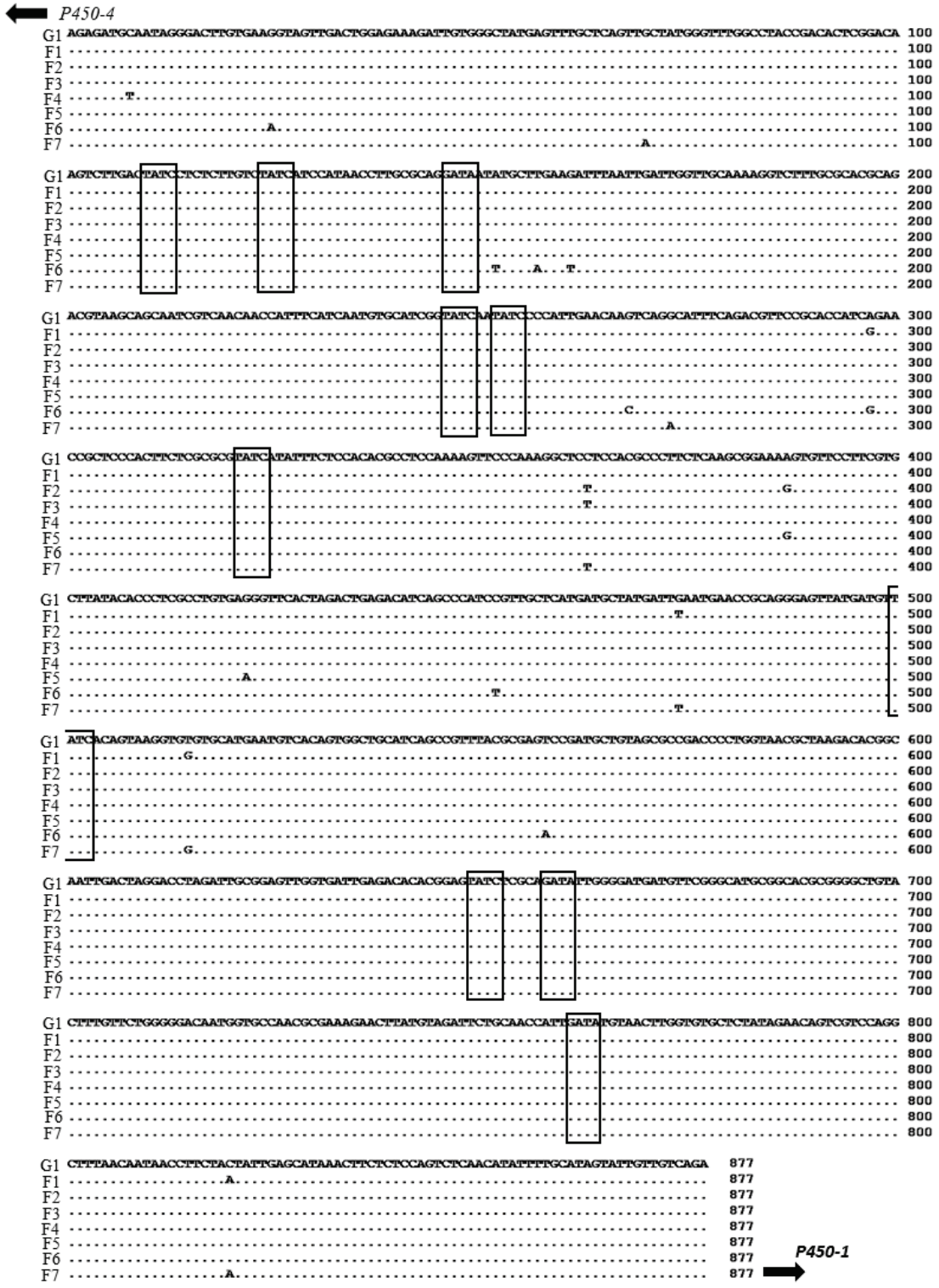

Figure 5. Sequence alignment of the bidirectional promoter region between *P450-4* and *P450-1* in G- and F-group. GATA motifs in Malonek *et al* (2005b) were indicated by boxes.

Table 4. Strains used in this CHAPTER.

Subgroup	Strain[a)]	Isolate source	Bidirectional promoter region between *P450-4* and *P450-1*		Reference
			Sequence type[b)]	Accession No.[c)]	
G-goup	Gfc0801001	*bakanae* diseased rice	G1	LC390205	Tateishi and Chida (2000)
	SMN86-2	*bakanae* diseased rice		LC521926	
	Gfc9424702	*bakanae* diseased rice		LC521927	
	Gfc0625001	*bakanae* diseased rice		LC521928	
	Gfc0625005	*bakanae* diseased rice		LC521929	
	Gfc0925005	*bakanae* diseased rice		LC521930	
	APF06083	unknown		LC521931	
	Gfc0825001	rice seed		LC521932	
	Gfc0901009	rice seed		LC521933	
	Gfc1034001	rice seed		LC521934	
	GL24	rice		LC521935	Proctor *et al* (2004)
F-group	Gfc0825009	maize	F1	LC390342	Imazaki and Kadota (2015)
	SL0271	tomato	F2	LC496066	
	MAFF235463	wheat		LC521936	
	Gfc0009063	strawberry		LC521937	
	41-79	wheat		LC521938	Busman *et al* (2012)
	Gfc0821004	rice seed	F3	LC521939	
	Gfc1004003	rice seed		LC521940	
	Gfc0921039	rice seed	F4	LC521941	
	Gfc0009105	strawberry		LC521942	
	Gfc1043032	rice seed		LC521943	
	Gfc0825007	maize	F5	LC521944	
	Gfc1019001	rice seed	F6	LC521945	
	Gfc0825011	maize	F7	LC521946	

a) All strains were used in Suga *et al* (2014) and Suga *et al* (2019), and reference indicated for strains which were not originally isolated in the previous researches.

b) Sequence of promoter in Gfc0801001, Gfc0825009 and SL0271 was obtained in CHAPTER I.

c) Accession No. in DDBJ/EMBL/GenBank database.

Table 5. Gibberellin production and *P450-1* and *P450-4* expression in G- and F-group.

Phylogenetic group	Strains	Sequence type	Gibberellin in post-induction (mg/l)[a]				Comparative expression level[b]			
							P450-4		*P450-1*	
			GA_3	GA_7	GA_4	GA_1	pre-induction	post-induction	pre-induction	post-induction
G-group	Gfc0801001	G1	>50.0	6.2(±0.7)	1.9(±0.2)	1.4(±0.1)	0.6**(±0.2)	13.5(±0.4)	1.0**(±0.0)	15.8(±1.0)
	Gfc0901009	G1	41.4(±2.0)	24.3(±5.5)	14.0(±0.0)	1.2(±0.1)	1.4(±0.5)	8.4(±5.1)	5.2(±1.3)	36.7(±7.6)
	GL24	G1	3.9 (±0.3)	1.3(±0.1)	7.3(±0.1)	0.6(±0.0)	0*	3.8(±2.3)	0.1(±0.0)	13.7(±3.2)
F-group	Gfc0825009	F1	trace	trace	trace	ND	0*	0.1(±0.1)	0.0(±0.0)	0.3(±0.2)
	SL0271	F2	ND	ND	trace	ND	0.0(±0.0)	0.0(±0.0)	0.0(±0.1)	0.0(±0.0)
	Gfc0821004	F3	ND	ND	ND	ND	0.0(±0.0)	0.0(±0.0)	0.0(±0.0)	0*
	Gfc0921039	F4	ND	trace	trace	trace	0.0(±0.0)	0.0(±0.0)	0.0(±0.0)	0.1(±0.0)
	Gfc0825007	F5	trace	trace	trace	trace	0*	0.4(±0.3)	0.0(±0.0)	0*
	Gfc1019001	F6	trace	trace	trace	trace	0.0(±0.0)	0.1(±0.0)	0.0(±0.0)	0.3(±0.0)
	Gfc0825011	F7	trace	trace	trace	trace	0.0(±0.0)	0(±0.0)	0.0(±0.0)	0.6(±0.5)

a) Mean of three replicates. Standard deviation was indicated in parenthesis.

In case of lower concentration than the limit of quantification, the peak with specific MS/MS spectrum is present (trace) or absent (ND).

b) One of the three biological replicates is used as a standard expression level (**).*means that all three replicates were less than detectable level.

SUMMARY

Rice *bakanae* is a fungal disease that results in huge yield and economical loss in rice growing regions all over the world. Diseased plants often exhibit yellowish leaves and abnormal stem elongation, especially at the seedling stage. During the crop growth, diseased seedlings die because all organs are attacked. *Fusarium fujikuroi* Nirenberg is the pathogen responsible for causing rice *bakanae* by producing gibberellins (GAs). GA is a family of diterpenoids containing 136 chemical forms whereas the most bioactive forms as plant hormone are GA_1, GA_3, GA_4 and GA_7. The main product in *F. fujikuroi* is GA_3 which is not essential for *F. fujikuroi* survival on medium or in rice, but promotes invasion of *F. fujikuroi* into cells of rice root.

Intriguingly, GA production has been proved to be diverse in *F. fujikuroi* although *F. fujikuroi* is well known as a GA producer. In addition, negative tendency of GA producibility and fumonisin (mycotoxin) producibility has been observed in *F. fujikuroi*. Phylogenetic analyses supported division of *F. fujikuroi* to gibberellin (G)-group and fumonisin (F)-group. G-group strains produced GA at high level and induced typical *bakanae* symtoms on rice. While, F-group strains produced GA at low or undetectable level and did not induce *bakanae* symptoms on rice.

In order to reveal the regulation of GA production in *F. fujikuroi*, gene diversity causing GA production difference between G- and F-group in *F. fujikuroi* was investigated in this study.

CHPATER I GA gene conplementation in F-group strains

In *F. fujikuroi*, GA biosynthesis is conferred by a gene cluster consisting of 7 adjacent genes: *P450-1*, *P450-2*, *P450-3*, *P450-4*, *DES*, *GGS2* and *CPS/KS*. Nitrogen

conditions have been shown to distinctly affect GA production in *F. fujikuroi*. GA yield is high under limited nitrogen conditions and *vice versa*. A GATA-type transcription factor (TF), AreA which mediates nitrogen metabolite repression is known to regulate GA gene expression except for *P450-3*. In addition, other TFs such as AreB, Ffsge1, and MeaB are essential to expression of the GA genes.

Linkage analysis was performed to clarify if GA production difference in G- and F-group attributes to the GA gene cluster. Forty-two progenies were obtained by crossing of G-group strain Gfc0801001 with high GA_3 production and F-group strain Gfc0825009 with low GA_3 production. GA_3 production assay and single nucleotide polymorphisms (SNP) analysis of the progenies indicated GA_3 producibility was completely linked with the SNP marker P4504_C842T located in the GA gene cluster. Reverse transcriptional-polymerase chain reaction (RT-PCR) analysis indicated all 7 GA genes were expressed in both Gfc0801001 and Gfc0825009. The nucleotide and amino acid sequences of the GA genes were compared between these strains. All the differences were substitutions and higher homology than 98.4% was observed in nucleotide and amino acid sequence of the GA genes.

We assumed that insufficient functionality of some GA genes might be the cause of GA low production in F-group strains and GA gene complementation were performed by transformation with the plasmid carrying the genes. The GA gene cluster was divided into DP2 (including *DES*, *P450-4*, *P450-1*, and *P450-2*) and GP3 region (including *GGS2*, *CPS/KS*, and *P450-3*) and complementation of each region was performed in F-group strain Gfc0825009 using G-group Gfc0801001 as a gene donor. We identified positive detection of a GA_3 spot on thin-layer chromatography (TLC) as GA production increase in transformant because the original strain did not display GA_3

spot on TLC. We assumed that either DP2 or GP3 complementation increase GA production in Gfc0825009. However, transformants with increased GA production were detected in both DP2 and GP3. GA production was detected in 14 of 32 DP2 transformants whereas in 1 of 32 GP3 transformants. Therefore, we focused on DP2 region for further analysis although contribution of GP3 region to the increase in GA production could not be excluded. Transformants of each *P450-1*, *P450-4*, *P450-2*, or *DES* were further created and the increase in GA production was detected in several transformants of each gene. These results suggested that each gene in the DP2 region of G-group Gfc0801001 could increase GA_3 production in F-group Gfc0825009. We defined transformant with positive GA_3 spot on TLC as GA recovery transformant and transformant without the spot as GA nonrecovery transformant. The GAs (GA_1, GA_3, GA_4 and GA_7) concentration and expression of the GA genes (*P450-1*, *P450-2* and *P450-4*) in GA recovery and nonrecovery transformants was investigated by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) and RT-qPCR, respectively. Obvious increase of GA_3 and slight increase of GA_7, GA_4 and GA_1 were observed in the GA recovery transformants. In GA recovery transformants of *P450-1*, expression increase of *P450-1* was observed, however, expression increase of *P450-2* and *P450-4* were also observed. Simultaneous increase in the expression of *P450-1*, *P450-4*, and *P450-2* were detected in GA recovery transformants of *P450-4*, *P450-2* and *DES*. Moreover, the same phenomenon was observed in GA recovery transformants obtained by complementation of each *P450-1* or *P450-4* in F-group strain SL0271. On the contrary, simultaneous increase of expression of the GA genes did not occur in GA nonrecovery transformants as F-group strains Gfc0825009 and SL0271. Recently, the association of histone modification and expression of the GA gene cluster

was revealed in *F. fujikuroi* and GA gene complementation might activate regulators of the GA gene cluster, such as modification of histone.

Although mechanisms of simultaneous increase of GA gene expression in GA recovery transformants remains unknown, linkage analysis and GA gene complementation analysis indicated that low expression of GA genes causes GA low production in F-group strains.

CHPATER II Bidirectional promoter region of *P450-4* and *P450-1*

In previous study, nucleotide substitutions located in GATA motifs in the bidirectional promoter region between *P450-4* and *P450-1* were determined to be associated with low expression of *P450-1* in the GA nonproducer, *F. proliferatum*. Low expression of *P450-4* and *P450-1* were observed in F-group strains Gfc0825009 and SL0271 in CHAPTER I and nucleotide substitutions compared to G-group strain Gfc0801001 were detected in the bidirectional promoter region.

In order to reveal association of nucleotide substitutions and expression level of *P450-4* and *P450-1*, sequence of the bidirectional promoter region were compared among 11 G-group strains and 13 F-group strains. The promoter sequence was identical among G-group strains while nucleotide substitutions compared to that of G-group strains were detected at various locations in F-group strains. Seven sequence types were detected in 13 F-group strains and most F-group strains did not share substitutions. GA low production and low expression of *P450-4* and *P450-1* were observed in the representative F-group strains of 7 sequence types. These results combined with the ancestral position of the F-group in the phylogenetic tree suggest that the population carrying highly expressed sequence generates the G-group rather than that the low

expression of *P450-1* and *P450-4* in the F-group is due to a specific mutation occured in G-group.

CONCLUSION

We aimed to reveal gene diversity causing GA production difference between G- and F-group in *F. fujikuroi*. GA gene cluster was retained in the F-group strains. Linkage analysis and GA gene complementation analysis indicated that GA low production in F-group strains attributes to insufficient functionality of the genes in the GA gene cluster. Expression level of *P450-4* and *P450-1* in the GA gene cluster were low in F-group strains. Nucleotide substitutions compared to G-group strains were detected at various locations in the bidirectional promoter region between *P450-4* and *P450-1* in F-group strains though low expression of *P450-4* and *P450-1* is considered not due to a particular substitution.

SUPPLEMENTARY FIGURE AND TABLE

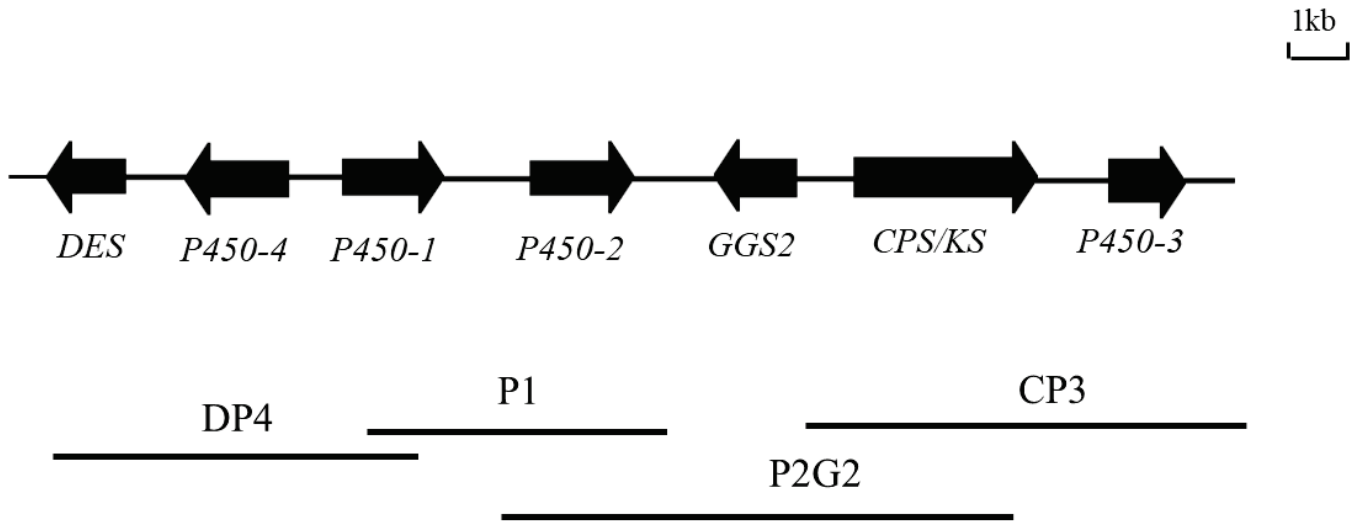

Figure S1. PCR and transformation vector region in the GA gene cluster in *F. fujikuroi*.

Amplified DNA fragments were used for sequencing. Primers for PCR amplification and sequencing were shown in Table S1 and Table S2.

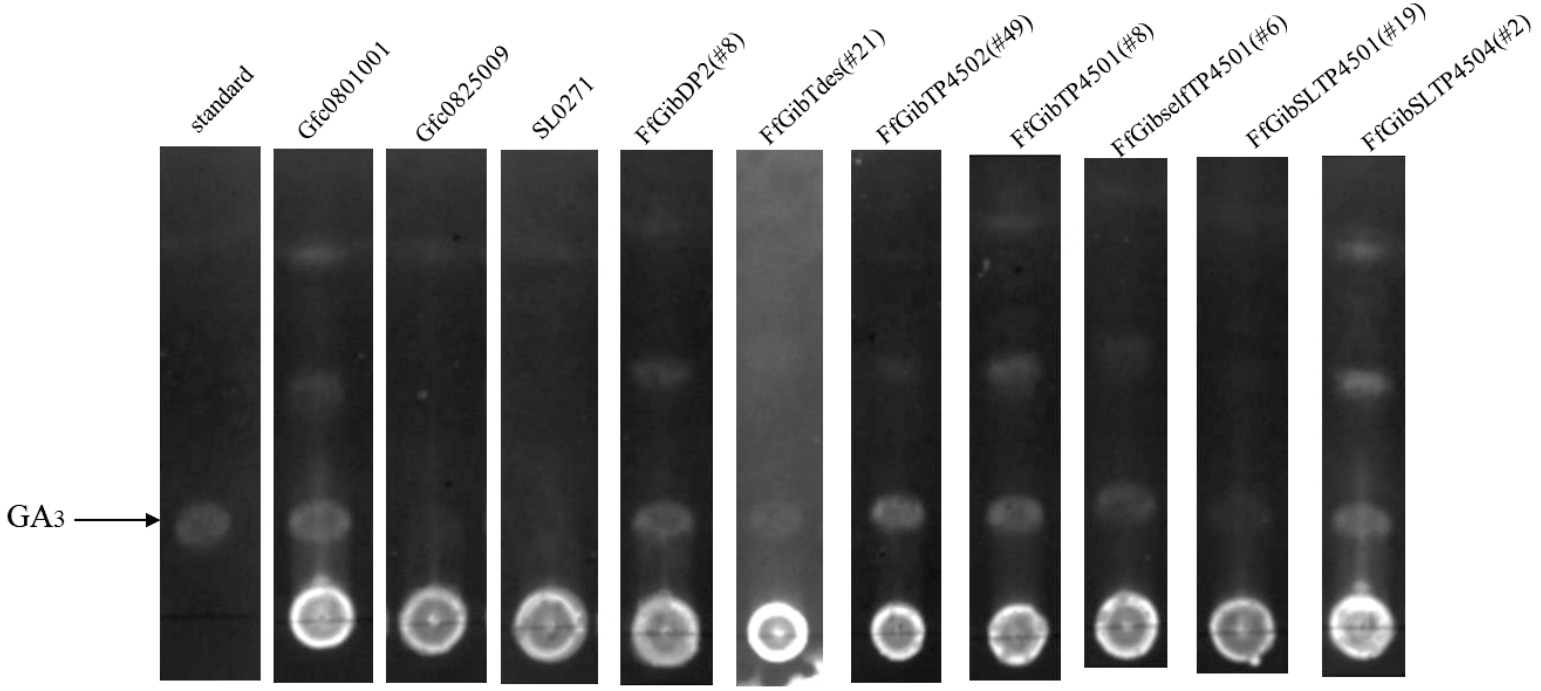

Figure S2. The TLC result of transformants

TLC was performed according to Suga *et al* (2019). Five μl GA_3 standard and 20 μl liquid culture were spotted on TLC.

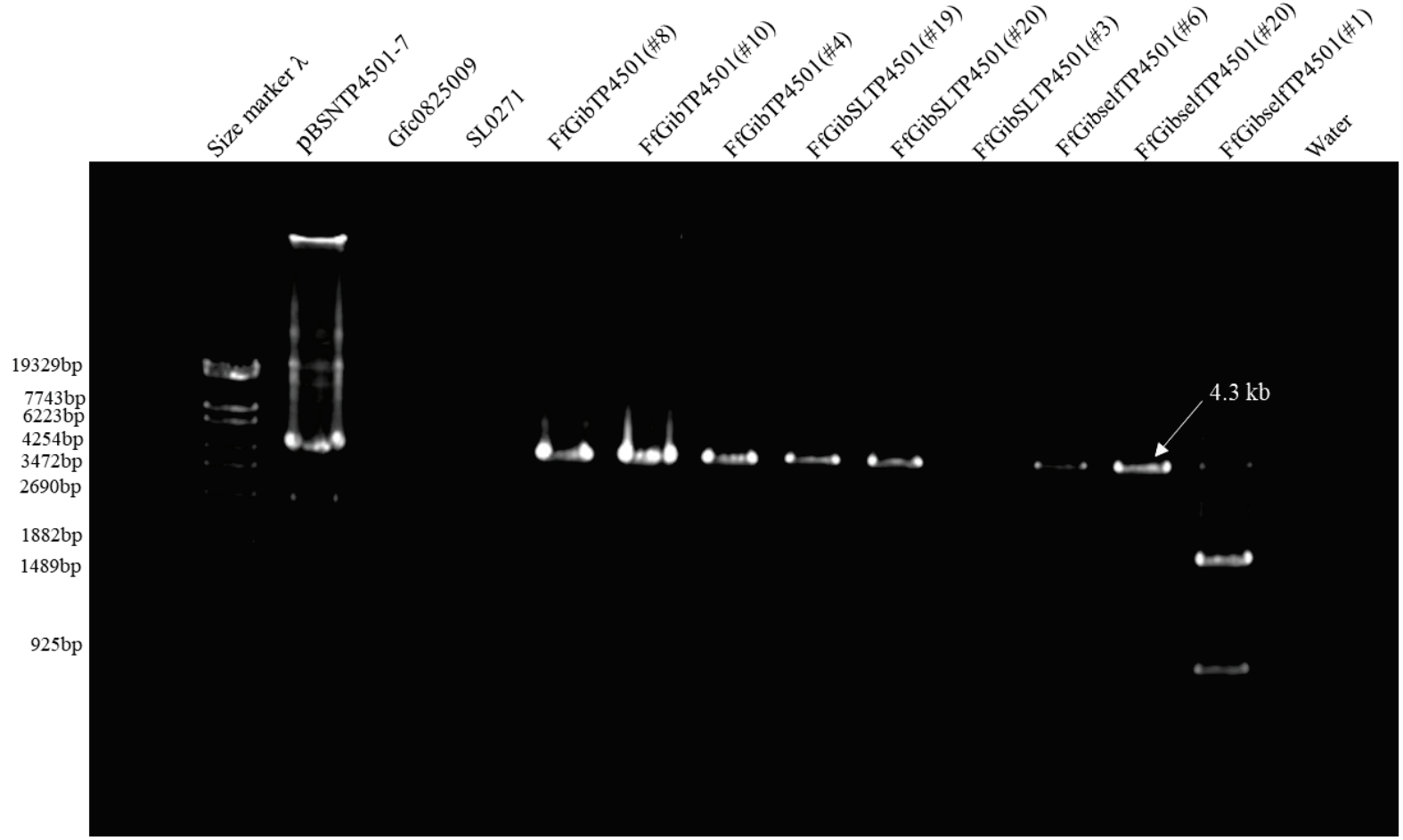

Figure S3. PCR of TP4501 full integration in *P450-1* transformants.

TP4501 (4.3kb) was amplified by PCR. pBSNTP4501-7 was the plasmid used as positive control. The original strains Gfc0825009 and SL0271, and water were used as negative control. FfGibTP4501(#4), FfGibSLTP4501(#3) and FfGibselfTP4501(#1) are GA nonrecovery transformants. Others are GA recovery transformants.

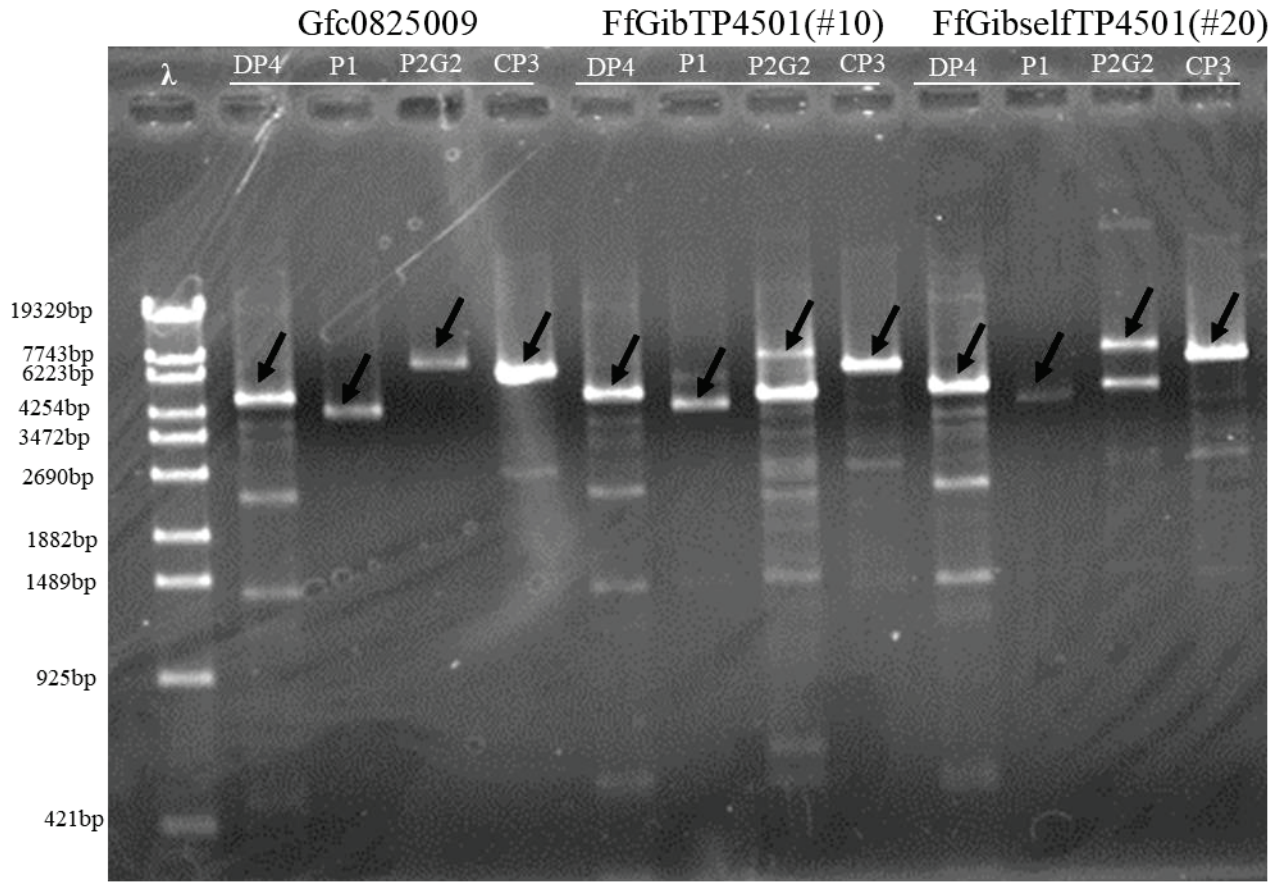

Figure S4. PCR amplification of the GA gene cluster.

λ: size marker. DP4 (4.9kbp), P1 (3.9kbp), P2G2 (7.6kbp) and CP3 (6.3kbp) in Figure S1 were amplified by PCR. F-group strain Gfc0825009 is the original strain of GA recovery transformants FfGibTP4501(#10) and FfGibTP4501(#20). The objective bands were indicated by arrows.

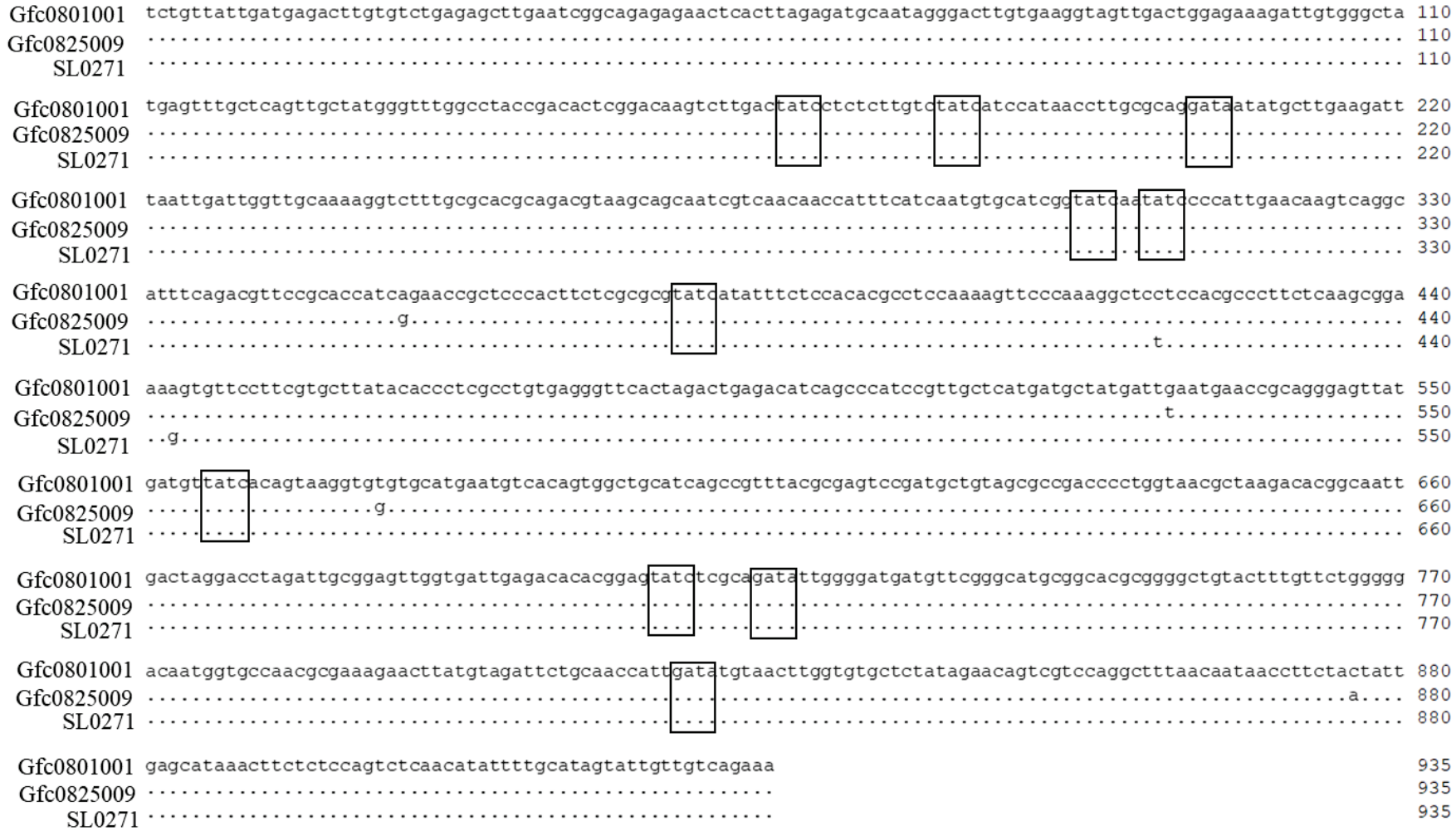

Figure S5. Sequence alignment of the bidirectional promoter region between the *P450-4* and *P450-1*

GATA motifs in Malonek *et al* (2005b) were indicated by black boxes.

Table S1. Primers for PCR of the GA gene cluster

Regions of the GA gene cluster (size)	Forward	Sequence (5'- -3')	Reverse	Sequence (5'- -3')
DP2 (10.0kb)[a)]	HS710	ATGCGGCCGCGAACGCATTTGGAATAGCGA	HS711	ATGCGGCCGCTGTAATTGCCTAATCCTC
GP3 (10.0kb)[a)]	HS712	ATGCGGCCGCTCTATAACCTTTCCCCTAA	HS713	ATGCGGCCGCTTTTGACCTCAGTGATCCTC
TDES (2.5kb)[b)]	HS752	ATGCGGCCGCCCCAGGGCGGTTCTATGC	HS710	same as above
TP4504 (4.2kb)[b)]	HS753	ATGCGGCCGCGTCAAGACGGTATGAGAGTGGTCC	HS754	ATGCGGCCGCTGCTGCCGCTGCTTGATTT
TP4501 (4.2kb)[b)]	HS755	ATGCGGCCGCGGCTGGTACTGTTCATGCTGTTGG	HS756	ATGCGGCCGCTTTTACCGGTGCCGAACAGTGA
SelfTP4501 (4.2kb)[c)]	HS755	same as above	HS756	same as above
TP4502 (5.2kb)[b)]	HS757	ATGCGGCCGCGGCGTCATCCTACCCAAGAACA	HS758	ATGCGGCCGCACAAGGTTCGATCCCCACAG

a) DP2 includes *DES*, *P450-4*, *P450-1*, and *P450-2*; GP3 includes *GGS2*, *CPS/KS* and *P450-3* in Gfc0801001 as showing in Figure 1.

b) "TXX" means "gene XX" in Gfc0801001.

c) SelfTP450-1means *P450-1* in Gfc0825009.

Table S2. Primers used for sequencing of the GA gene cluster.

Amplification fragment (size)	Primer[a)]	Sequence (5'- -3')	Reference
DP4 (4.9kb)	HS726*	ATATTATCGCATGGCATKAGRCATC	
	P1-Fus2*	CGTCAAGACGGTATGAGAGTGGTCC	Malonek *et al* 2005b
	HS766	TCTCACTCTCGGTGTCCA	
	orf3-Fus1	TGCTGCCGCTGCTTGATTT	Malonek *et al* 2005b
	HS767	CGTAGTGACACCAGCGAT	
	HS768	GAAAGCCTAAAATGTGAC	
	P450-4-GD2	CGTGGTCTTCCTTTCCCATCTGGC	Malonek *et al* 2005b
	P450-4-Fus2	CCCAGGGCGGTTCTATGC	Malonek *et al* 2005b
	HS769	ATGCGTCATCGTCATTGC	
	P450-4-GD1	TTTCTCGGTCCAGAGCACTGCCGC	Malonek *et al* 2005b
	HS770	ACTTCAACCCATTCATCA	
	HS771	GCTTACTTTGTTGTAGCC	
	P4-Fus1	GGCTGGTACTGTTCATGCTGTTGG	Malonek *et al* 2005b
P1 (3.9kb)	P4-Fus1**	GGCTGGTACTGTTCATGCTGTTGG	Malonek *et al* 2005b
	P450-2-Fus4*	TTTTACCGGTGCCGAACAGTGA	Malonek *et al* 2005b
	HS772	GGGATGATGTTCGGGCAT	
	HS773	CGAACTGTCCAACGTCCG	
	HS774	GTAGCTCAAAGTACGGGA	
	HS775	CTGTGCTTTGCTCTCTTT	
	HS776	TGCTTTTGATCTATTCTC	
	P450-1-Fus3	GGCGTCATCCTACCCAAGAACA	Malonek *et al* 2005b
	HS777	ATCTAGTGCCCCTCCATA	
P2G2 (7.6kb)	P450-1-Fus3**	GGCGTCATCCTACCCAAGAACA	Malonek *et al* 2005b
	HS723cps-Fus5*	TGGCCACCCAAGCAGTGTCATATACC	Malonek *et al* 2005b
	HS797	CTTCTTCTCTTGAACGTC	
	HS798	GCAGATGAGATTAGAAAT	
	HS780	TCGTCCAGAATCTATCT	
	HS781	ACGATGATTGACCTTGGT	
	P450-2-Fus5	GCTTCGGCCACGGTCAG	Malonek *et al* 2005b
	HS782	TTAGGCAATTACAGCTAA	
	HS783	AAACGGCAGCTTAGACTG	
	HS784	TGGTTAGCAGAGGGTCTG	
	HS785	GATATCCTGTATTTTCTC	
	HS786	CTTCCACCTGTGATTGAG	
	ggs2-Fus3	GGATACAAGAGCGAAGAATGACGTG	Malonek *et al* 2005b
CP3 (6.3kb)	ggs2-Fus3**	same as above	Malonek *et al* 2005b
	HS727*	CCACTAAAAAGCCTCSRYCAGTATT	
	HS787	CCAAAGACCTCAAGACAG	
	HS788	TACTTTCCATGCTTGAGA	
	HS789	TTGAAACAAATCGATGGTGA	
	HS799	AGCGACCATTGCGTCAAA	
	HS791	GCTACTATTGGCCACAGC	
	HS793	GATATGTATTTTCCTCTAG	
	HS792	GGCAATGGTCATCAGCAT	
	cps-Fus7	TTCGCTTTCTCCAACTGCCTAATGT	Malonek *et al* 2005b

(Continue to the next page)

	HS794	CCGATGCATGGGACCTTA	
	P450-3-Fus8	CCGAACGGACGCTGGGTAAAAA	Malonek *et al* 2005b
	HS795	CCCTTGTGTCGCGATGAA	
	HS796	CAGGTCGGGCTAGATCAA	

a) Single asterisk means primers for PCR and sequencing. Double asterisk means primers only for PCR.

Table S3. Plasmid created in the study.

Regions of the GA gene cluster (size)	Before insertion of PCR product	After insertion of PCR product	Antibiotic resistance gene	
			for *Escherichia coli*	for *Fusarium fujikuroi*
DP2 (10.0kb)[a)]	pCB1004	pCBDP2-1	CmlR	HygR
GP3 (10.0kb)[a)]	pBSNII99-3	pBSNGP3-1	AmpR	GenR
TDES (2.5kb)[b)]	pCB1004	pCBTDES-1	CmlR	HygR
TP4504 (4.2kb)[b)]	pCB1004	pCBTP4504-2	CmlR	HygR
TP4501 (4.2kb)[b)]	pBSNII99-3	pBSNTP4501-7	AmpR	GenR
SelfTP4501 (4.2kb)[c)]	pBSNII99-3	pBSNSelfTP4501-1	AmpR	GenR
TP4502 (5.2kb)[b)]	pDNAT1	pDTP4502-1	AmpR and KanR	NouR

a) DP2 includes *DES*, *P450-4*, *P450-1*, and *P450-2*; GP3 includes *GGS2*, *CPS/KS* and *P450-3* in Gfc0801001 as showing in Figure 1.

b) "TXX" means "gene XX" in Gfc0801001.

c) SelfTP450-1means the *P450-1* in Gfc0825009.

Table S4. Primers for RT-PCR of the GA gene cluster

Gene	Forward[a)]	Sequence (5'- -3')	Reverse[a)]	Sequence (5'- -3')	Size of expected amplification (bp)
DES	HS802	CTCTTCCAACCTCCCCGAT	HS714orf3-Fus1	TGCTGCCGCTGCTTGATTT	351
P450–4	HS803*	CAACCCATTCATCATTCTTCA	HS804*	CGGCCAAGGCTACAACAAATT	320
P450–1	HS805*	CATGGCAGCTTTCAAGTGGTT	HS806*	TGTCGTGCCATTCTGGACTAT	200
P450–2	HS807*	CGGTAAAACCAGGAGGAGTTT	HS808	ATGCTTGGTCAACTGCTTTCG	289
GGS2	HS809	TCACTTCCAAAACCAGCATCT	HS810*	TGGTCAATGACAAGACTGGTG	338
CPS/KS	HS811*	TAATTGGCTTTGCACCCCGTA	HS812	ACCAGAAGCATGGTTGGGTAT	334
P450–3	HS813*	ACACTACGACCATGGCCCT	HS814*	TTTCCTCCGACATGTGGATT	318
Histone H3	HS899	TCTACCGGAGGTGTCAAGAA	HS801*	AATCTCACGGACCAGACGCT	132

a) * indicates that primer sequence split over two exons.

Table S5. Primers for RT-qPCR

Gene	Forward[a)]	Sequence (5'- -3')	Reverse[a)]	Sequence (5'- -3')	Size of expected amplification (bp)
P450-4	HS901	TCAGGCATCTCTTTGGTCAA	HS804*	CGGCCAAGGCTACAACAAATT	297
P450-1	HS805*	CATGGCAGCTTTCAAGTGGTT	HS806*	TGTCGTGCCATTCTGGACTAT	200
P450-2	HS807*	CGGTAAAACCAGGAGGAGTTT	HS808	ATGCTTGGTCAACTGCTTTCG	289
Histone H3	HS800	TCTAAGGCTGCCCGCAAGT	HS801	AATCTCACGGACCAGACGCT	159

a) * indicates that primer sequence split over two exons.

Table S6. SNP analyses and gibberellin production of the progenies[a)].

Progeny	SNP data[a)]					MAT type[a)]	GA_3[b)]
	TEF_T618G	CPR_C1152A	P4504_C842T	FUM1_G423A	FUM18_G51T		
Gfc0801001 (parent)	G	A	T	A	T	1-2	+
Gfc0825009 (parent)	T	C	C	G	G	1-1	-
Gfc①CP91002	G	A	C	G	G	1-2	-
Gfc①CP91005	T	C	C	A	T	1-1	-
Gfc①CP91007	T	C	T	A	T	1-1	+
Gfc①CP91008	G	A	C	A	T	1-2	-
Gfc①CP91009	G	A	T	G	G	1-1	+
Gfc①CP91011	T	C	T	G	G	1-1	+
Gfc①CP91017	T	C	T	G	G	1-1	+
Gfc①CP91019	T	A	T	A	T	1-1	+
Gfc①CP91020	G	C	C	G	G	1-2	-
Gfc①CP91022	T	C	C	A	T	1-1	-
Gfc①CP91023	T	A	C	G	G	1-1	-
Gfc①CP91024	T	A	T	G	G	1-2	+
Gfc①CP91027	T	C	T	A	T	1-1	+
Gfc①CP91029	T	C	T	G	G	1-1	+
Gfc①CP91033	T	A	T	G	G	1-1	+
Gfc①CP91034	G	C	C	A	T	1-2	-
Gfc①CP91035	G	C	T	G	G	1-2	+
Gfc①CP91041	G	A	T	A	T	1-2	+
Gfc①CP91045	G	C	C	A	T	1-2	-
Gfc①CP91049	G	A	C	G	G	1-2	-
Gfc①CP91051	T	A	T	A	T	1-1	+
Gfc①CP91053	T	A	T	G	G	1-2	+
Gfc①CP91054	G	C	C	G	G	1-1	-
Gfc①CP91055	G	C	T	G	G	1-2	+
Gfc①CP91059	G	C	C	A	T	1-2	-
Gfc①CP91062	G	A	C	A	T	1-2	-
Gfc①CP91063	T	C	T	A	T	1-1	+
Gfc①CP91065	T	C	T	A	T	1-1	+
Gfc①CP91066	T	C	C	A	T	1-1	-
Gfc①CP91067	T	C	C	A	T	1-1	-

(Continue to the next page)

Gfc①CP91068	T	A	T	A	T	1-1	+
Gfc①CP91070	T	A	C	G	G	1-1	-
Gfc①CP91071	T	A	C	G	G	1-1	-
Gfc①CP91076	T	C	T	G	G	1-1	+
Gfc①CP91077	G	C	C	G	G	1-1	-
Gfc①CP91078	T	C	C	G	G	1-1	-
Gfc①CP91079	G	C	C	G	G	1-2	-
Gfc①CP91082	T	C	T	A	T	1-1	+
Gfc①CP91084	T	C	C	G	G	1-1	-
Gfc①CP91086	T	A	C	G	G	1-1	-
Gfc①CP91089	T	A	C	G	G	1-1	-
Gfc①CP91099	G	C	T	G	G	1-2	+

a) Data in Sulnata *et al* (2019) was used.

b) "+" means GA_3 spot was present in TLC, "-" means GA_3 spot was absent.

Table S7. Transformants created in CHPATER I.

Original strain	Plasmid used for transformation	Created transformant	Gene of Gfc0801001 integrated in the F-group strains	Antibiotic resistance of created transformant	GA recovery transformant/investigated transformants
Gfc0825009	pCBDP2-1	FfGibDP2	*DES, P450-4, P450-1, P450-2*	HygR	14/32
	pBSNGP3-1	FfGibGP3	*GGS2, CPS/KS, P450-3*	GenR	1/32
	pCBTDES-1	FfGibTDES	*DES*	HygR	4/30
	pCBTP4504-2	FfGibTP4504	*P450-4*	HygR	2/70
	pBSNTP4501-7	FfGibTP4501	*P450-1*	GenR	3/20
	pDTP4502-1	FfGibTP4502	*P450-2*	NouR	1/50
	pBSNSelfTP4501-1	FfGibSelfTP4501	*P450-1* of Gfc0825009	GenR	8/20
SL0271	pCBTP4504-2	FfGibSLTP4504	*P450-4*	HygR	2/28
	pBSNTP4501-7	FfGibSLTP4501	*P450-1*	GenR	2/32

ACKNOWLEDGEMENT

I would like to express my gratitude to professor Haruhisa Suga for professional, patient, meticulous and wholehearted instructions in my doctoral duration. I harvested the spirit of a researcher which I benefit the whole life under his influence. I am also grateful for valuable advice and instructions provided by professors Masafumi Shimizu, Takashi Ikka and Koji Kageyama in my research procedure. Thanks to my lab-mates: Ms. Sharmin Sultana, Ms. Fangjing Li, Ms. Rina Okumura, Ms. Mao Asai, Ms. Natsumi Suzuki, Ms. Maki Hanamoto, Ms. Reina Ueno, Ms. Saki Kurachi, Ms. Hina Kikuchi, Ms. Yuri Sakahara, Ms. Natsune Mokoyama, Ms. Sarara Matsumoto, Mr. Shin Inagaki and Mr. Sho Tatebayashi. I could not finish my doctor course smoothly and happily without your help. It is a treasure for me to meet all of you. Thanks to Ms. Tomomi Katsu, Ms. Ayako Usui and Ms. Shoko Wakihara for technical support. Thanks to clerks of UGSAS: Ms. Etsuko Tomoto, Ms. Asuko Takahashi, Ms. Terumi Ito, Ms. Miku Nagata, Ms. Ayano Imai and Mr. Kentaro Imai for dealing with daily affairs with patience and earnest. Finally, I am grateful to get carefulness, support and love from my partner, my friends and my family. Thanks for everything in Gifu University, it is a precious memory for me in the whole life.

REFERENCES

Abbas, H.K., Tanaka, T., Duke, S.O., Porter, J.K., Wray, E.M., Hodges, L., Sessions, A.E., Wang, E., Merril, A.H.Jr., and Riley, R.T. (1994). Fumonisin- and AAL-toxin-induced disruption of sphingolipid metabolism with accumulation of free sphingoid bases. Plant Physiol., 106, 1085-1093.

Bacon, C.W., Porter, J.K., Norred, W.P., and Leslie, J.F. (1996). Production of fusaric acid by *Fusarium* species. Appl. Environ. Microbiol., 62, 4039-4043.

Barrero, A.F., Sánchez, J.F., Oltra, J.E., Tamayo, N., Cerdá-Olmedo, E., Candau, R., and Avalos, J. (1991). Fusarin C and 8Z-fusarin C from *Gibberella fujikuroi*. Phytochemistry, 30, 2259-2263.

Bömke, C., Rojas, M.C., Hedden, P., and Tudzynski, B. (2008). Loss of gibberellin production in *Fusarium verticillioides* (*Gibberella fujikuroi* MP-A) is due to a deletion in the gibberellic acid gene cluster. Appl. Environ. Microbiol., 74, 7790-7801.

Bömke, C., and Tudzynski, B. (2009). Diversity, regulation, and evolution of the gibberellin biosynthetic pathway in fungi compared to plants and bacteria. Phytochemistry, 70, 1876-1893.

Busman, M., Desjardins, A. E., and Proctor, R. H. (2012). Analysis of fominisin contamination and presence of *Fusarium* in wheat with kernel black point disease in the

United States. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess., 29, 1092-1100.

Caroll, A.M., Sweigard, J.A., and Valent, B. (1994). Improved vectors for selecting resistance to hygromycin. Fungal Genet. Rep., 41, Article 5.

Colebrook, E.H., Thomas, S.G., Phillips, A.L., and Hedden, P. (2014). The role of gibberellin signaling in plant responses to abiotic stress. J. Exp. Biol., 217, 67-75.

Cumagun, C.J.R., Arcillas, E., and Gergon, E. (2011). UP-PCR analysis of the seedborne pathogen *Fusarium fujikuroi* causing bakanae disease in rice. Int. J. Agric. Biol. (Pakistan), 13, 1029-1032.

Finch-Savage, W.E., and Leubner-Metzger, G. (2006). Seed dormancy and the control of germination. New Phytol., 171, 501-523.

Fourie, G., van der Merwe, N.A., Wingfield, B.D., Bogale, M., Tudzynski, B., Winfield, M.J., and Steenkamp, E.T. (2013). Evidence for inter-specific recombination among the mitochondrial genomes of *Fusarium* species in the *Gibberella fujikuroi*. BMC Genomics, 14, 605.

Fox, E.M., and Howlett, B.J. (2008). Secondary metabolism: regulation and role in fungal biology. Curr. Opin. Microbiol., 11, 481-487.

García-Martínez, J., Adám, A.L., and Avalos, J. (2012). Adenylyl cyclase plays a regulatory role in development, stress resistance and secondary metabolism in *Fusarium fujikuroi*. PLoS ONE, 7, e28849.

Gupta, A.K., Solanki, I.S., Bashyal, B.M., Singh, Y., and Srivastava, K. (2015). *Bakanae* of rice-an emerging disease in Asia. J. Anim. Plant Sci., 25, 1499-1514.

Hedden, P., Phillips, A.L., Rojas, M.C., Carrera, E., and Tudzynski, B. (2001). Gibberellin biosynthesis in plants and fungi: a case of convergent evolution? J. Plant Growth Regul., 20, 319-331.

Imazaki, I., and Kadota, I. (2015). Molecular phylogeny and diversity of *Fusarium* endophytes isolated from tomato stems. FEMS Microbiol. Ecol., 91, 1-15.

Ito, S., and Kimura, J. (1931). Studies on the 'bakanae' disease of the rice plant. Res. Bull. Hokkaido Natl. Agric. Exp. Sta., 27, 1-95.

Kawaide, H. (2006). Biochemical and molecular analyses of gibberellin biosynthesis in fungi. Biosci. Biochem., 70, 583-590.

Keyser, K., Vismer, H.F., Klaasen, J.A., Snjiman, P.W., and Marasas, W.F.O. (1999). The antifungal effect of fumonisin B1 on *Fusarium* and other fungal species. S. Afr. J. Sci., 95, 455-458.

Klaasen, J.A., and Nelson, P.E. (1996). Identification of a mating population, *Gibberella nygamai* sp. nov., within the *Fusarium nygamai* anamorph. Mycologia, 88, 965-969.

Klittich, C.J.R., Leslie, J.F., Nelson, P.E., and Marasas, W.F.O. (1997). *Fusarium thapsinum*, (*Gibberella thapsina*): a new species in section *Liseola* from sorghum. Mycologia, 89, 643-652.

Leslie, J.F., and Summerell, B.A. (2006). Species concepts in *Fusarium*. *In*: The *Fusarium* Laboratory Manual. pp. 87-96. Blackwell Publishing, Ames.

Leslie, J. F., Zeller, K. A., Logrieco, A., Mule, G., Moretti, A., and Ritieni, A. (2004). Species diversity of and toxin production by *Gibberella fujikuroi* species complex strains isolated from native prairie grasses in Kansas. Appl. Environ. Microbiol., 70, 2254-2262.

Li, Y.H., Wu, Y.J., Wu, B., Zou, M-H., Zhang, Z., and Sun, G-M. (2011). Exogenous gibberellic acid increases the fruit weight of 'Comte de Paris' pineapple by enlarging flesh cells without negative effects on fruit quality. Acta Physiol. Plant., 33, 1715-1722.

Lima, C.S., Pfenning, L.H., Costa, S.S., Abreu, L.M., and Lesile, J.F. (2012). *Fusarium tupiense* sp. nov., a member of the *Gibberella fujikuroi* complex that causes mango malformation in Brazil. Mycologia, 104, 1408-1419.

Logrieco, A., Moretti, A., Castella, G., Kostecki, M., Golinski, P., Ritieni, A., and Chelkowski, J. (1998). Beauvericin production by *Fusarium* species. Appl. Environ. Microbiol., 64, 3084-3088.

Malonek, S., Rojas, M.C., Hedden, P., Gaskin, P., Hopkins, P., and Tudzynski, P. (2004). The NADPH-cytochrome P450 reductase gene from *Gibberella fujikuroi* is essential for gibberellin biosynthesis. J. Biol. Chem., 279, 25075-25084.

Malonek, S., Bömke, C., Bornberg-Bauer, E., Rojas, M.C., Hedden, P., Hopkins, P., and Tudzynski, B. (2005a). Distribution of gibberellin biosynthetic genes and gibberellin production in the *Gibberella fujikuroi* species complex. Phytochemistry, 66, 1296-1311.

Malonek, S., Rojas, M.C., Hedden, P., Gaskin, P., Hopkins, P., and Tudzynski, B. (2005b). Functional characterization of two cytochrome P450 monooxygenase genes, *P450-1* and *P450-4* of the gibberellic acid gene cluster in *Fusarium proliferatum* (*Gibberella fujikuroi* MP-D). Appl. Environ. Microbiol., 71, 1462-1472.

Malonek, S., Rojas, M.C., Hedden, P., Hopkins, P., and Tudzynski, B. (2005c). Restoration of gibberellin production in *Fusarium proliferatum* by functional complementation of enzymatic blocks. Appl. Environ. Microbiol., 71, 6014-6025.

Marzluf, G.A. (1997). Genetic regulation of nitrogen metabolism in the fungi. Mol. Biol. Rev., 61, 17-32.

Michielse, C.B., Pfannmüller, A., Macios, M., Rengers, P., and Dzikowska, A. (2014a). The interplay between the GATA transcription factor AreA, the global nitrogen regulator and AreB in *Fusarium fujikuroi*. Mol. Microbiol., 91, 472-493.

Michielse, C.B., Studt, L., Janevska, S., Sieber, C.M.K., Arndt, B., Espino, J.J., Humpf, H-U., Güldener, U., and Tudzynski, B. (2014b). The global regulator FfSge1 is required for expression of secondary metabolite gene clusters but not for pathogenicity in *Fusarium fujikuroi*. Environ. Microbiol., 17, 2690-2708.

Mihlan, M., Homann, V., Liu, T.W.D., and Tudzynski, B. (2003). AREA directly mediates nitrogen regulation of gibberellin biosynthesis in *Gibberella fujikuroi*, but its activity is not affected by NMR. Mol. Microbiol., 47, 975-991.

Nelson, P.E., Desjardins, A.E., and Plattner, R.D. (1993). Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry and significance. Annu. Rev. Phytopathol., 31, 233-252.

Niehaus, E-M., Kim, H-K., Münsterkötter, M., Janevska, S., Arndt, B., Kalinina, S.A., Houterman, P.M,. Ahn, II-P., Alberti, I., Tonti, S., Kim, D-W., Sieber, C.M.K., Humpf, H-U., Yun, S-H., Güldener, U., and Tudzynski, B. (2017). Comparative genomics of geographically distant *Fusarium fujikuroi* isolates revealed two distinct pathotypes correlating with secondary metabolite profiles. PLoS Pathog., 13, e1006670.

O'Donnell, K., Cigelnik, E., and Nirenberg, H.I. (1998). Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia, 90, 465-493.

Park, A.R., Son, H., Min, K., Park, J., Goo, J.H., Rhee, S., Chae, S-K., and Lee, Y-W. (2015). Autoregulation of *ZEB2* expression for 2 zearalenone production in *Fusarium graminearum*. Mol. Microbiol., 97, 942-956.

Piombo, E., Bosio, P., Acquadro, A., Abbruscato, P. and Spadaro, D. (2020). Different phenotypes, similar genomes: three newly sequenced *Fusarium fujikuroi* strains induce different symptoms in rice depending on temperature. Phytopathology, 110, 656-665.

Prado-Cabrero, A., Schaub, P., Díaz-Sánchez, V., Estrada, A.F., Al-Babili, S., and Avalos, J. (2009). Deviation of the neurosporaxanthin pathway towards beta-carotene biosynthesis in *Fusarium fujikuroi* by a point mutation in the phytoene desaturase gene. FEBS J., 276, 4582-4597.

Proctor, R.H., Plattner, R.D., Brown, D.W., Seo, J.A., and Lee, Y.W. (2004). Discontinuous distribution of fumonisin biosynthetic genes in the *Gibberella fujikuroi* species complex. Mycol. Res., 108, 815-822.

Rabie, C.J., Marasas, W.F.O., Thiel, P.G., Lubben, A., and Vleggaar, R. (1982). Moniliformin production and toxicity of different *Fusarium* species from southern Africa. Appl. Environ. Microbiol., 43, 517-521.

Rim, S-O., Lee, J-H., Choi, W-Y., Hwang, S-K., Suh, S-J., Lee, I-J., Rhee, I-K., and Kim, J-G. (2005). *Fusarium proliferatum* strain KGL0401 as a new gibberellin-producing fungus. J. Microbiol. Biotechnol., 15, 303-314.

Rodríguez-Oriz, R., Limn, M.C., and Avalos J. (2009). Regulation of carotenogenesis and secondary metabolism by nitrogen in wild-type *Fusarium fujikuroi* and carotenoid-overproducing mutants. Appl. Environ. Microbiol., 75, 405-413.

Rojas, M.C., Urrutia, O., Cruz, C., Gaksin, P., Tudzynski, B., and Hedden, P. (2001). Kaurenolides and fujenoic acids are side products of the gibberellin P450-1 monooxygenase in *Gibberella fujikuroi*. Phytochemistry, 65, 821-830.

Rosewich, U.L., and Kistler, H.C. (2000). Role of horizontal gene transfer in the evolution of fungi. Annu. Rev. Phytopathol., 38, 325-363.

Seto, F. (1928). Studies on the “bakanae” disease of the rice plant: A consideration of the occurrence of the “bakanae” disease and the “bakanae” phenomenon. Jpn. J. Phytopathol., 2, 118-139.

Sharma, R.R., and Singh, R. (2009). Gibberellic acid influences the production of malformed and button berries, and fruit yield and quality in strawberry (*Fragaria* × *ananassa* Duch.). Sci. Hortic., 119, 430-433.

Sponsel, V.M., and Hedden, P. (2004). Gibberellin biosynthesis and inactivation. *In*: Plant hormones, Biosynthesis, Signal transduction, Action! Davis, P.J. [ed], pp. 63-94. Kluwer Academic Publishers. Dordrecht.

Studt, L., Humpf, H-U., and Tudzynski, B. (2012a). Signaling governed by G proteins and cAMP is crucial for growth, secondary metabolism and sexual development in *Fusarium fujikuroi*. PLoS ONE, 8, e58185.

Studt, L., Wiemann, P., Kleigrewe, K., Humpf, H-U., and Tudzynski, B. (2012b). Biosynthesis of fusarubins accounts for pigmentation of *Fusarium fujikuroi* perithecia. Appl. Environ. Microbiol., 78, 4468-4480.

Suga, H., Arai, M., Fukasawa, E., Motohashi, K., Nakagawa, H., Tateishi, H., Fuji, S., Shimizu, M., Kageyama, K., and Hyakumachi, M. (2019). Genetic differentiation associated with fumonisin and gibberellin production in Japanese *Fusarium fujikuroi*. Appl. Environ. Microbiol., 85, e02414-18.

Suga, H., Kitajima, M., Nagumo, R., Tsukiboshi, T., Ugeki, R., Nakajima, T., Kushiro, M., Nakagawa, H., Shimizu, M., Kageyama, K., and Hyakumachi M. (2014). A single nucleotide polymorphism in the translation elongation factor 1α gene correlates with ability to produce fumonisin in Japanese *Fusarium fujikuroi*. Fungal Biol., 118, 402-412.

Suga, H., Karugia, G.W., Ward, T., Gale, L.R., Tomimura, K., Nakajima, T., Miyasaka, A., Koizumi, S., Kageyama, K., and Hyakumachi, M. (2008). Molecular characterization of the *Fusarium graminearum* species complex in Japan. Phytopathology, 98, 159-166.

Sultana, S., Kitajima, M., Kobayshi, H., Nakagawa, H., Shimikzu, M., Kageyama, K., and Suga H. (2019). A natural variation of fumonisin gene cluster associated with fumonisin production difference in *Fusarium fujikuroi*. Toxins, 11, 200.

Tateshi, H., and Chida, T. (2000). Sensitivity of *Fusarium moniliforme* isolates to ipconazole. J. Gen. Plant Pathol., 66, 353-359.

Teichert, S., Schönig, B., Richter, S., and Tudzynski, B. (2004). Deletion of the *Gibberella fujikuroi* glumine synthetase gene has significant impact on transcriptional control of primary and secondary metabolism. Mol. Microbiol., 53, 1661-1675.

Troncoso, C., González, X., Bömke, C., Tudzynski, B., Gong, F., Hedden, P., and Rojas, M.C. (2010). Gibberellin biosynthesis and gibberellin oxidase activities in *Fusarium sacchari*, *Fusarium konzum* and *Fusarium subglutinans* strains. Phytochemistry, 71, 1322-1331.

Tsavkelova, E.A., Bömke, C., Netrusov, A.I., Weiner, J., and Tudzynsk,i B. (2008). Production of gibberellic acids by an orchid-associated *Fusarium proliferatum* strain. Fungal Genet. Biol., 45, 1393-1403.

Tudzynski, B. (2014). Nitrogen regulation of fungal secondary metabolism in fungi. Front. Microbiol., 5, 656.

Tudzynski, B., and Höler, K. (1998). The gibberellin biosynthetic pathway in *Gibberella fujikuroi* evidence or a gene cluster. Fungal Genet. Biol., 25, 157-170.

Tudzynski, B., Mihlan, M., Rojas, M.C., Linnemannstöns, P., Gaskin, P., and Hedden, P. (2003). Characterization of the final two genes of the gibberellin biosynthesis gene cluster of *Gibberella fujikuroi*. J. Biol. Chem., 278, 28635-28643.

Tudzynski, B., Rojas, M.C., Gaksin, P., and Hedden, P. (2002). The gibberellin 20-oxidase of *Gibberella fujikuroi* is a multifunctional monooxygenase. J. Biol. Chem., 277, 21246-21253.

Wagner, D., Schmeinck, A., Mos, M., Morozov, I.Y., Caddick, M.X., and Tudzynski, B. (2010). The bZIP transcription factor MeaB mediates nitrogen metabolite repression at specific loci. Eukaryotic Cell, 9, 1588-1601.

Wiemann, P., Brown, D.W., Kleigrewe, K., Bok, J.W., Keller, N.P., Humpf, H-U. and Tudzynski, B. (2010). FfVel1 and FfLae1, components of avelvetlike complex in *Fusarium fujikuroi*, affect differentiation, secondary metabolism and virulence. Mol. Microbiol., 77, 972-994.

Wiemann, P., Seiber, C.M.K., von Bargen, K., Studt, L., Niehaus, E-M., Espino, J.J., Huß, K., Michielse, C.B., Albermann, S., Wagner, D., Bergner, S.V., and Connolly, L.R. (2013). Deciphering the cryptic genome: genome-wide analyses of rice pathogen *Fusarium fujikuroi* reveal complex regulation of secondary metabolism and novel metabolites. PLoS Pathog., 9, e1003475.

Wiemann, P., Willmann, A., Straeten, M., Kleigrewe, K., Beyer, M., Humpf, H-U., and Tudzynski, B. (2009). Biosynthesis of the red pigments bikaverin in *Fusarium fujikuroi*: genes, their function and regulation. Mol. Microbiol., 72, 931-946.

Wulff, E.G., Sørensen, J.L., Lübeck, M., Nielsen, K.F., Thrane, U., and Trop, J. (2010). *Fusarium* spp. associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxigenicity. Environ. Microbiol., 12, 649-657.

Yoshizawa, T., Yamasaki, M., Nanba, N., Yamashita, A., Ueda, S., and Shibata, H. (1994). Fumonisin producibility of fungicide resistant *Gibberella fujikuroi*, a pathogen of Bakanae disease. Mycotoxins, 40, 33-37.

Zainudin, N.A.I.M., Razak, A.A., and Salleh, B. (2008). Secondary metabolite profiles and mating populations of *Fusarium* species in section Liseola associated with bakanae disease of rice. Malays. J. Microbiol., 4, 6-13.