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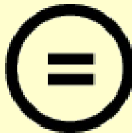

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THESIS FOR DEGREE OF MASTER OF SCIENCE

Regulatory roles of *OsGATA16* in chloroplast development in rice

벼 엽록체 발달에 관여하는 *OsGATA16*의 조절 기작 규명

BY

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FEBRUARY, 2022

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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UNDER THE DIRECTION OF DR. NAM-CHON PAEK
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
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Regulatory roles of *OsGATA16* in chloroplast development in rice

YOUNGOH KIM

ABSTRACT

Chloroplast is an important organ that performs photosynthesis, which is important for the growth and development of plants. Since photosynthetic efficiency greatly affects plant productivity, understanding the underlying regulatory mechanisms in rice plants can be useful for strategies to increase grain and biomass yields. In Arabidopsis thaliana, GNC (GATA, NITRATE INDUCIBLE, CARBON-METABOLISM INVOLVED) and GNL/CGA1 (GNC-LIKE/CYTOKININ-RESPON SIVE GATA FACTOR1) have been well documented for the direct, positive regulator of chlorophyll and chloroplast biogenesis and are candidates for master controllers for chloroplast development. However, in rice, the regulatory roles of *OsGATA16*, orthologous gene of Arabidopsis *GNC*, are largely unknown. Here, we show that the T-DNA insertion knockdown *osgata16* mutant show a pale-green phenotype and *OsGATA16* overexpression lines have dark-green phenotype and accumulate chlorophyll also in tissues such as mid vein, root, callus and lamina joint, where chlorophyll does not visibly accumulate in the wild type. To identify the molecular mechanism, we performed RT-qPCR analysis, revealing that most of chlorophyll biosynthesis and photosynthesis genes are down-regulated in osgata16 mutant and up-regulated when *OsGATA16* was transiently expressed in protoplasts. Using luciferase assays and Chromatin immunoprecipitation (ChIP) assays, we found that OsGATA16 bound to the promoters of two chlorophyll biosynthetic genes, *OsPORA* and *OsPORB*, and promotes their transcription. In addition, we also found by transmission electron microscopy analysis that overexpression of *OsGATA16* enhanced chloroplast development in vascular bundle sheath cells and it was a first step toward engineering C4 rice. Taken

together, our results demonstrate that like Arabidopsis *GNC*, *OsGATA16* functions to promote chlorophyll synthesis and chloroplast development.

Keywords: rice, *OsGATA16*, transcription factor, Chloroplast, Chlorophyll, *OsPORA, OsPORB*, TEM, BS, C4

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ABBREVIATION

WT	Wild type
Chl	Chlorophyll
Car	Carotenoid
ABA	Abscisic acid
ACC	1-aminocyclo-propane-1-carboxylic acid
MeJA	Methyl jasmonate
GA	Gibberellic acid
BA	6-benzyladenine
BS	Bundle sheath

INTRODUCTION

Chloroplast, which accumulates chlorophyll (Chl) and develop photosynthetic machinery in thylakoid membranes, is one of the most essential organelles, since the growth of sessile plants largely depends on the chemical energy produced by photosynthesis in the chloroplast. Chlorophyll and its intermediates are strong photosensitizers, plant cells must strictly regulate their metabolism to coincide with the construction of the photosynthetic machinery [1]. Indeed, the expression profiles of genes involved in Chl biosynthesis are highly co-expressed with photosynthetic proteins suggests that there is a central transcriptional regulation system governing Chl metabolism, as well as photosynthetic proteins [2]. In addition, based on the profoundly different phenotypes found in chloroplasts compared to proplastids and plastids from non-photosynthetic tissues, the existence of chloroplast-specific “master controllers” has been proposed [3]. Transcription factors known as positive regulators of chloroplast differentiation include LONG-HYPOCOTYL5 (HY5), GOLDEN2-LIKE (GLK), and class B GATA transcription factors (B-GATAs), including GATA, NITRATE-INDUCIBLE CARBON METABOLISM INVOLVED (GNC) and GNC-LIKE/CYTOKININ RESPONSIVE GATA1 (GNL/CGA1) have been suggested to candidates for such master controllers in Arabidopsis (Arabidopsis thaliana) [4].

In rice, a major cereal crop worldwide, the transcription factor

associated with chloroplast development have also been studied. For example, *OsbZIP48*, one of the three *AtHY5*, could functionally complement the *hy5* Mutant [5]. The overexpression of *OsGLK1* induced chloroplast development in vascular bundle (VB) and vascular bundle sheath (VBS) cells of young leaves and was synchronized with greening of calli [6]. Phytochrome-Interacting Factor-Like1 (OsPIL1), a basic helix-loop-helix transcription factor, directly up-regulates the expression of two transcription factor genes, *OsGLK1* and *OsGLK2* [7]. *OsGATA11*, orthologous to Arabi *CGA1*, regulates chloroplast development and plant architecture; Strong overexpression of *OsGATA11* produces dark green, semidwarf plants with reduced tillering, whereas RNA interference knockdown results in reduced chlorophyll and increased tillering [8]. However, it is still unknown whether *OsGATA16*, orthologous to Arabidopsis *GNC*, is involved in regulation of chloroplast development and up-regulates the expression of genes for photosynthetic apparatus components and Chl biosynthesis. Even the Populus *GNC* overexpression plants exhibited darker green leaves and faster growth than the control wild type [9].

The study of chloroplast in rice is important for increasing the grain yields by improving the efficiency of photosynthesis and can be used as an early marker for efficient identification and elimination of false hybrids in commercial hybrid rice production [10-12]. In addition, the installation of a C4 photosynthetic system into rice has the theoretical potential to double current rice yields [13] and one of the first steps of toward engineering C4 rice is to

induce proto-Kranz by activating chloroplast and mitochondrial biogenesis in vascular sheath cells [14]. Therefore, to boost rice chloroplast development and photosynthesis, increase in bundle sheath cells chloroplast size, photorespiratory bypasses and C4 cycle enzymes have been introduced into rice chloroplast. For example, four of the maize core C4 cycle enzymes required for a functional NADP-ME C4 cycle, ZmPEPC, ZmNADP-MDH, ZmNADP-ME, and ZmPPDK, introduced into the rice [15]. The photorespiratory bypasses, as opposed to previous attempts that sought to suppress photorespiration, sought to metabolize the glycolate produced by ribulose-1,5-bisphosphate (RuBP) oxygenation while minimizing the loss of carbon, nitrogen, and energy, and avoiding the accumulation of photorespiratory intermediate. Bypass engineered plants showed increased photosynthesis and biomass yield and reduced photorespiration [16]. Overexpressing maize ZmGLK1 and ZmGLK2 in rice, with constitutive promoters or maize promoters, induced chloroplast development in both mesophyll cell and bundle sheath cells and improved rice photosynthetic performance and productivity [17-19].

GATA transcription factors, as the name suggest, constitute a family of transcription factors that recognize a target site conforming to the consensus WGATAR (W = A or T and R = A or G). In rice, 28 gene loci encoding GATA proteins have been reported [20]. In rice, GATA factors function in various processes. For example, *OsGATA15* is an important regulator of leaf development as suggest by OsGATA15 disruption led to

development of short and narrow leave [21]. Rice seedlings overexpressing *OsGATA8* showed an improved phenotype under salinity stress in terms of higher biomass and higher photosynthetic efficiency [22]. Overexpression of *OsGATA12* regulates chlorophyll content, delays plant senescence [23].

In this study, we found that OsGATA16 is a positive regulator for Chloroplast development. The T-DNA insertion *osgata16* knockdown mutant exhibited a pale-green leaf phenotype, with significantly reduced levels of chlorophyll content compared to the wild-type. However, *OsGATA16*-Overexpressing transgenic rice plants exhibit dark green leaf phenotype. Using RT-qPCR analysis, we confirmed that the genes for Chl biosynthetic enzymes and the photosynthetic apparatus were significantly down-regulated in *osgata16* mutants and up-regulated when *OsGATA16* was transiently expressed in protoplasts. Furthermore, Luciferase assay and Chromatin Immunoprecipitation (ChIP) assays revealed that OsGATA16 binds to the promoters of *OsPORA* and *OsPORB* and up-regulates their expression.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Phytohormone

The T-DNA insertion mutant *osgata16* (PFG_3A-06147) was obtained from Crop Biotech Institute at Kyung Hee University, Republic of Korea [24]. The Oryza sativa japonica cultivar 'Dongjin' (parental line) and the *osgata16* mutant were cultivated in a paddy field under natural long day (NLD) conditions (>14 h sunlight/day, 37°N latitude) in Suwon, Republic of Korea. The germinated rice seedlings were transplanted in a paddy soil and grown in a growth chamber under long day (LD) conditions (14.5 h light/9.5 h dark, 37°N latitude) in Seoul, Republic of Korea. For phytohormone treatments, the sterilized seeds were germinated on half-strength Murashige and Skoog (1/2 MS) solid medium under continuous light (90 μmol m-2 s-1) at 30°C. The 10-day-old seedlings were transferred to 1/2 MS liquid medium containing 100 μM 1-aminocyclo-propane-1-carboxylic acid (ACC), 100 μM MeJA, 100 μM ABA, 100μM GA and 100μM BA. Rice seedlings incubated in 1/2 MS liquid medium without additional phytohormones were used as a mock control.

Determination of Total Chlorophyll and carotenoid

To measure the total chlorophyll and carotenoid were extracted from

leaves tissues using 80% ice-cold ace. The extracts were centrifuged at 10,000 g for 10 min at 10°C and then the absorbance of the supernatants was measured at 647 and 663 nm using an UV/VIS spectrophotometer (BioTek). The concentration of chlorophyll was calculated as previously described [25].

Measurements of agronomic traits

To measure the agronomic traits of the WT and *osgata16*, the plants that were grown in a paddy field under NLD conditions (>14-h sunlight/day in Suwon, South Korea, 37°N latitude). The yield components of eight plants per each line were measured.

Subcellular localization of OsGATA16

To determine the localization of OsGATA16, the full-length *OsGATA16* cDNA was cloned into the pGA3651 vector containing the superfolder green fluorescent protein (*sGFP*) gene. The plasmids were transformed into rice protoplasts isolated from rice suspension cultured cells [26] using PEG-mediated protoplast transformation [27]. Protoplasts were then transferred into multi-well plates and cultured in the dark at room temperature for 6–16 h. After incubation, green fluorescence signals from transfected protoplasts were observed using a Carl Zeiss Axioskop 2 confocal microscope and the image acquisition software ZEN blue edition (Carl Zeiss, Oberkochen, Germany). A sGFP fluorescence was detected with 488 nm

excitation and 505–530 nm emission wavelengths.

Transmission Electron Microscopy

To perform transmission electron microscopy, a previously described method [28] was used, with some modifications. The middle part of the second leaves of fully expanded plants were used for this experiment. Small leaf pieces were fixed with modified Karnovsky fixative (2 % paraformaldehyde, 2 % glutaraldehyde, and 50 mM sodium cacodylate buffer, pH 7.2). After fixation, samples were washed with 0.05 M sodium cacodylate buffer, pH 7.2 at 4 ℃, three times for 10 min each. The samples were post-fixed with 1 % osmium tetroxide in 50 mM sodium cacodylate buffer, pH 7.2, at 4 ℃ for 2 h and washed twice with distilled water at room temperature. Samples were stained en bloc in 0.5 % uranyl acetate at 4 ℃ overnight and dehydrated in an ethanol gradient solution with propylene oxide, then infiltrate with Spurr's resin. Samples were polymerized at 70 ℃ for 24 h and sectioned with an Ultramicrotome (MT-X). The sections were mounted on copper grids and stained with 2 % uranyl acetate for 7 min and with Reynolds′ lead citrate for 7 min. Micrographs were made using a LIBRA 120 transmission electron microscope.

Reverse Transcription and Quantitative Real-Time PCR (RT-qPCR) Analysis

Total RNA was extracted from rice leaf tissues or transfected protoplast with the MG Total RNA Extraction Kit (Macrogen, Korea). First-strand cDNA was synthesized with 2 μg of total RNA in a 25 μl volume using M-MLV reverse transcriptase and oligo(dT)15 primer (Promega), and diluted with 75 μl water. The 20 μl of qPCR mixture was prepared including 2 μl of the first-strand cDNA mixture, 10 ul of 2X GoTaq PCR Mix (Roche), and 1 μl of 10pM qRT primer pairs. qPCR was conducted on the LightCycler 2.0 instrument (Roche Diagnostics). The qPCR conditions were 95 ℃ for 2 min, followed by 50 cycles at 95 ℃ for 5 s, 59 ℃ for 15 s, and 72 ℃ for 10 s. *OsGAPDH* (glyceraldehyde-3-phosphate dehydrogenase; Os04g0486600) was used as an internal control. Primers used for qRT-PCR analysis are listed in Supplemental Table 1.

Plasmid Construction and Transformation

A full-length cDNA of *OsGATA16* was ligated into the pMDC32 gateway binary vector containing the 35S promoter and then *35S:OsGATA16* were introduced into calli generated from the mature embryos of Dongjin seeds by

Agrobacterium (strain LBA4404)-mediated transformation method [29]. Agrobacterium-infected calli were transferred to solid media containing cytokinin and auxin and plantlets were regenerated from the calli grown under continuous light condition. Transgenic plants overexpressing *OsGATA16* were confirmed by qRT-PCR using the specific primers listed in Supplemental Table 1.

Y1H assays

The *OsGATA16* coding sequence was subcloned into the EcoRI and XhoI sites of the pGADT7 vector (Clontech) as prey. To determine whether OsGATA16 interacted with the GATA or GATC motif, the construct was cloned into the pLacZi vector (Clontech) as baits. The resulting plasmids or empty vectors were transformed into yeast strain YM4271 using the PEG/LiAc method, and yeast cells were incubated on SD/–Ura/–Leu liquid medium. β-Galactosidase activity was determined based on the absorbance of chloramphenicol red, a hydrolysis product of chlorophenol red-β-D-galactopyranoside, at 595 nm using a ultra violet-visible spectroscopy (UV/VIS) spectrophotometer (BioTek Instruments, USA) according to the Yeast Protocol Handbook (Clontech).

Luciferase assays in protoplasts

To construct the plasmids containing the LUC reporter gene under the

control of various promoters, a fragment of the *OsPORA* and *OsPORB* promoter region (up to -2kb) was cloned into the pJD301 vector, which contains the LUC reporter gene at the C-terminus. For the effector plasmids, the cDNA of *OsGATA16* was cloned upstream of a sequence encoding one copy of a GFP epitope tag in the pGA3651 vector[30]. The reporter (2 μg), effector plasmids (4 μg), and internal control (1 μg) plasmids were co-transfected into 5 × 10^4 rice protoplasts by PEG-mediated transfection[27]. Transfected protoplasts were then suspended in protoplast culture medium (0.4-mM mannitol, 4-mM MES buffer, and 15-mM MgCl2, pH 5.8) and kept in darkness for 12 h. The LUC activity in each cell lysate was determined using the LUC reporter assay system kit (PR-E1910, Promega, USA).

Chromatin Immunoprecipitation (ChIP) Assays

For the ChIP assay, the *Ubi : OsGATA16-Myc* and *Ubi : Myc* were transfected into rice protoplasts [31]. Protoplasts were then subjected to cross-linking with 1% formaldehyde for 30 min under vacuum. Then, nuclei were isolated and lysed, and chromatin complexes were isolated and sonicated, as described [32]. DNA was sonicated using a BIORUPTORII (COSMO BIO). Anti-Myc monoclonal antibody (Abcam, Cambridge, UK) and protein A agarose beads (Merck Millipore) were used for immunoprecipitation. DNA recovered from agarose beads was purified using the DNeasy Plant Mini Kit (Qiagen). Quantitative PCR was performed using the KAPA SYBR FAST qPCR Kit (KAPA Biosystems) and gene-specific primer listed in Supplemental Table 1.

Table1. Information of primers used in this study.

Marker	Forward primer (5'→3')	Reverse primer(5'→3')
A. Primers for verification of transgenic plants		
PFG 3A-06147.R	AGGGAAACTGTTGGAACCG	GGCAGCAACTGCCTAGCTAC
B. Primers used for gene cloning		
OsGATA16 (Y1H)	AAAGAATTCATGTCTACCATCTACATG	AAACTCGAGTCAGCTCCGGACAAGCCCG
GATA x 7 *(Y1H)*	AATTCGATAGATAGATAGATAGATAGATAGATAC	TCGAGTATCTATCTATCTATCTATCTATCTATCG
GATC x 7 *(Y1H)*	AATTCGATCGATCGATCGATCGATCGATCGATCC	TCGAGGATCGATCGATCGATCGATCGATCGATCG
CGATAA x 7 *(Y1H)*	AATTCCGATAACGATAACGATAACGATAACGATAACGATAACGATAAC	TCGAGTTATCGTTATCGTTATCGTTATCGTTATCGTTATCGTTATCGG
TGATAA x 7 *(Y1H)*	AATTCTGATAATGATAATGATAATGATAATGATAATGATAATGATAAC	TCGAGTTATCATTATCATTATCATTATCATTATCATTATCATTATCAG
CGATCA x 7 *(Y1H)*	AATTCCGATCACGATCACGATCACGATCACGATCACGATCACGATCAC	TCGAGTGATCGTGATCGTGATCGTGATCGTGATCGTGATCGTGATCGG
TGATCA x 7 *(Y1H)*	AATTCTGATCATGATCATGATCATGATCATGATCATGATCATGATCAC	TCGAGTGATCATGATCATGATCATGATCATGATCATGATCATGATCAG
CCATCA x 7 *(Y1H)*	AATTCCCATCACCATCACCATCACCATCACCATCACCATCACCATCAC	TCGAGTGATGGTGATGGTGATGGTGATGGTGATGGTGATGGTGATGGG
CGTTCA x 7 *(Y1H)*	AATTCCGTTCACGTTCACGTTCACGTTCACGTTCACGTTCACGTTCAC	TCGAGTGAACGTGAACGTGAACGTGAACGTGAACGTGAACGTGAACGG
CGAGCA x 7 *(Y1H)*	AATTCCGAGCACGAGCACGAGCACGAGCACGAGCACGAGCACGAGCAC	TCGAGTGCTCGTGCTCGTGCTCGTGCTCGTGCTCGTGCTCGTGCTCGG
CGATCT x 7 *(Y1H)*	AATTCCGATCTCGATCTCGATCTCGATCTCGATCTCGATCTCGATCTC	TCGAGAGATCGAGATCGAGATCGAGATCGAGATCGAGATCGAGATCGG
proPORA	AAAAAGCTTCTTCGCCCATCATCTCAATTTAA	AAAGTCGACTGGTGTGAGTGTTTGAGTTTGG
proPORB	AAAAAGCTTAAGCTGGGGAAGAAGCTAGC	AAAGTCGACCGGAGATTTTTCTCTCTTTTTTTTC
C. Primers used for qRT-PCR		
OsGATA16	GAG GAT CAC GGC CAT GGC	CCG CAC GCG TTG CAA AG
OsGATA11	TGGGTGTCGTTAGAGTGTGC	CTTTGCCTGATGCCACACG
OsGATA12	GGGAGTGGGTCGATAGAT	CCTCTTGCTCTTCTTCTTCT
OsGLK1	ACACATGATTGCGAGAGAGG	GAGGAGGAGGGAAGCCAAT
OsGLK2	GCCTCTAAATCCAGCAGACG	GCATCCCAAAATCTCTCCCC
OsPIL1	ATGCCAAATCACATCCCTCTAA	GCTTGCCTCTCTCAGTTGAA
OsbZIP48	GAAGGACCTGGAGAGGAGCA	TGTCTGGCCCTCTTCTGTTTG
GS	GCCGACATCAACACCTTCAAAT	GACGACGTATGGGTCCATGTT
Fd-GOGAT	GCATACTTGTGAAGCACCGAAGTG	CTGCAAATAGCAACCTAGCGTCAG

HEMA	CGCTATTTCTGATGCTATGGGT	TCTTGGGTGATGATTGTTTGG
CHLH	AACTGGATGAGCCAGAAGAGA	AAATGCAAAAGACTTGCGACT
PORA	ATGGCTCTCCAAGTTCAGG	CTTCTGGCTCACGCTAAGGAA
PORB	AGCTCCTCGCCGACCTCA	TCCCCCAGGTTCGCCTT
CAO1	AGCCCAGGTTCATCAAGGT	TGATCACCCTCTCGAAGAACT
CAO2	ACTTTGCTCCCTGGCTCAAG	GCGCCATTTATCATCCGCTC
SIG1	ACGAGCGTGAGAGGGACATT	AGTCCTACTTGCCTCACCCT
SIG2B	GCCTCCCAAAATACACCGTT	CGTCTTCCTTCATCAGCCACC
SIG6	CTGGTCAGATGAGGGTGTCA	GATCCCCAGGAAGTCCCTCA
PsbA		
PsbR	CACCGGTGACACCTACGTT	GTGAAATTTAGCCGGCGAGG
LHCA4	CTCAACTTCGAGCCCACCCT	CTGGATGATGGTGTTGTGCCA
LHCB1A	AGTAGCTGAGCTTGAAGCAG	GAACTCGCCGGTCAGGTAG
LHCB2	CAGGTGGTGCTCATGGGATT	CCTGGGTACACCTTGTCGAG
LHCB5	AACAGCATCCCCATCAACCTC	ACCTGGGTGGAGCTTGTCCT
FtsZ	TCACTGGTGGAAAGGACATAAC	TACCGGTCATCAACAACAGC
RbcS	TCCGCTGAGTTTTGGCTATTT	GGACTTGAGCCCTGGAAGG
OsGAPDH	AAGCCAGCATCCTATGATCAGATT	CGTAACCCAGAATACCCTTGAGTTT

D. Primers used for ChIP-qPCR

PORA-a	CAAACGATAAGGACGAGTGTGC	CAGGATCTCGCTGGAAAGTCC
PORA-b	AACTGAGACTGGTGTACGCA	TCTGACCAGTAACTTGACCCA
PORA-c	CATCTTCCCACGCAAAACGAC	GGTGGGATATTTTTTCGTAACCG
PORB-a	TCGTTTTGCTTTTTGCCTACGTG	AGGACAAGCAGTAGCAGAGTAA
PORB-b	CAGGCCCATATCTCATCCGT	GGCCGGTTTTGTTTGGTCTC
GAPDH	CCTTAGAAACGGAGGGAAGGT	TTGGTAAGACGCTCATTTTGCG

RESULTS

Expression of OsGATA16 in Rice

Rice OsGATA16 (LOC_Os06g37450) consists of 1173 bp of CDS containing three exons and encoding a 390-amino acid protein belonging to the GATA transcription factor family. To investigate the expression of *OsGATA16*, we measured OsGATA16 transcript levels in various organs (panicle, leaf sheath, flag leaf, leaf blade, culm, lamina joint, internode, node, root and tiller base). We used wild-type (WT; japonica rice cultivar 'Dongjin') plants grown in a growth chamber under long-day (LD) conditions (14.5 h light/9.5 h dark) at 21 days after germination (DAG, seedling stage) or in the paddy field under natural LD (NLD) conditions (≥14 h light/day in Suwon, South Korea, 37°N latitude) at 120 DAG (heading stage). Reverse transcription and quantitative real-time PCR (qRT-PCR) analysis revealed that the *OsGATA16* transcripts were most abundant in panicle, and then (in order of decreasing abundance), in leaf sheath, flag leaf, leaf blade, culm and lamina joint (Figure 1A).

To test whether phytohormones affect the expression of *OsGATA16*, we examined the *OsGATA16* transcript levels in 10-day-old WT seedlings treated with ABA, ACC, MeJA, GA and BA for 1.5 h. qRT-PCR analysis showed that *OsGATA16* expression significantly increased in response to the Cytokinin (BA) and *OsGATA16* transcript levels were significantly reduced by

MeJA (Figure 1B). The strong induction of gene expression by cytokinin is highly similar to results in the Arabidopsis orthologous gene *GNC*, as well as the paralogous transcription factor *OsGATA11* in rice which indicates conservation between the dicot model and a monocot crop species [8, 33].

Rice GATA genes were categorized into seven and Subfamily II is the largest with nine GATA members viz. OsGATA8, OsGATA9, OsGATA10, OsGATA11, OsGATA12, OsGATA13, OsGATA14, OsGATA15, and OsGATA16 [34]. Therefore, we examined the differential expression of OsGATA16, OsGATA11 and OsGATA12, belongs to the same subfamily of GATA TF, depending on leaf age and de-etiolation. The results showed that *OsGATA16* and *OsGATA11* mRNA levels were highly expressed in younger leaves such as L5 and L4, whereas *OsGATA12* mRNA levels were highly expressed in the older leaves (L2) than younger leaves (L5) (Figure 2A-D). Next, we examined the effect of light on GATA expression during de-etiolation. *OsGATA12* was rapidly downregulated after illumination, whereas *OsGATA16* and *OsGATA11* expression drastically increased (Figure 2E-G). This result suggests that, in contrast with OsGATA12, OsGATA16 and OsGATA11 was responsible for Chl synthesis during early leaf development.

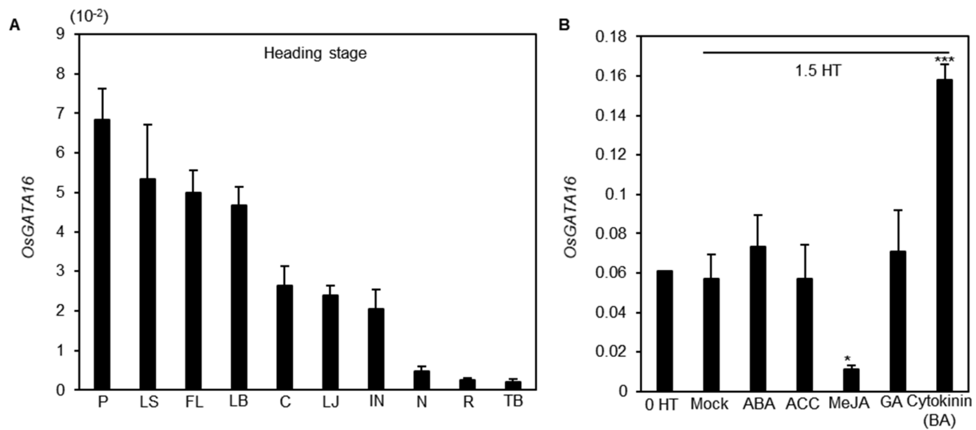

Figure 1. Expressional profile of *OsGATA16*

(A) *OsGATA16* was differentially expressed in various japonica cultivar ′Dongjin′ (hereafter wild type; WT) tissues from heading stage. P, panicle; LS, leaf sheath; FL, flag leaf; LB, leaf blade; C, culm; LJ, lamina joint; IN, inter node; N, node; R, root; TB, tiller base. (B) Expression patterns of *OsGATA16* in response to phytohormones. WT seedlings grown in half-strength Murashige and Skoog (1/2 MS) solid medium for 10 d under continuous light at 28°C were incubated in 1/2 MS liquid medium supplemented with 100 μM ABA, 100 μM ACC, 100 μM MeJA, 100μM GA and 100μM BA. Seedlings incubated in 1/2 MS liquid medium without treatment were used as a mock control. Total RNA was isolated from the leaves at 0 and 1.5 h of treatment (HT). Asterisks indicate statistically significant differences between treated samples and the mock control, as determined by Student′s t-test (**p < 0.01, ***p < 0.001).

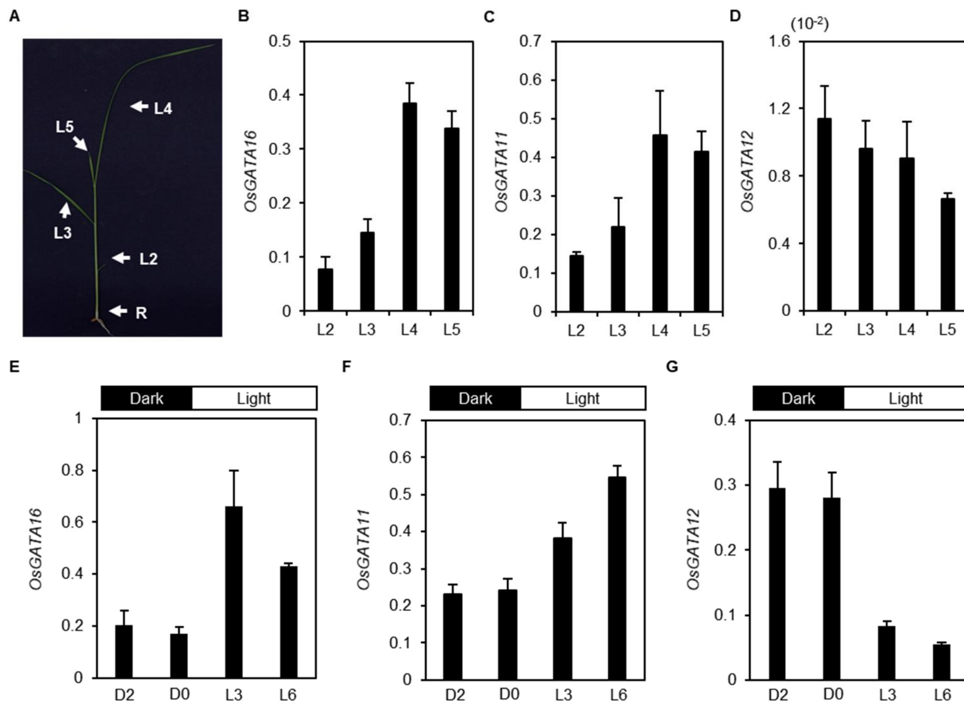

Figure 2. Expression patterns of GATA genes depending on leaf age and de-etiolation

(A) Wild-type(‘Dongjin’) plants at the five-leaf stage were used for the qRT-PCR analysis in (B), (C) and (D). L2-L5, second to fifth leaves; R, root. (B-D) Transcript levels of *OsGATA16*, *OsGATA11* and *OsGAGTA12* depending on leaf age. (E-G) The 10–day-old etiolated WT seedlings were exposed to light (90 μmol m-2 s-1) for 0 h (D0) up to 6h (L6). Transcript levels of *OsGATA16*, *OsGATA11* and *OsGATA12* during de-etiolation.

Subcellular localization and Identification of OsGATA16 binding Motif

To determine the subcellular localization of OsGATA16, we transiently expressed the *Ubi: OsGATA16-sGFP* construct in rice protoplasts isolated from rice suspension cultured cells. As expected for a TF, we detected green fluorescence in nuclei (Figure 3), indicating that OsGATA16 localizes to the nucleus.

A previous study defined the 4-bp core sequence of the GATA binding motif as GATA and GATC in Arabidopsis [35]. Therefore, it is possible that OsGATA16 also binds the GATA or GATC motif. To examine this hypothesis, we first determined whether OsGATA16 interacts with GATA or GATC motif by yeast one hybrid (Y1H) assays (Figure 4). The reporter plasmid contained the six tandem repeats of the GATA or GATC sequence and OsGATA16 was used as a bait. OsGATA16 binds to the GATC but not to the GATA. If add additional nucleotide on both sides of the motif, OsGATA16 still does not bind to GATA motif but more strongly binds to the GATC motif. We subsequently examined whether OsGATA16 also interacts with the GATC variants by point mutation analysis of the GATC in Y1H assays. We found that point mutations of the "GATC" nucleoitde significantly decreased the binding capacity of OsGATA16, while mutations in the nucleotides flanking "GATC" did not significantly change the binding efficiency, indicating that OsGATA16 has binding capacity for the GATC core sequence, but the flanking nucleotides of the GATC core sequence are relatively flexible for interacting with OsGATA16.

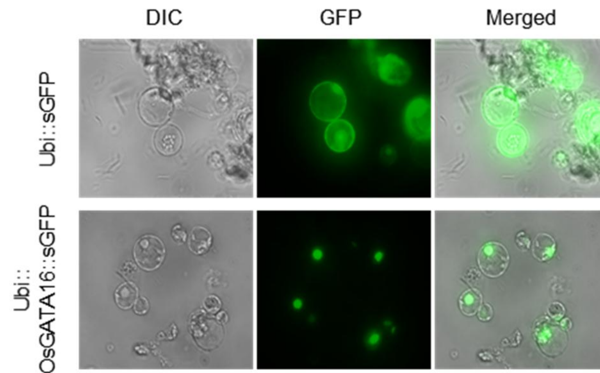

Figure 3. Subcellular localization of OsGATA16

Subcellular localization of OsGATA16 in rice protoplasts isolated from rice suspension cultured cells. OsGATA16, fused to sGFP, was transiently expressed in rice protoplasts. The upper panels show the fluorescent signal from the unfused sGFP control, which is distributed throughout the cell. The bottom panels show the fluorescent signal from OsGATA16-sGFP, which localizes exclusively to the nucleus

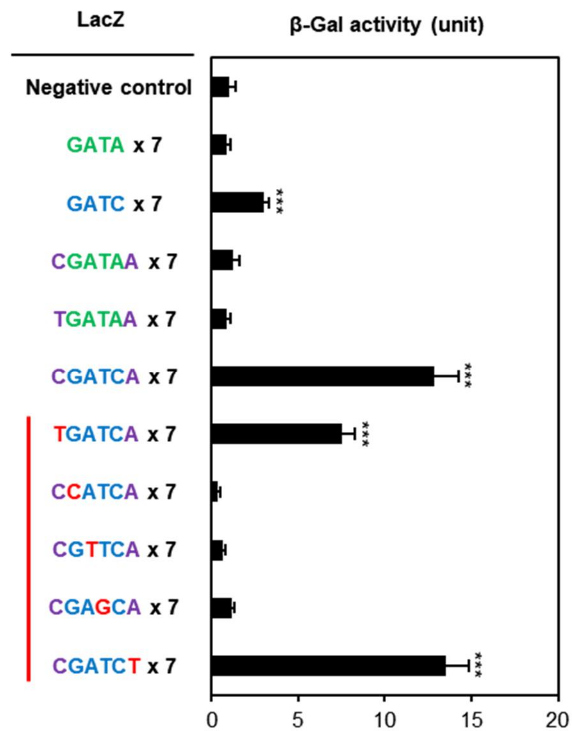

Figure 4. Identification of OsGATA16 binding Motifs

Interaction of OsGATA16 with the GATA, GATC, and its substituted sequences in Y1H assays. Empty bait and prey plasmids (−) were used for the negative controls. b-Galactosidase (b-Gal) activity was measured by liquid assays (see "Methods"). Asterisks indicate statistically significant differences compared to empty prey vector, as determined by Student's t-test (***$p < 0.001$).

Phenotypic characterization of the *osgata16* knockdown mutant and *OsGATA16* overexpression line

To examine the role of the *OsGATA16* TF in regulating chloroplast development, we searched for mutant lines in the RiceGE database (http://signal.salk.edu/cgi-bin/RiceGE) and found one T-DNA insertion line (PFG_3A-06147.R), which harbors a T-DNA fragment in the promoter region of *OsGATA16* (Figure 3A). To verify the effect of the T-DNA insertion, we performed semi-quantitative RT-PCR analysis and found that the *OsGATA16* transcript levels were much lower in the PFG_3A-06147.R line than its wild-type parental line, japonica cultivar 'Dongjin' (hereafter WT; Figure 3B), indicating that this line is a knockdown mutant of *OsGATA16* (hereafter *osgata16*). To examine the possible phenotypic effect of the *osgata16* mutation, we grew *osgata16* plants in a paddy field under natural long-day (NLD) conditions (>14h light/day at 37° N latitude, Suwon, Korea). The *osgata16* leaf blades exhibited a pale-green phenotype compared to the WT (Figure 5C, D). To verify this phenotype, we measured the contents of photosynthetic pigments at two different stages. At 50 d after sowing (DAS), the Chl contents in *osgata16* leaves were reduced by 27.2% compared to the WT, while those of carotenoids (Car) were similar (Figure 5E, F). At 125DAS heading stage, the *osgata16* mutant leaves still showed pale-green phenotype compared with WT (Figure 6).

To examine the pale-green phenotype of *osgata16* leaves in more

detail, we grew the plants in a growth chamber under LD conditions (14.5 h light, 30 °C / 9.5 h dark, 24 °C). Similar to the phenotype under NLD conditions, young leaves of 3-week-old *osgata16* plants were pale green (Figure 7A, B), with lower levels of Chl compared to the WT (Figure 7C).

To determine whether *OsGATA16* gene function is sufficient to promote enhanced chloroplast development in rice, Using Agrobacterium-mediated transformation, we obtained four independent transgenic lines containing *35S: OsGATA16* cDNA. Whether T-DNA is present in transgenic plants, PCR analysis was conducted to amplify the *hptII* gene and the fragments surrounding the target sites (Figure 8A). To validate the overexpression of OsGATA16 transcripts in the leaves of transgenic lines by RT-qPCR (Figure 8C) and only overexpression line of *OsGATA16* had dark green leaves and lamina joint (Figure 8D-M). Differences in plant architecture were also evident in the Overexpression lines. At maturity, overexpression lines show reductions in overall plant height compared with wild-type plants (Figure 9A). We also found not only leaves but also culm, lamina joint and panicle were more dark green in overexpression line than WT (Figure 9B-H). These results imply that *OsGATA16* functions to regulate chloroplast development and to alter aspects of plant architecture in rice.

Roots of several plant species have the potential to turn green when exposed to light. Some researchers used root greening to unravel the mechanism that regulates chloroplast development [36, 37]. OsGATA16-overexpression (OX) plants were grown in the growth chamber on half-

strength MS phytoagar medium under LD conditions for 4 weeks to induce root greening, finding that overexpression of *OsGATA16* caused ectopic chloroplast development in the root than those of the WT (Figure 10). A phenotypic consequence of constitutive expression was immediately apparent in that transgenic calli were dark green, compared to wild type calli (Figure 11). Constitutive expression of *OsGATA16* in rice is, therefore, sufficient to induce greening in tissues where chloroplasts don't usually develop (lamina joint, root, and callus) and to enhance greening in shoots

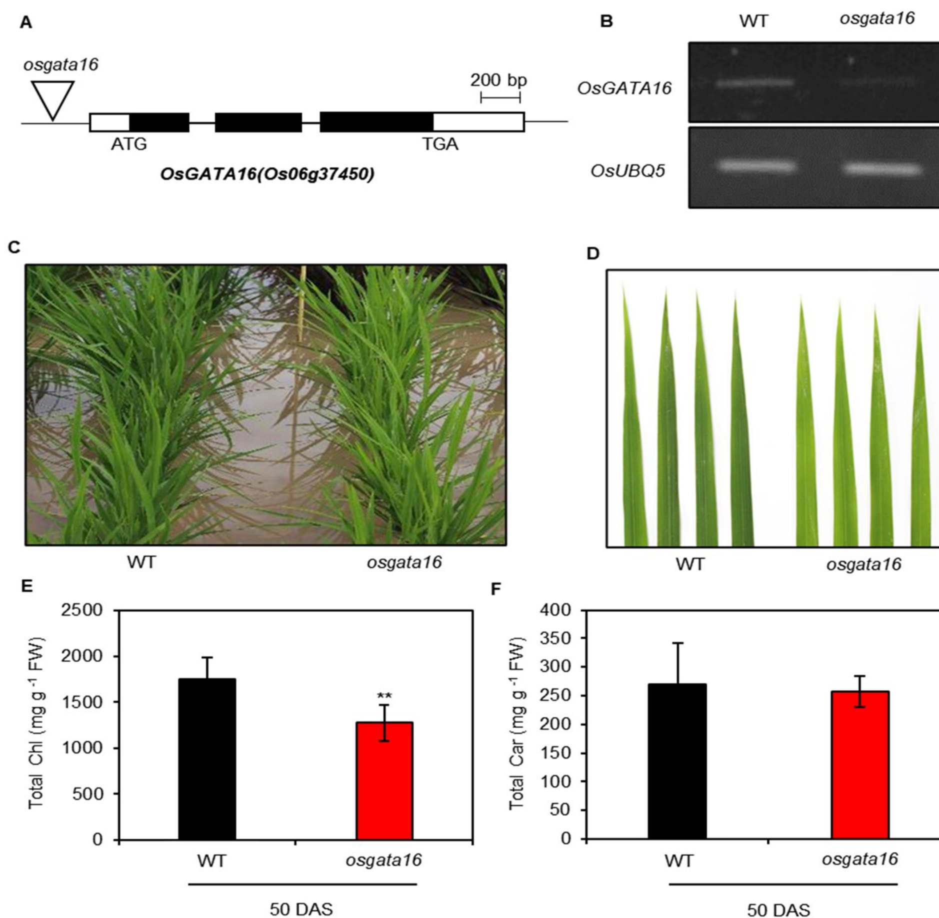

Figure 5. The T-DNA insertion osgata16 knockdown mutant exhibited a pale-green leaf phenotype

(A) Gene structure and T-DNA insertion site (inverted triangle) in the promoter region of *OsGATA16* (PFG_3A-06147.R). (B) Decrease in OsGATA16 transcript levels in the osgata16 mutant confirmed by RT-PCR. UBQ5 was used for an internal control. (C, D) Color difference in whole plants (C) and the second leaf of the main culm (D) between WT and osgata16 at 50 d after sowing (DAS). (E, F) Reduced levels of total Chl (E) and Car (F) in osgata16. Means and SD were obtained from 6 biological replicates. Significant differences between WT and *osgata16* was determined by Student's t-test (** P<0.01)

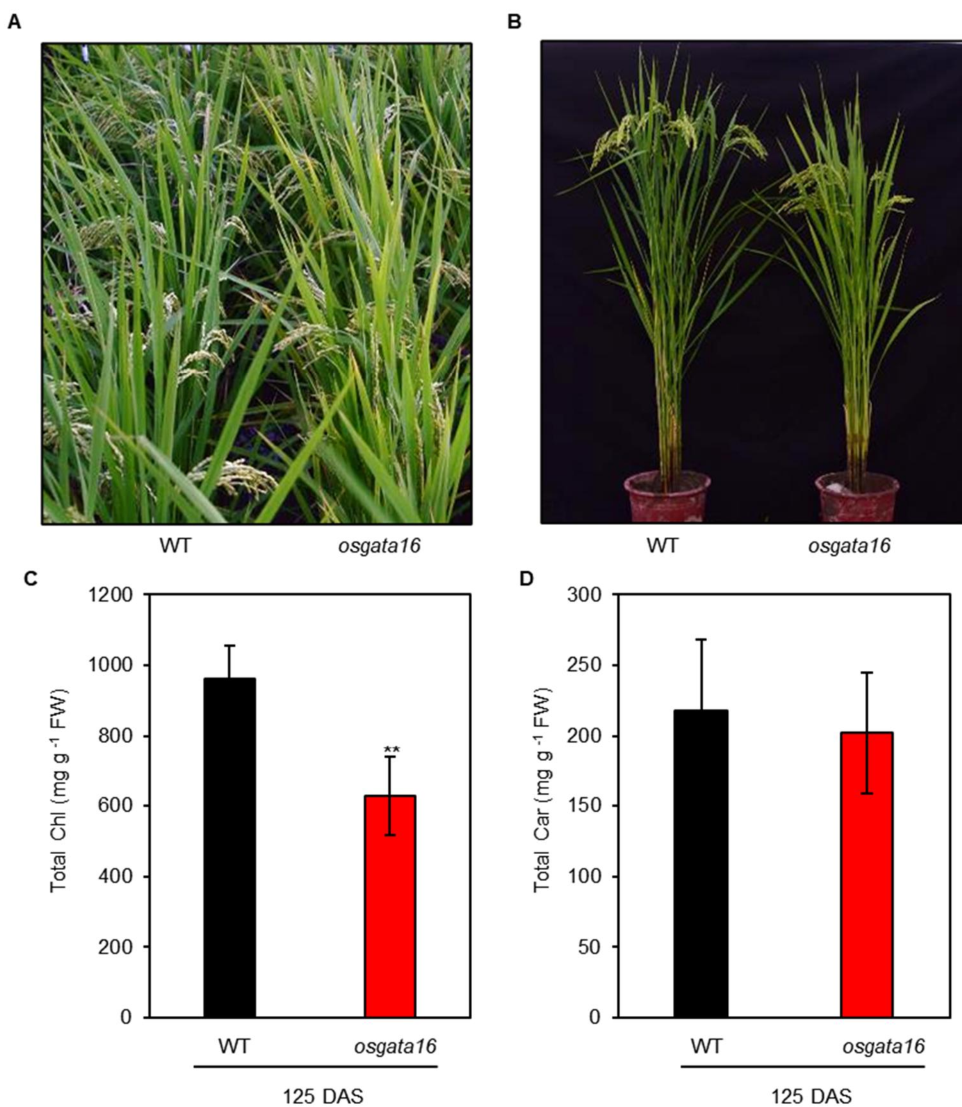

Figure 6. Pale-green phenotype of *osgata16* mutant at heading stage

(A, B) Phenotypes of 125 DAS heading stage plants in a paddy field for wild-type (WT) and *osgata16* mutant lines. (C, D) Total Chl (E) and Car (F) levels were measured using the middle part of the second leaves in the main culm. Mean and SD values were obtained from five biological replicates. Student's t-test (** $P < 0.01$).

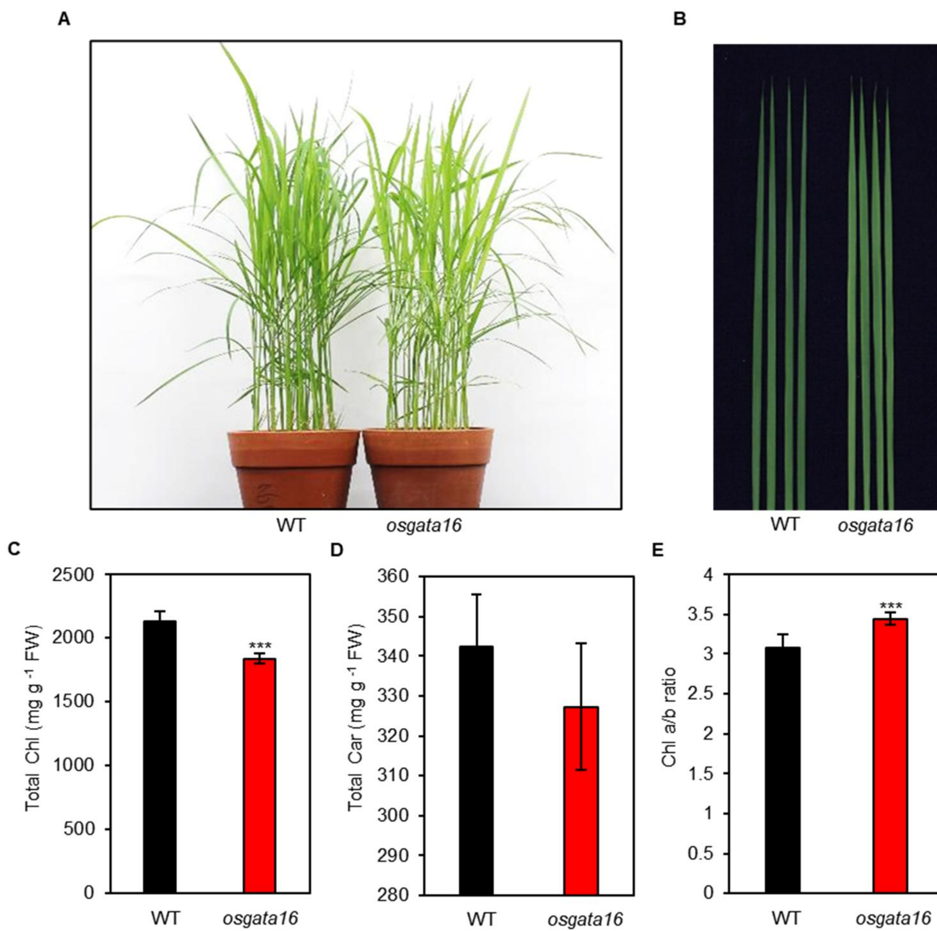

Figure 7. Pale-green phenotype of *osgata16* mutant at seedling stage

(A, B) Phenotypes of 15-day-old WT and *osgata16* plants grown in the greenhouse under NLD conditions (A) and first leaves of WT and *osgata16* plants (B). (C–E) Changes in total Chl (C), total Car (D), and the Chl a/b ratio. (C, D, E) Pigment levels in the first leaves of 15-day-old WT and *osgata16* plants. Mean and SD values were obtained from five biological replicates (n=5). Student's t-test (*** $P < 0.001$).

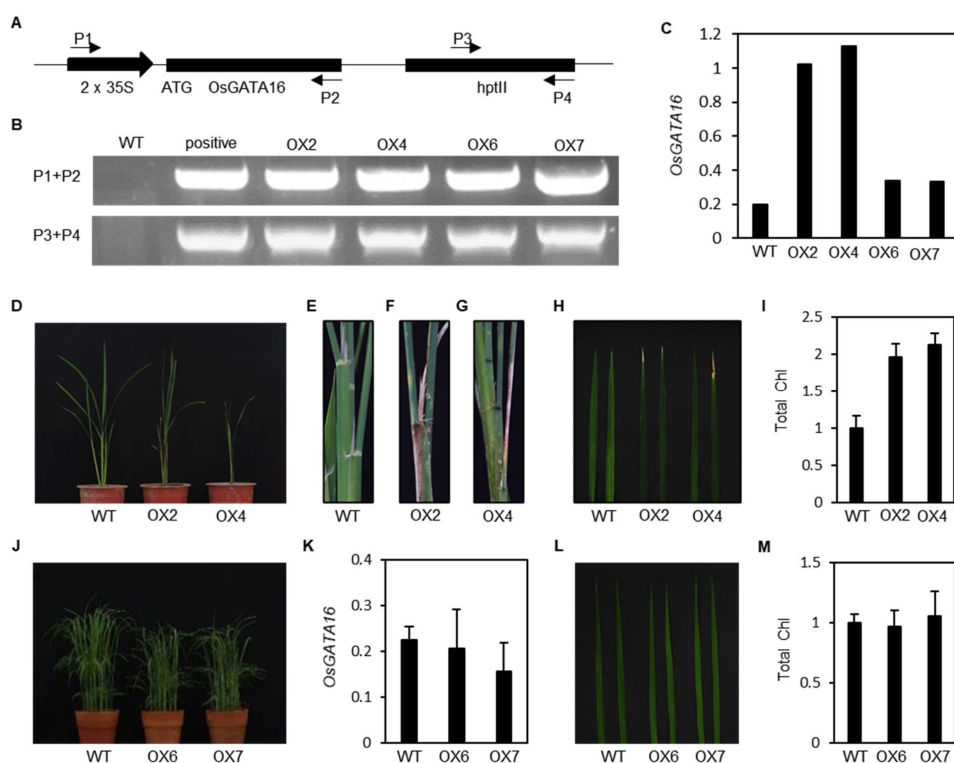

Figure 8. The *35S: OsGATA16* transgenic plants phenotype

(A) Schematic representation of the transfer DNA (T-DNA) in pMDC32 used for constitutive overexpression of the *OsGATA16* coding sequence and the primers used in (B). (B) Genotyping of T0 plants by PCR. Upper bands are product of primers P1 and P2. Lower bands are product of primer P3 and P4. 2 × 35S, constitutive dual CaMV 35S promoter; hptII, hygromycin phosphotransferase II. (C) *OsGATA16* expression levels, as measured by RT-qPCR and normalized to the transcript levels of *GAPDH*. (D-I) phenotype of T0 OX2 and OX4 plants and total chlorophyll content (I). (J, L) phenotype of T1 OX6 and OX7 plants, *OsGATA16* expression levels (K) and total chlorophyll content (M).

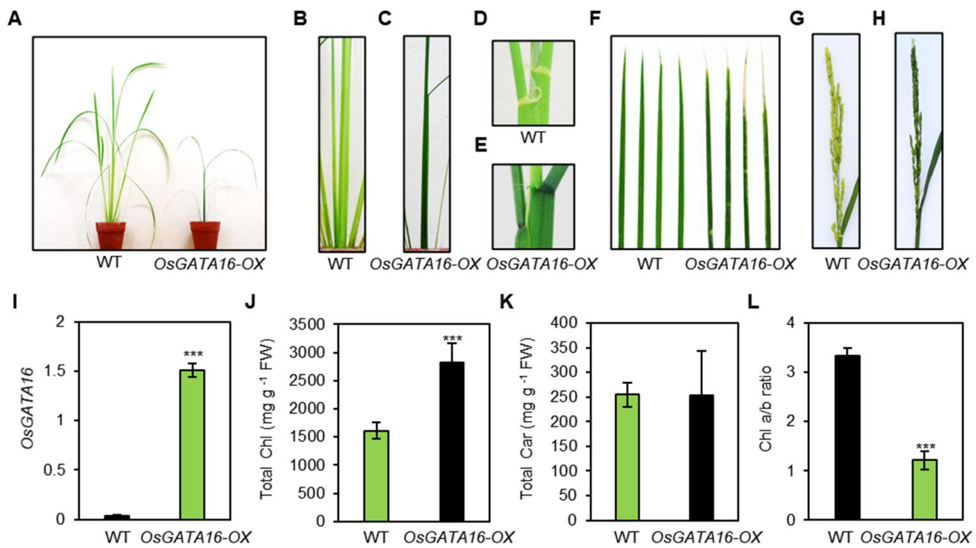

Figure 9. Phenotype of *OsGATA16* overexpression line

(A) Phenotypes of 6-week-old WT and *OsGATA16* overexpression line plants grown in the greenhouse under NLD conditions. (B-H) culm (B, C), lamina joint (D, E), leaves (F) of 6 weeks of WT and *OsGATA16* overexpression line and panicle (G, H) of WT and OsGATA16 overexpression line at heading stage. (I-L) *OsGATA16* expression levels, as measured by RT-qPCR and normalized to the transcript levels of *GAPDH* (I), total chlorophyll content (J), total carotenoid content (K) and chlorophyll a / b ratio (L) shown in (A). Means and SD were obtained from four biological replicates. Significant differences between WT and *OsGATA16* overexpression line was determined by Student's t-test (*** P<0.001)

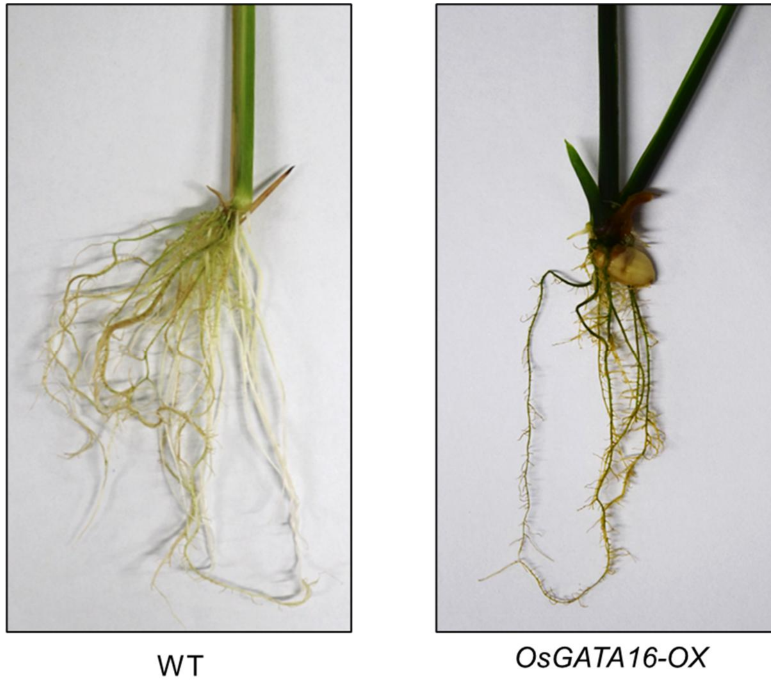

Figure 10. Overexpression of *OsGATA16* enhances greening of Root

WT and *OsGATA16-OX* plants were grown in the growth chamber on half-strength MS phytoagar medium under LD conditions for 4 weeks to induce root greening.

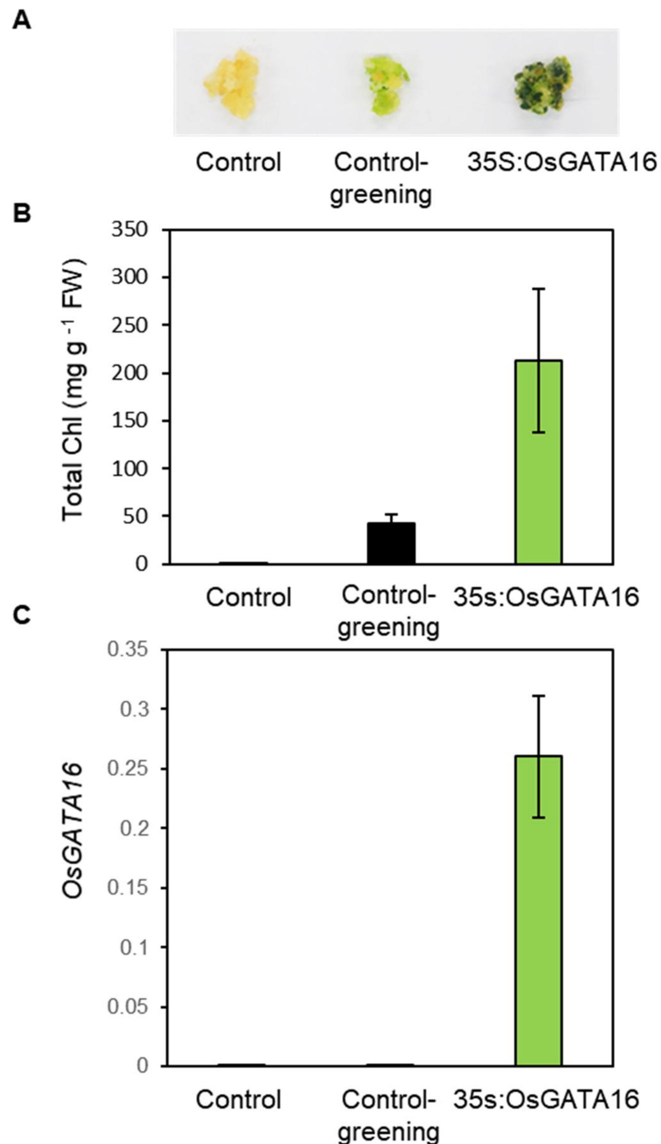

Figure 11. Overexpression of *OsGATA16* enhances greening of Callus

(A) Color of calli from a vector control and *35S: OsGATA16* lines. (B) total chlorophyll content of the control, control-greening and the *35S: OsGATA16* calli shown in (A). (C) RT–PCR analysis of calli from the control, control greening and the *35S: OsGATA16* lines as in (A) and normalized to the transcript levels of *GAPDH*.

OsGATA16 Expression Increases the Chloroplasts development in Bundle Sheath Cells of Rice

The installation of a C4 photosynthetic system into rice has the theoretical potential to double current rice yields [38, 39] through improved photosynthetic efficiencies while reducing water and nitrogen demands. Unlike the BS plastids in a C3 plant, in a C4 context, the BS plastids serve an essential role in photosynthesis and therefore the number and or size of the BS plastids in rice must be increased to support the C4 cycle. Recently, to manipulate chloroplast architecture specifically in the BS of rice. This was achieved by driving *OsGATA11*, paralogous gene of *OsGATA16*, expression with the use of the glycine decarboxylase p-subunit promoter (pFtGLDp) [40] and achieved through the introduction of one of the two heterologous maize GLK genes (either ZmG2 or ZmGLK1) [17-19]. Likewise, we found by light microscopy analysis that the *OsGATA16*-OX plants enhanced chloroplast development in vascular sheath cells of large vein, small vein and even mid vein compared with the WT (Figure 12). Transmission electron micrographs of minor veins in leaf of WT, *osgata16* and *OsGATA16*-OX line also confirmed that bundle sheath chloroplast volume was increased, and highly organized thylakoid systems were apparent when *OsGATA16* is overexpressed (Figure 13).

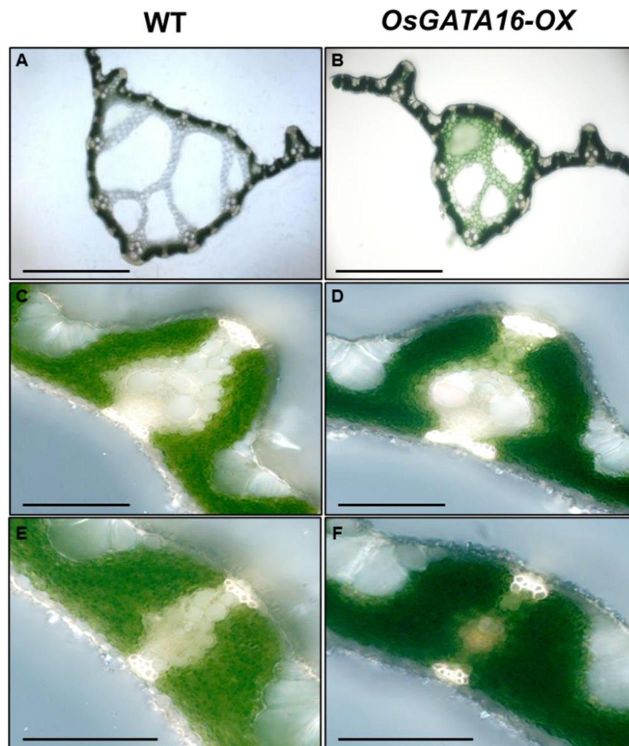

Figure 12. Light microscopy of veins in leaf of wild-type and *OsGATA16* overexpression line

(A-F) Transverse section of mid vein (A, B), large vein (C, D) and small vein (E, F) at the middle of the second leaf from the top at 2-week-old plant, Bars = 1 mm in (A, B); 100μm in (C-F).

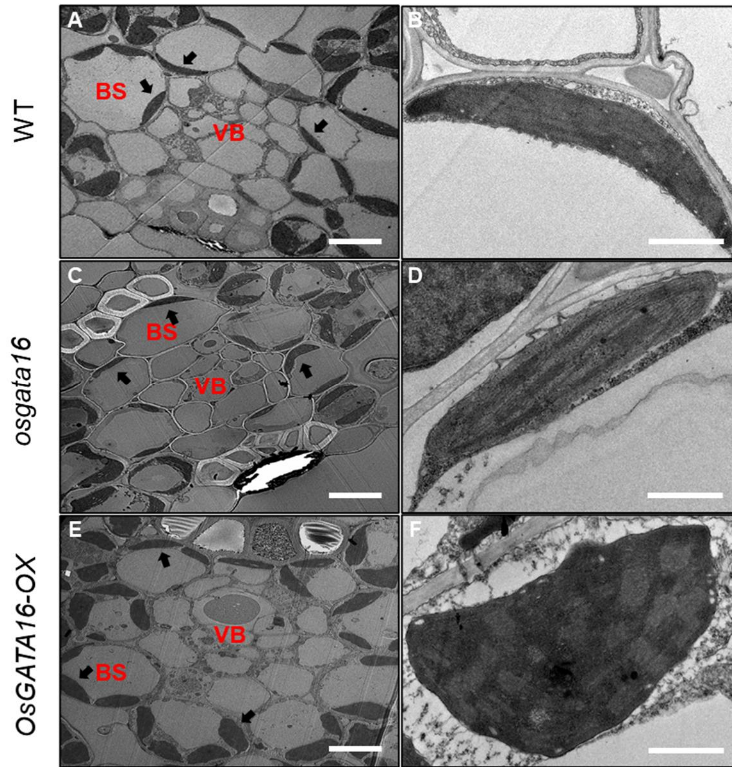

Figure 13. Transmission electron microscopy of minor veins in leaf of wild-type and *OsGATA16* overexpression line

(A, C, E) Transmission electron micrographs of minor veins in leaf of WT (A), osgata16 (C) and *OsGATA16* overexpression (E) lines. (B, D, F) Transmission electron micrographs of WT (B), *osgata16* (D) and *OsGATA16* overexpression (F) lines showing chloroplast ultrastructure in BS cells. VB, vascular bundle; BS, bundle sheath cell; arrows, chloroplast. Bars = 1 μm in (A, C, E); 5μm in (B, D, E).

Mutation of OsGATA16 decreases agronomic performance in rice

Since reduced photosynthetic pigment levels and photosynthetic activity negatively affect plant production, many leaf color-associated mutants show poor agronomic traits compared to the WT [7, 41]. To examine the relationship between the mutation in *osgata16* and crop production, we evaluated several agronomic traits in this mutant, including plant height, panicle length, number of panicles per plant, number of spikelets per panicle, spikelet fertility, and 500-grain weight under NLD conditions (Figure 14). The height of *osgata16* plants was significantly smaller than that of the WT (Figure 14A). WT and *osgata16* plants produced a similar number of spikelets per panicle (Figure 14D); however, the *osgata16* mutant had significantly lower values for other agronomic traits compared to the WT, including panicle length (Figure 14B), the number of panicle per plant (Figure 14C), seed fertility (Figure 14F) and 500-grain weight (Figure 14G). These results indicate that the *osgata16* mutation has negative effects on agronomic traits, ultimately reducing grain yield per plant (Figure 14H). However, very few seeds were obtained from T0 *OsGATA16*-OX lines and T1 *OsGATA16*-OX lines died while growing.

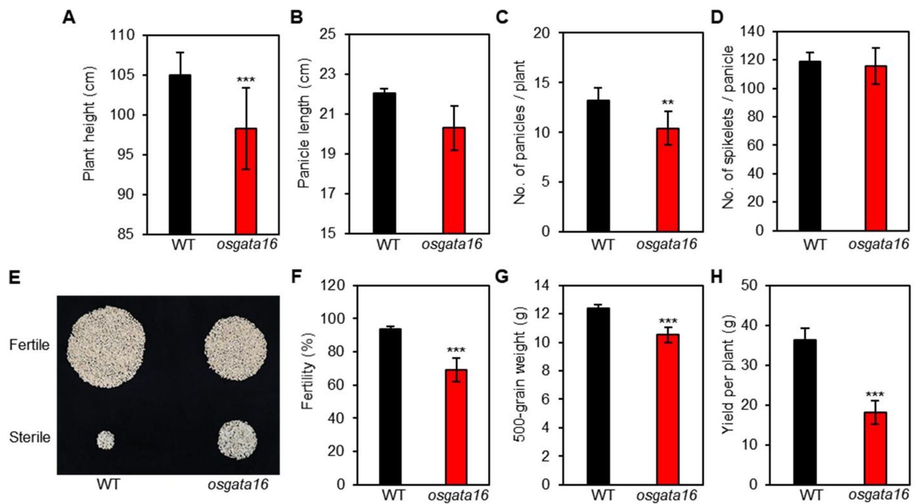

Figure 14. Agronomical traits of *osgata16* mutant

(A) plant height, (B panicle length, (C) number of panicles per plant, (D) number of spikelets per panicle, (E) Photographs of the relative abundances of fertile and sterile spikelets, (F) fertility, (G) 500-grain weight, and (H) yield in WT and *osgata16* grown in paddy field. Means and SD were obtained from at least 8 biological replicates. Significant differences were determined by Student's t-test (** P<0.01, *** P<0.001).

OsGATA16 altered the transcription expression of Chlorophyll biosynthetic and photosynthetic genes

To identify the effect of *OsGATA16* on chlorophyll biosynthesis and photosynthesis, first we investigated the transcript levels of Chl biosynthetic and photosynthetic related genes in the detached leaves of 10-day-old WT and *osgata16* seedlings by RT-qPCR (Figure 15). The expression of transcription factor, associated with chloroplast development, (Figure 15A) (*OsGLK1*, *OsGLK2* and *OsPIL1*), most of Chl biosynthesis genes and photosynthetic genes were significantly downregulated in *osgata16* (Figures 15B, C). whereas the expression levels of *OsGS*, *OsCAO2* and *OsFtsZ* were not significantly altered. Because Car contents in *osgata16* leaves were similar to those WT, we examined carotenoid biosynthetic gene, finding that carotenoid biosynthetic genes were not down-regulated in *osgata16* (Figure 16).

However, Chl biosynthesis genes were not altered in *OsGATA16*-OX plants, even the expression levels of *OsFd-GOGAT*, *OsHEMA*, *OsCHLH*, *OsPORB* and *OsCAO1* were down-regulated in *OsGATA16*-OX compared to WT (Figure 17). This phenomenon has been reported in the past [42, 43], and authors report three factors of this phenomenon. First, expression in a wild-type background would reduce the sensitivity of the experiment in that the plants are already photosynthetically competent. Second, strong constitutive gene expression leads to pleiotropic effects. Third, constitutive gene

expression confounds primary and secondary effects. Or it may be due to inappropriate sampling timing, which has already increased chlorophyll as much as possible, and this cause Chl biosynthesis gene to be down regulated. Meanwhile the expression of *OsRbcS*, *OsPsbR*, *OsLhca4* and *OsLhcb1a* in lamina joints of *OsGATA16*-OX were higher than those of WT (Figure 18), which were reported that *OsRbcS* and light-harvesting chlorophyll a/b-binding protein in Lamina joint were not detected by in situ hybridization [44].

Instead of using the leaves of *OsGATA16*-OX lines, we applied transient gene expression system using WT rice green tissues, according to the protocol of Zhang and Su [31]. After transfection with the OsGATA16-Myc plasmid by using the PEG-mediated transfection approach and incubation for 12 h, total RNA was extracted from rice protoplast and relative expression levels of *OsGATA16*, Chl biosynthetic and photosynthetic related genes were measured by RT-qPCR. In contrast to expression patterns in *osgata16* mutant, transient expression of *OsGATA16* upregulated the transcript levels of various of chlorophyll biosynthetic and photosynthetic genes. Especially *OsPORA*, *OsPORB* and *OsLhcb1a* transcript level drastically increased. Taken together, these results indicate *OsGATA16* gene responsible for Chl biosynthesis and photosynthesis (Figure 19).

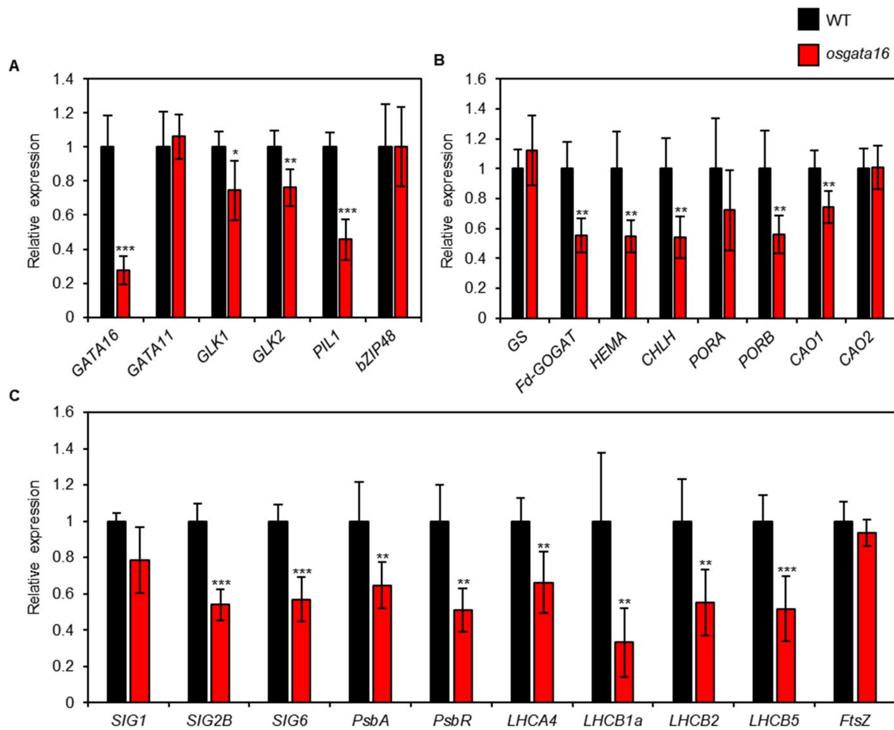

Figure 15. Expression of chlorophyll biosynthetic and photosynthetic genes in osgata16 mutant

Total RNA was extracted from the first leaves of 10-day-old WT and *osgata16* plants grown in a growth chamber under LD conditions. Relative transcript levels of transcription factor (A), chlorophyll biosynthesis genes (B) and photosynthesis-associated genes (C) were obtained by normalizing to the transcript level of *GAPDH*. Mean and SD values were obtained from six biological replicates. Student's t-test (* $P < 0.05$, ** $P<0.01$, *** $P<0.001$).

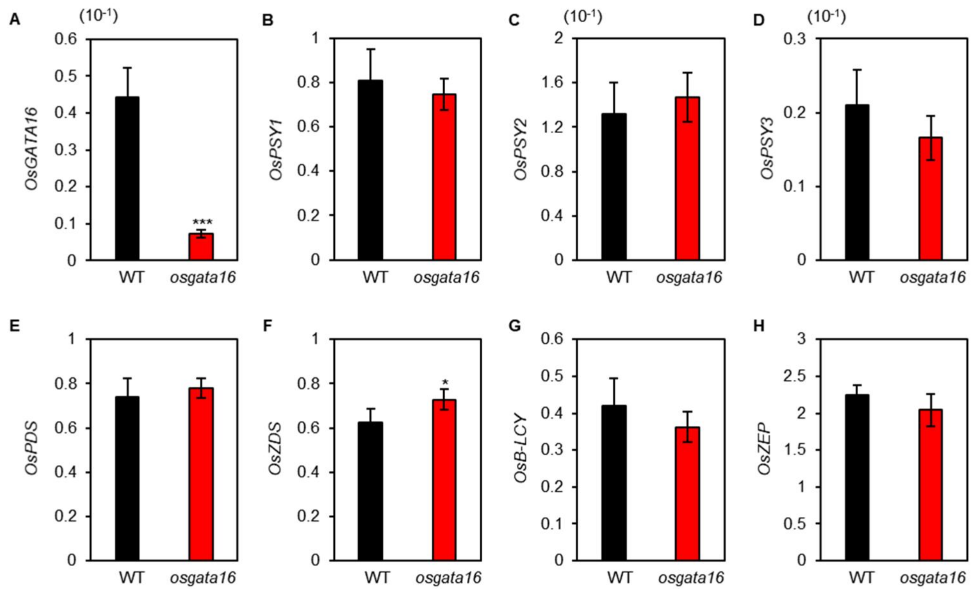

Figure 16. Expression of carotenoid biosynthetic genes in *osgata16* mutant Total RNA was extracted from the first leaves of 10-day-old WT and *osgata16* plants grown in a growth chamber under LD conditions. Relative transcript levels *OsGATA16* (A), *OsPSY1* (B), *OsPSY2* (C), *OsPSY3* (D), *OsPDS* (E), *OsZED* (F), *OsB-LCY* (G) and *OsZEP* (H) were obtained by normalizing to the transcript level of *GAPDH*. Mean and SD values were obtained from six biological replicates. Student's t-test (* P < 0.05, ** P<0.01, *** P<0.001).

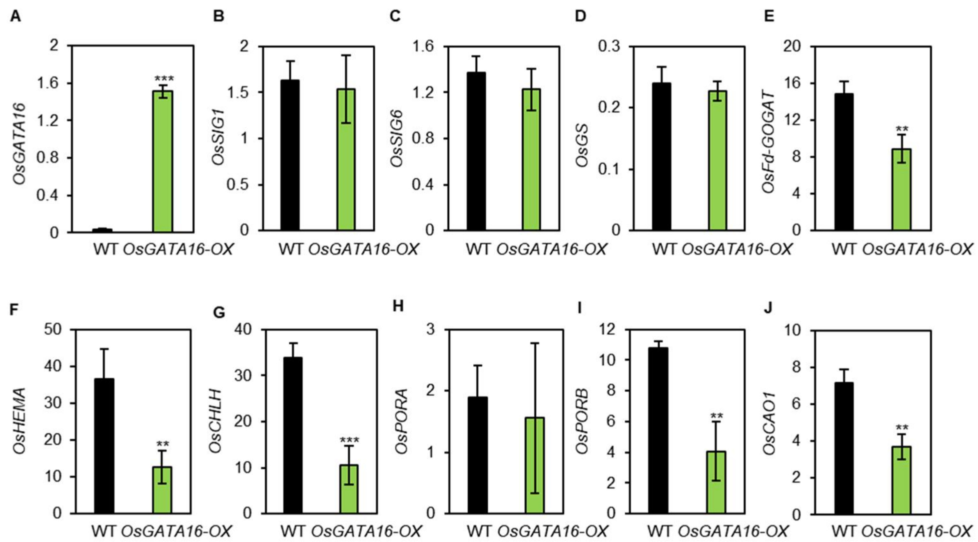

Figure 17. Expression of chlorophyll biosynthetic genes in leaf of wild-type and *OsGATA16* overexpression line

Total RNA was extracted from the first leaves of 10-day-old WT and *OsGATA16*-OX plants grown in the greenhouse under NLD conditions. Relative transcript levels *OsGATA16* (A), *OsSIG1* (B), *OsSIG6* (C), *OsGS* (D), *OsFd-GOGAT* (E), *OsHEMA* (F), *OsCHLH* (G), *OsPORA* (H), *OsPORB* (I) and *OsCAO1* (J) were obtained by normalizing to the transcript level of *GAPDH*. Mean and SD values were obtained from four biological replicates. Student's t-test (** P<0.01, *** P<0.001).

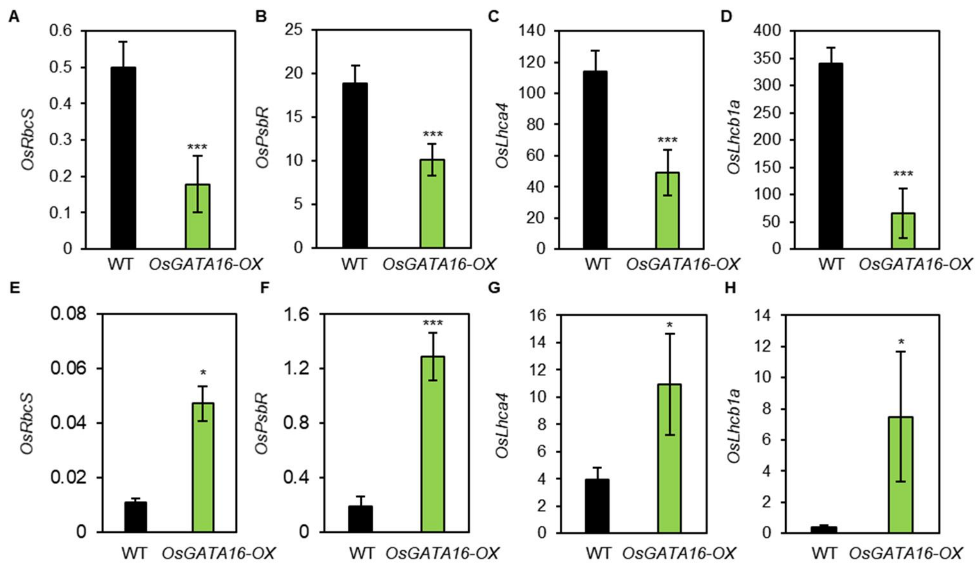

Figure 18. Expression of photosynthetic genes in lamina joint of wild-type and *OsGATA16* overexpression line

(A-H) Total RNA was extracted from the first leaves (A-D) and lamina joint (E-H) of 10-day-old WT and *OsGATA16*-OX plants grown in the greenhouse under NLD conditions. Relative transcript levels *OsRbcS* (A, E), *OsPsbR* (B, F), *OsLHCA4* (C, G) and *OsLhcb1a* (D, H) were obtained by normalizing to the transcript level of *GAPDH*. Mean and SD values were obtained from four biological replicates. Student's t-test (* $P<0.05$, *** $P<0.001$).

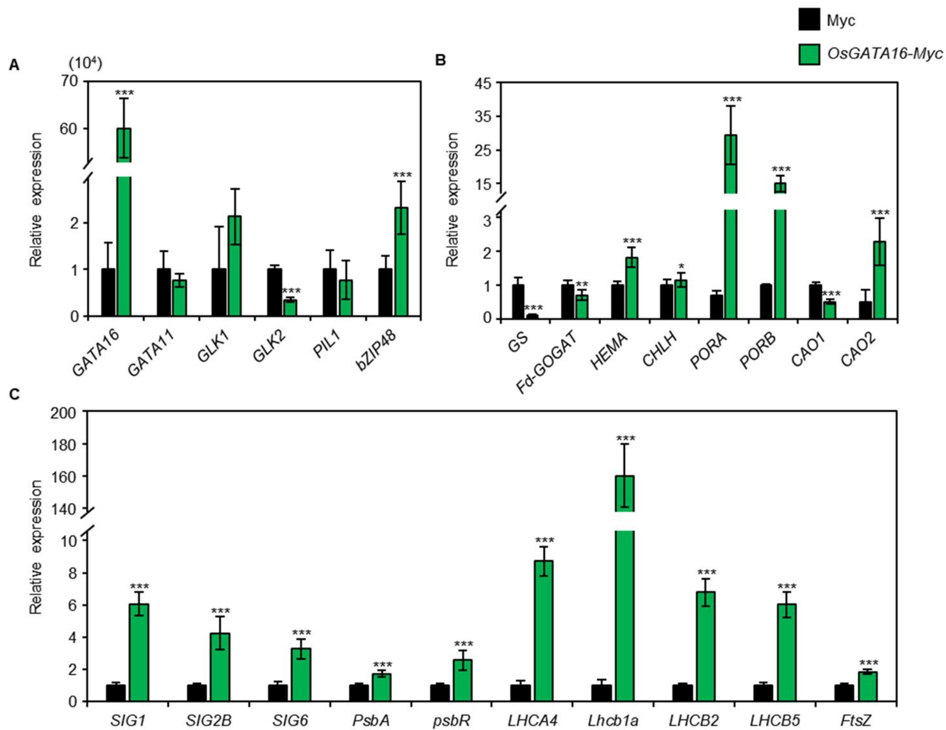

Figure 19. Transient expression of *OsGATA16* upregulated the transcript levels of various chlorophyll biosynthetic and photosynthetic genes

Total RNA was extracted from the protoplast transfected with OsGATA16-Myc or Myc as a control, treated with continuous light conditions for 12h. Relative transcript levels of transcription factor (A), chlorophyll biosynthesis genes (B) and photosynthesis-associated genes (C) were obtained by normalizing to the transcript level of *GAPDH*. Mean and SD values were obtained from six biological replicates. Student's t-test (* P < 0.05, ** P<0.01, *** P<0.001).

OsGATA16* up-regulates the expression of *OsPORA* and *OsPORB

In the RT-qPCR analysis, we found that *OsPORA* and *OsPORB* were down-regulated in *osgata16* mutant and significantly up-regulated in protoplasts in which *OsGATA16*-Myc was transiently expressed. In addition, we found that OsGATA16 proteins bind to 'GATC' motifs and Repeated GATC elements were found in the promoter regions of *OsPORA* and *OsPORB* genes (Figure 20A).

Therefore, we examined whether *OsGATA16* act as a transcriptional activator of *OsPORA* and *OsPORB* by luciferase (LUC) assay using rice protoplasts. Protoplasts were isolated from rice suspension cultured cells and transfected with a plasmid containing *Ubi: OsGATA16-Myc*, together with plasmids containing the LUC reporter gene behind the promoter regions of *OsPORA* and *OsPORB*. *OsPORA* promoter-directed and *OsPORB* promoter-directed LUC activity were significantly enhanced in the cells expressing *OsGATA16*, compared with the vector control (Figure. 20B, C). To further investigate whether OsGATA16 binds to the promoter region of *OsPORA* and *OsPORB*, we performed chromatin immunoprecipitation (ChIP) assay using WT protoplast in which Ubi : OsGATA16-Myc and Ubi : Myc transiently expressed. ChIP assays showed that OsGATA16 bound to amplicon-a of the *OsPORA* promoter and amplicon-b of the *OsPORB* promoter

containing the repeated GATC motif (Figure 20D, E). Taken together, these results indicate that *OsPORA* and *OsPORB* are the one of the targets genes of OsGATA16 among genes encoding Chl biosynthetic enzymes.

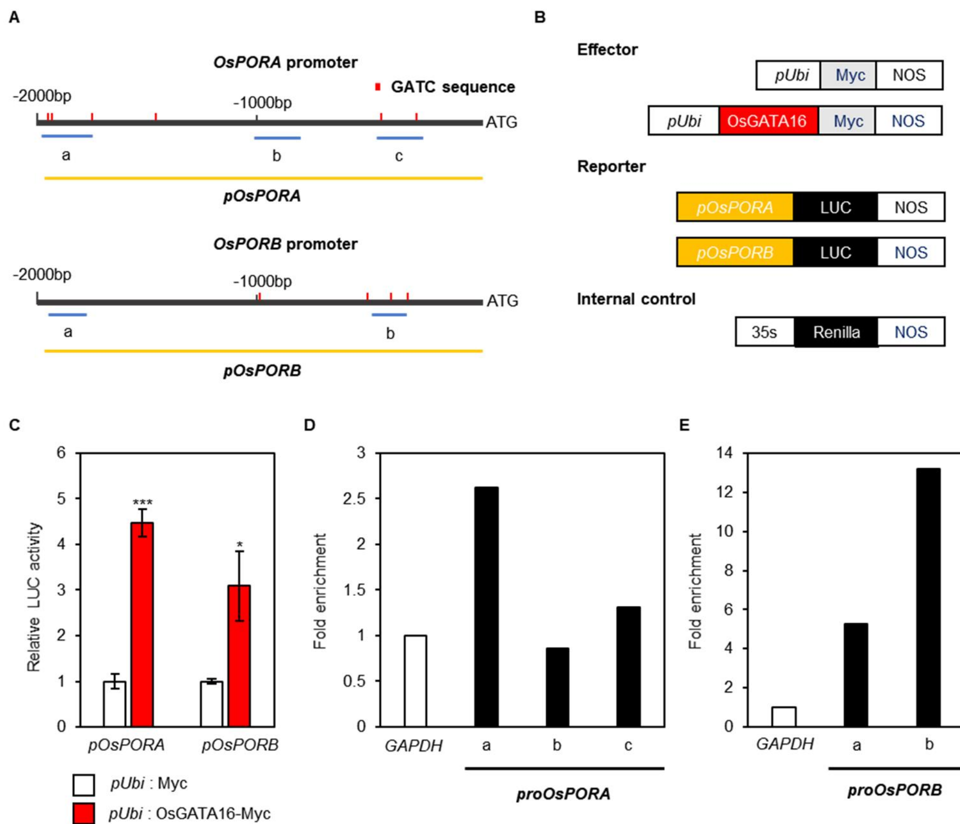

Figure 20. *OsGATA16* up-regulates the expression of *OsPORA* and *OsPORB*

(A) The red boxes in the promoter of *OsPORA* and *OsPORB* (gray horizontal line) represent the positions of the OsGATA16 binding sequence (GATC). The position of fragments used for ChIP (lowercase letters a–c with blue horizontal lines) assay, and transient LUC reporter (orange horizontal line) assay, respectively. (B) Effector, reporter, and internal control constructs used in the transient LUC reporter assay. pUbi, Ubiquitin promoter; 35S, 35S promoter; LUC, luciferase. Each construct also contains the NOS terminator. (C) The activation of the *OsPORA* and *OsPORB* promoter by OsGATA16-Myc expression in a protoplast transient assay. (D, E) OsGATA16 binding affinity to the promoter regions of *OsPORA* (D) and *OsPORB* (E) in planta examined by ChIP assays. *OsGATA16-Myc* was transiently expressed in protoplasts

isolated from 10-d-old WT rice seedlings. Fold enrichment of the promoter fragments (lowercase letters a–c with blue horizontal lines) was measured by immunoprecipitation with an anti-Myc antibody (see “Materials and methods”). The *OsGAPDH* gene (Os04g40950) encoding glyceraldehyde 3-phosphate dehydrogenase was used as the negative control. The mean and SD values were obtained from four biological replicates (C). Asterisks above bars (C–F) indicate a statistically significant difference compared to the negative controls, as determined by Student’s t test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). These experiments were repeated twice with similar results.

DISCUSSION

Recent study revealed that OsGATA16 confer Cold Tolerance by repressing *OsWRKY45-1* in early flowering rice cultivar Kitaake rice [45]. However, there was no difference between Kitaake *OsGATA16* overexpression lines and Kitaake WT in chloroplast development and plant growth and *OsGATA16* expression was repressed by cytokinin. Like genetic analysis of F2 progeny from a cross between cv. Kitaake and cv. Dongjin revealed that *Ghd7* and *OsPRR37* mutations additively contribute to the early-flowering phenotype in cv. Kitaake [46]. Further investigation of the genetic analysis between Dongjin and Kitaake will help to find out the different mechanism of *OsGATA16*.

In Arabidopsis thaliana, transcriptional network in which several transcription factors associated with chloroplast development (HY5, GNC, GNL, GLK and PIF) have been reported. For example, overexpression of *GNC* and *GNL* greatly enhanced Chl accumulation in intact roots but overexpression of *GNC* or *GNL* in the *hy5* background (*hy5 GNCox* and *hy5 GNLox*) did not increase Chl content in roots. These data demonstrate that *HY5* also is essential for the conditional root greening mediated by GATA factors [37]. In addition, when expressed in a *glk1 glk2* mutant background, *35S: GNC* partially rescued the mutant phenotype in terms of rosette mass, mesophyll chloroplast size, and chlorophyll levels, although these parameters remained substantially reduced compared to the WT and Interestingly, *GLK2* could be overexpressed in the wild-type background, but they were unable to obtain overexpression in the *gnc cga1* background. Thus, the expression level of *GLK2* appears to be dependent on the *GNC* family. Also, *GNC* Inhibits expression of *PIF* to Facilitate Chloroplast Biogenesis [4]. Furthermore, Enrichment of diverse motifs in the peak binding regions for *GNC* and *CGA1* suggests the possibility that these two transcription factors also interact with other transcription factor and the number of overlapping binding-associated

genes between *GNC* and *CGA1* was extremely low, indicating the possibility that most target genes are not shared by *GNC* and *CGA1* [35]. Therefore, we examined the relationship between *OsGATA16* and other TFs in rice. In *osgata16* mutant, the expression of *OsGLK1*, *OsGLK2* and *OsPIL1* were down-regulated and *OsGATA11* and *OsbZIP48* were similar to WT (Figure 15). However, in transient expression of *OsGATA16*, the expression of *OsGLK1*, *OsPIL1* and *OsGATA11* were similar to WT and *OsbZIP48* was up-regulated (Figure 19). Therefore, there was no intersection of osgata16 mutant and transiently expressed protoplast. Further investigation of the molecular connection between *OsGATA16* and other TFs will be necessary to understand chloroplast development regulation in rice.

The overexpression of *OsGATA16* in rice showed a lethal phenotype (Figure 12). *OsGATA11*, paralogous gene of *OsGATA16*, overexpression resulted in a semidwarf plants with reduced tillering [8]. In Arabidopsis, overexpression of *GNC* and *GNL* also leads to growth defects [47]. However, strong overexpression *OsGATA11* lines actually show the highest harvest index with reduced nitrogen. Likewise, specifically regulated expression of *OsGATA16* may be useful for increasing chloroplast activity, especially under low N. Furthermore Increasing the Chl content in rice may be an effective way to increase biomass production and grain yield [48].

Since reduced photosynthetic pigment levels and photosynthetic activity negatively affect plant production, many leaf color-associated mutants show poor agronomic traits compared to the WT [7, 41], but it was not only reason for *osgata16* mutant's poor agronomic traits. There was research about mutant with reduced chlorophyll synthesis exhibit higher photosynthetic rate and nitrogen-use efficiency, improved canopy light distribution, and greater yields than its wild type [49]. However, they also tested the yield of the mutant in a shading environment, the mutant plants performed worse compared to the wild type, with decreases in seed production up to 50%. The *OsGATA16* overexpression can activate chloroplast development in BS cells

(Figure 15, 16). As reported previously [40], the chloroplast development of BS cells by ectopic expression of *OsGATA11*, could not enhance photosynthetic performance and yield and they suggest, it was possible to improve BS photosynthesis, if a mechanism to deliver CO2 to the BS was also installed. Taken together, we postulated that if overall chlorophyll contents were defective, but chloroplast were developed in BS cells, higher photosynthesis efficiency and yield might be achieved.

The developmental processes were divided into five successive stages (S1-5) based on the morphological features of developing seedlings and LJs and the *OsGATA16* expression was not down-regulated during LJ development [50]. Therefore, it was not because of the decrease in *OsGATA16* transcript levels in LJs that WT had white color of LJs. In addition, Dark green color of the LJ formed before leaf blade bending in *OsGATA16*-OX plants. At S1 to S3, the cell size and cell number significantly increased and this may be reason for darker color of LJ than other parts in *OsGATA16*-OX plants. This feature of LJs may be used as an early marker for efficient identification.

In this study, we found that *OsGATA16* functions to promote Chl biosynthesis and chloroplast development, similar to arabidopsis *GNC*. Furthermore, ChIP assays and luciferase assays revealed that OsGATA16 bound to the promoters of *OsPORA* and *OsPORB* and up-regulates their expression (Figure 20). The presence of chloroplasts in cells where they are not normally developed (callus, lamina joint, mid vein and roots) (Figure 9-12) confirms that *OsGATA16* expression is sufficient to increase chloroplast development in rice. Especially chloroplast development in BS can be used to C4 rice research. Although the *OsGATA16*-OX plants showed a lethal phenotype, the use of more specialized, tissue-specific promoters may be useful for preventing or removing the detrimental problems and improve agronomic trait.

Accession number

Sequence data generated in this study can be found in the National Center for Biotechnology Information (NCBI): OsGATA16, Os06g0571800; OsGATA11, Os02g022040; OsGATA12, Os03g0831200; OsGLK1, Os06g0348800; OsGLK2, Os01g0239000; OsPIL1, Os03g0782500; OsbZIP48, Os06g0601500; OsGS, Os03g0223400; OsFd-GOGAT, Os07g0658400; OsHEMA, Os10g0502400; OsCHLH, Os03g0323200; OsPORA, Os04g0678700; OsPORB, Os10g0496900; OsCAO1, Os10g0567400; OsCAO2, Os10g0567100; OsSIG1, Os08g0163400; OsSIG2B, Os03g0271100; OsSIG6, Os08g0242800; OsPsbA, NP_039360.1; OsPsbR, Os08g0200300; OsLHCA4, Os08g0435900; OsLHCB1a, Os01g0600900; OsLHCB2, Os03g0592500; OsLHCB5, Os11g0242800; OsFtsZ, Os04g0665400; OsPSY1, Os06g0729000; OsPSY2, Os12g0626400; OsPSY3, Os09g0555500; OsPDS, Os03g0184000; OsZDS, Os07g0204900; OsB-LCY, Os02g0190600; OsZEP, Os04g0448950; OsRbcS, Os12g0274700

REFERENCES

1. Tanaka R, Kobayashi K, Masuda T: **Tetrapyrrole Metabolism in Arabidopsis thaliana**. *Arabidopsis Book* 2011, **9**:e0145.
2. Masuda T, Fujita Y: **Regulation and evolution of chlorophyll metabolism**. *Photochem Photobiol Sci* 2008, **7**(10):1131-1149.
3. López-Juez E: **Plastid biogenesis, between light and shadows**. *Journal of Experimental Botany* 2006, **58**(1):11-26.
4. Zubo YO, Blakley IC, Franco-Zorrilla JM, Yamburenko MV, Solano R, Kieber JJ, Loraine AE, Schaller GE: **Coordination of Chloroplast Development through the Action of the GNC and GLK Transcription Factor Families**. *Plant Physiol* 2018, **178**(1):130-147.
5. Burman N, Bhatnagar A, Khurana JP: **OsbZIP48, a HY5 Transcription Factor Ortholog, Exerts Pleiotropic Effects in Light-Regulated Development**. *Plant Physiol* 2018, **176**(2):1262-1285.
6. Nakamura H, Muramatsu M, Hakata M, Ueno O, Nagamura Y, Hirochika H, Takano M, Ichikawa H: **Ectopic overexpression of the transcription factor OsGLK1 induces chloroplast development in non-green rice cells**. *Plant Cell Physiol* 2009, **50**(11):1933-1949.
7. Sakuraba Y, Kim EY, Han SH, Piao W, An G, Todaka D, Yamaguchi-Shinozaki K, Paek NC: **Rice Phytochrome-Interacting Factor-Like1 (OsPIL1) is involved in the promotion of chlorophyll biosynthesis through feed-forward regulatory loops**. *J Exp Bot* 2017, **68**(15):4103-4114.
8. Hudson D, Guevara DR, Hand AJ, Xu Z, Hao L, Chen X, Zhu T, Bi YM, Rothstein SJ: **Rice cytokinin GATA transcription Factor1 regulates chloroplast development and plant architecture**. *Plant Physiol* 2013, **162**(1):132-144.
9. An Y, Zhou Y, Han X, Shen C, Wang S, Liu C, Yin W, Xia X: **The GATA transcription factor GNC plays an important role in photosynthesis and growth in poplar**. *J Exp Bot* 2020, **71**(6):1969-1984.
10. Deng XJ, Zhang HQ, Wang Y, He F, Liu JL, Xiao X, Shu ZF, Li W, Wang GH, Wang GL: **Mapped clone and functional analysis of leaf-color gene Ygl7**

in a rice hybrid (Oryza sativa L. ssp. indica). *PLoS One* 2014, **9**(6):e99564.

11. Xiang-sheng Z-qS, Dian-xing Wu, Jun-wei Sun, Qing-yao Shu: **Introduction of a xantha mutation for testing and increasing varietal purity in hybrid rice**. *Field Crops Research* 2006, **96**(1):71-79.
12. Su N, Hu ML, Wu DX, Wu FQ, Fei GL, Lan Y, Chen XL, Shu XL, Zhang X, Guo XP *et al*: **Disruption of a rice pentatricopeptide repeat protein causes a seedling-specific albino phenotype and its utilization to enhance seed purity in hybrid rice production**. *Plant Physiol* 2012, **159**(1):227-238.
13. Julian M Hibberd JES, Jane A Langdale: **Using C4 photosynthesis to increase the yield of rice-rationale and feasibility**. *Current Opinion in Plant Biology* 2008, **11**(2):228-231.
14. Sage RF, Khoshravesh R, Sage TL: **From proto-Kranz to C4 Kranz: building the bridge to C4 photosynthesis**. *J Exp Bot* 2014, **65**(13):3341-3356.
15. Lin H, Arrivault S, Coe RA, Karki S, Covshoff S, Bagunu E, Lunn JE, Stitt M, Furbank RT, Hibberd JM *et al*: **A Partial C4 Photosynthetic Biochemical Pathway in Rice**. *Front Plant Sci* 2020, **11**:564463.
16. Shen BR, Wang LM, Lin XL, Yao Z, Xu HW, Zhu CH, Teng HY, Cui LL, Liu EE, Zhang JJ *et al*: **Engineering a New Chloroplastic Photorespiratory Bypass to Increase Photosynthetic Efficiency and Productivity in Rice**. *Mol Plant* 2019, **12**(2):199-214.
17. Wang P, Khoshravesh R, Karki S, Tapia R, Balahadia CP, Bandyopadhyay A, Quick WP, Furbank R, Sage TL, Langdale JA: **Re-creation of a Key Step in the Evolutionary Switch from C3 to C4 Leaf Anatomy**. *Curr Biol* 2017, **27**(21):3278-3287 e3276.
18. Li X, Wang P, Li J, Wei S, Yan Y, Yang J, Zhao M, Langdale JA, Zhou W: **Maize GOLDEN2-LIKE genes enhance biomass and grain yields in rice by improving photosynthesis and reducing photoinhibition**. *Commun Biol* 2020, **3**(1):151.
19. Su-Ying Y, Lin HH, Chang YM, Chang YL, Chang CK, Huang YC, Ho YW, Lin CY, Zheng JZ, Jane WN *et al*: **Maize Golden2-like transcription factors boost rice chloroplast development, photosynthesis and grain yield**. *Plant Physiol* 2021.

20. Reyes JC, Muro-Pastor MI, Florencio FJ: **The GATA family of transcription factors in Arabidopsis and rice**. *Plant Physiol* 2004, **134**(4):1718-1732.
21. He P, Wang X, Zhang X, Jiang Y, Tian W, Zhang X, Li Y, Sun Y, Xie J, Ni J *et al*: **Short and narrow flag leaf1, a GATA zinc finger domain-containing protein, regulates flag leaf size in rice (Oryza sativa)**. *BMC Plant Biol* 2018, **18**(1):273.
22. Nutan KK, Singla-Pareek SL, Pareek A: **The Saltol QTL-localized transcription factor OsGATA8 plays an important role in stress tolerance and seed development in Arabidopsis and rice**. *J Exp Bot* 2020, **71**(2):684-698.
23. Lu G, Casaretto JA, Ying S, Mahmood K, Liu F, Bi YM, Rothstein SJ: **Overexpression of OsGATA12 regulates chlorophyll content, delays plant senescence and improves rice yield under high density planting**. *Plant Mol Biol* 2017, **94**(1-2):215-227.
24. Jeong DH, An S, Kang HG, Moon S, Han JJ, Park S, Lee HS, An K, An G: **T-DNA insertional mutagenesis for activation tagging in rice**. *Plant Physiol* 2002, **130**(4):1636-1644.
25. Porra RJ, Thompson, W. A., Kriedemann, P. E.: **Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy**. *Biochim Biophys Acta (BBA) - Bioenerg* 1989:975, 384–394.
26. Toriyama K, and Kokichi Hinata: **Cell suspension and protoplast culture in rice**. *Plant Science* 1985(41.3):179-183.
27. Yoo SD, Cho YH, Sheen J: **Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis**. *Nat Protoc* 2007, **2**(7):1565-1572.
28. Inada N, Sakai A, Kuroiwa H, Kuroiwa T: **Three-dimensional analysis of the senescence program in rice (Oryza sativa L.) coleoptiles. Investigations of tissues and cells by fluorescence microscopy**. *Planta* 1998, **205**(2):153-164.
29. Jeon JS, Lee S, Jung KH, Jun SH, Jeong DH, Lee J, Kim C, Jang S, Yang K, Nam J *et al*: **T-DNA insertional mutagenesis for functional genomics in**

rice. *Plant J* 2000, **22**(6):561-570.

30. Kim S-R LD-Y, Yang J-I, Moon S, An G: **Cloning vectors for rice**. *J Plant Biol* 2009:52: 73–78.
31. Zhang Y, Su J, Duan S, Ao Y, Dai J, Liu J, Wang P, Li Y, Liu B, Feng D *et al*: **A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes**. *Plant Methods* 2011, **7**(1):30.
32. Saleh A, Alvarez-Venegas R, Avramova Z: **An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in Arabidopsis plants**. *Nat Protoc* 2008, **3**(6):1018-1025.
33. Ranftl QL, Bastakis E, Klermund C, Schwechheimer C: **LLM-Domain Containing B-GATA Factors Control Different Aspects of Cytokinin-Regulated Development in Arabidopsis thaliana**. *Plant Physiol* 2016, **170**(4):2295-2311.
34. Gupta P, Nutan KK, Singla-Pareek SL, Pareek A: **Abiotic Stresses Cause Differential Regulation of Alternative Splice Forms of GATA Transcription Factor in Rice**. *Front Plant Sci* 2017, **8**:1944.
35. Xu Z, Casaretto JA, Bi YM, Rothstein SJ: **Genome-wide binding analysis of AtGNC and AtCGA1 demonstrates their cross-regulation and common and specific functions**. *Plant Direct* 2017, **1**(4):e00016.
36. Kobayashi K, Baba S, Obayashi T, Sato M, Toyooka K, Keranen M, Aro EM, Fukaki H, Ohta H, Sugimoto K *et al*: **Regulation of root greening by light and auxin/cytokinin signaling in Arabidopsis**. *Plant Cell* 2012, **24**(3):1081-1095.
37. Kobayashi K, Ohnishi A, Sasaki D, Fujii S, Iwase A, Sugimoto K, Masuda T, Wada H: **Shoot Removal Induces Chloroplast Development in Roots via Cytokinin Signaling**. *Plant Physiol* 2017, **173**(4):2340-2355.
38. Hibberd JM, Sheehy, J.E. and Langdale, J.A: **Using C4 photosynthesis to increase the yield of rice—rationale and feasibility**. *plant Biol* 2008, **11(2)**:228-231.
39. von Caemmerer S, Quick, W. P. and Furbank, R.T: **The Development of C4 Rice: Current Progress and Future Challenges**. *science* 2012, **336(6089)**:1671-1672.

40. Lee DY, Hua L, Khoshravesh R, Giuliani R, Kumar I, Cousins A, Sage TL, Hibberd JM, Brutnell TP: **Engineering chloroplast development in rice through cell-specific control of endogenous genetic circuits**. *Plant Biotechnol J* 2021, **19**(11):2291-2303.
41. Sakuraba Y, Rahman ML, Cho SH, Kim YS, Koh HJ, Yoo SC, Paek NC: **The rice faded green leaf locus encodes protochlorophyllide oxidoreductase B and is essential for chlorophyll synthesis under high light conditions**. *Plant J* 2013, **74**(1):122-133.
42. Savitch LV, Subramaniam R, Allard GC, Singh J: **The GLK1 'regulon' encodes disease defense related proteins and confers resistance to Fusarium graminearum in Arabidopsis**. *Biochem Biophys Res Commun* 2007, **359**(2):234-238.
43. Waters MT, Wang P, Korkaric M, Capper RG, Saunders NJ, Langdale JA: **GLK transcription factors coordinate expression of the photosynthetic apparatus in Arabidopsis**. *Plant Cell* 2009, **21**(4):1109-1128.
44. Koichi Tsutsumi YT, Michio Kawasaki,Mitsutaka Taniguchi &Hiroshi Miyake: **Expression of photosynthesis-related genes during the leaf development of a C3 plant rice as visualized by in situ hybridization**. *Plant Production Science* 2006, **9**(3):232-241.
45. Zhang H, Wu T, Li Z, Huang K, Kim NE, Ma Z, Kwon SW, Jiang W, Du X: **OsGATA16, a GATA Transcription Factor, Confers Cold Tolerance by Repressing OsWRKY45-1 at the Seedling Stage in Rice**. *Rice (N Y)* 2021, **14**(1):42.
46. Kim SL, Choi M, Jung KH, An G: **Analysis of the early-flowering mechanisms and generation of T-DNA tagging lines in Kitaake, a model rice cultivar**. *J Exp Bot* 2013, **64**(14):4169-4182.
47. Richter R, Behringer C, Muller IK, Schwechheimer C: **The GATA-type transcription factors GNC and GNL/CGA1 repress gibberellin signaling downstream from DELLA proteins and PHYTOCHROME-INTERACTING FACTORS**. *Genes Dev* 2010, **24**(18):2093-2104.
48. Wang F, Wang G, Li X, Huang J, Zheng J: **Heredity, physiology and mapping of a chlorophyll content gene of rice (Oryza sativa L.)**. *J Plant Physiol* 2008, **165**(3):324-330.

49. Junfei Gu* ZZ, Zhikang Li, Ying Chen, Zhiqin Wang, Hao Zhang: **Rice (Oryza sativa L.) with reduced chlorophyll content exhibit higher photosynthetic rate and efficiency, improved canopy light distribution, and greater yields than normally pigmented plants**. *Field Crops Research* 2017, **200**:58-70.
50. Wang R, Liu C, Li Q, Chen Z, Sun S, Wang X: **Spatiotemporal Resolved Leaf Angle Establishment Improves Rice Grain Yield via Controlling Population Density**. *iScience* 2020, **23**(9):101489.

초 록

엽록체는 엽록소 축적을 통한 광합성을 하는 중요한 기관으로, 벼에서의 엽록체 연구는 광합성 효율 개선을 통한 수량성 증가나 hybrid-rice 육종시에 편리한 마커로서 사용될 수 있기에 중요하다. 특히 C3인 벼를 C4로 바꾸었을 때, 두 배가량의 수확량 증가가 예상되기에 이를 위해 오랫동안 많은 연구들이 있어왔다. 본 연구에서는 애기장대에서 많은 연구가 이루어진, 엽록체 발달에 있어 Master regulator 역할을 할 것이라고 예상되는 GNC (AtGATA21) 유전자를 토대로 벼에서도 상동유전자인 OsGATA16 유전자의 엽록체 발달 관련 기능에 대해 분석하였다. 엽록체 발달에 중요한 역할을 한다고 알려진 호르몬인 cytokinin을 처리했을 때와 식물체를 암조건에서 기른 후 빛조건으로 바꾸었을 때 OsGATA16의 발현이 늘어남을 확인하였고 잎발달에 따른 OsGATA16의 발현패턴을 분석하였을 때 상위의 갓나온 잎에서 오래된 잎보다 발현량이 높음을 확인하였다. Yeast one hybrid assay를 통해 OsGATA16의 DNA결합서열이 'GATA' 가 아니라 'GATC' 서열에 결합함을 밝혀내었다. 애기장대 GNC 유전자와 마찬가지로, T-DNA가 삽입된 osgata16 knockout 돌연변이체의 경우, 야생형에 비해 전체적으로 연녹색 표현형을 보이고 OsGATA16 과발현체는 야생형에 비해 매우 진한 녹색을 가짐을 확인하였다. 그뿐만 아니라, 과발현체의 경우 원래 엽록체가 잘 발달되지 않는 잎의 주맥, 뿌리 그리고 캘러스에서 엽록체가 발달되고 투과전자현미경을 통해 유관속초 세포에서도 야생형에 비해 엽록체가 더 크고 발달됨을 확인하였다. 유관속초 세포에서의 엽록체발달은 C3인 벼를 C4로 만들기 위한 첫번째 과정으로, OsGATA16이 C4 벼를 만들기위한 연구에 쓰일 수 있음을 보여준다. 전사인자인 OsGATA16이 어떤 하위 유전자들의 발현을 조절하는지 확인하기위해 상위 첫번째 잎에서 RNA를 추출해 cDNA를 합성한 후 qRT-PCR을 통해 엽록체 및 엽록소 관련 유전자들의 발현량을 확인하였다. 전반적으로 osgata16 돌연변이체에서 발현이 낮음을 확인하였고, 과발현체를 사용하는 대신 야생형 잎에서 추출한 원형질체에 OsGATA16의 일시적 과발현(transient overexpresssion)에서는 발현이 올

라감을 확인하였다. 특히 이러한 유전자들 중 프로모터 서열에 OsGATA16 단백질의 DNA 결합 모티프가 연속되게 많이 있는 유전자인 OsPORA와 OsPORB에 대해 luciferase assay와 Chromatin Immunoprecipitation (ChIP) assay를 통해 OsGATA16 단백질이 두 유전자 프로모터에 결합하여 upregulation 함을 확인하였다. 이를 통해 애기장대와 마찬가지로 벼에서도 OsGATA16이 엽록체 및 엽록소 발달에 중요한 역할을 함을 밝혀내었다.