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Ectopic Overexpression of The Transcription Factor *OsGLK1* Induces Chloroplast Development in Non-Green Rice Cells

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For systematic and genome-wide analyses of rice gene functions, we took advantage of the full-length cDNA overexpresser (FOX) gene-hunting system and generated >12 000 independent FOX-rice lines from >25 000 rice calli treated with the rice-FOX *Agrobacterium* library. We found two FOX-rice lines generating green calli on a callus-inducing medium containing 2,4-D, on which wild-type rice calli became ivory yellow. In both lines, *OsGLK1* cDNA encoding a GARP transcription factor was ectopically overexpressed. Using rice expression-microarray and northern blot analyses, we found that a large number of nucleus-encoded genes involved in chloroplast functions were highly expressed and transcripts of plastid-encoded genes, *psaA*, *psbA* and *rbcL*, increased in the *OsGLK1*-FOX calli. Transmission electron microscopy showed the existence of differentiated chloroplasts with grana stacks in *OsGLK1*-FOX calli cells. However, in darkness, *OsGLK1*-FOX calli did not show a green color or develop grana stacks. Furthermore, we found developed chloroplasts in vascular bundle and bundle sheath cells of coleoptiles and leaves from *OsGLK1*-FOX seedlings. The *OsGLK1*-FOX calli exhibited high photosynthetic activity and were able to grow on sucrose-depleted media, indicating that developed chloroplasts in *OsGLK1*-FOX rice calli are functional and active. We also observed that the endogenous *OsGLK1* mRNA level increased synchronously with the greening of wild-type calli after transfer to plantlet regeneration medium. These results strongly suggest that OsGLK1 regulates chloroplast development under the control of light and phytohormones, and that it is a key regulator of chloroplast development.

Keywords: Chloroplast development • FOX hunting system • GARP transcription factor • OsGLK1 • *Oryza sativa* • Rice.

Abbreviations: ALA, 5-aminolevulinic acid; CAO, chlorophyllide a oxygenase; Chl, chlorophyll; DIG, digoxigenin; FL-cDNA, full-length cDNA; FOX, full-length cDNA overexpresser; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GltX, glutamyl-tRNA synthetase; HemA, glutamyl tRNA reductase; HemL, glutamate-1-semialdehyde aminotransferase; Hyg, hygromycin B; ICS, isochorismate synthase; NEP, nuclear-encoded plastid RNA polymerase; PEP, plastid-encoded plastid RNA polymerase; PSI, photosystem I; PSII, photosystem II; RT–PCR, reverse transcription–PCR; SAM, shoot apical meristem; SAR, systemic acquired resistance; VB, vascular bundle; VBS, vascular bundle sheath.

A complete set of microarray data was deposited to the Gene Expression Omnibus (GEO) repository under accession number GSE11451.

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Introduction

Proplastids develop into mature organelles, such as chloroplasts, amyloplasts and chromoplasts, during the life cycle of land plants, and the mature organelles perform important functions in various plant vegetative and reproductive organs (Possingham 1980, Link 1991, Kobayashi 1991, López-Juez and Pyke 2005). Among the plastids, the chloroplast 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. In darkness, proplastids are converted into etioplasts, while etioplasts in leaves rapidly differentiate into chloroplasts upon exposure to light, and the leaves exhibit photosynthetic activity. This indicates that plastid development is regulated by a light-related factor(s). Wild-type rice calli grown on 2,4-D-containing media are usually ivory yellow, and proplastids do not develop into chloroplasts in these cells, even under illuminated conditions. However, when such calli are transferred to regeneration medium containing a low level of auxin and a high concentration of cytokinin, chloroplast development is induced and the calli gradually turn green. Therefore, in addition to light, chloroplast development may be regulated by an ingredient(s) such as phytohormone.

Such environmental and hormonal signals are thought to activate transcription factors, which positively regulate gene expression required for chloroplast development. Because components in the photosynthetic apparatus of the chloroplast are encoded in the nuclear and plastid genomes, such transcription factors may coordinate gene expression in both nuclei and plastids. Currently, only a few transcription factors have been described that positively regulate photosynthesis-related genes or plastid differentiation, e.g. HY5 (Oyama et al. 1997, Chattopadhyay et al. 1998, McCormac and Terry 2002), the HY5 homolog HYH (Holm et al. 2002) and HFR1/REP1/RSF1 (Fairchild et al. 2000, Soh et al. 2000, Spiegelman et al. 2000) from Arabidopsis. However, we have not yet clarified the overall mechanism(s) underlying chloroplast development induced by environmental and endogenous factors.

Gain-of-function gene-hunting systems are thought to be powerful tools to explore positively regulating factors involved in chloroplast development. The full-length cDNA overexpresser (FOX) gene-hunting system is a recently developed genome-wide gene screening procedure (Ichikawa et al. 2006, Nakamura et al. 2007, Fujita et al. 2007, Kondou et al. 2009). The Rice Full-Length cDNA Consortium (2003) collected rice full-length cDNAs (FL-cDNAs) and clustered them into 28 469 non-redundant clones. Subsequently, the clones were used to develop the FOX hunting system in rice for systematic functional analysis of rice genes and generated >12 000 FOX-rice lines (Nakamura et al. 2007, Hakata, Nakamura, Okada, Miyao, Kajikawa, Amano, Toki, Pang, Horikawa, Tsuchida-Mayama, Song, Igarashi, Kitamoto, Ichikawa, Matsui, Nagamura, Hirochika and Ichikawa. unpublished). All transgenic rice lines produced ivory-yellow calli, except two independent lines that produced pale-green calli in the presence of 2,4-D. In both exceptions, a FL-cDNA encoding *OsGLK1* was integrated and highly expressed (Nakamura et al. 2007).

OsGLK1 is orthologous to maize *Golden2-like 1* (*ZmGLK1*) gene (Rossini et al. 2001). *ZmGLK1* is homologous to *Golden2* (*G2*), and the maize *g2* mutant shows pale-green leaf blades (Hall et al. 1998). *GLK* genes encode transcription factors that belong to the plant-specific GARP family (Riechmann et al. 2000, Fitter et al. 2002). Arabidopsis GLKs (AtGLK1/GPRI1 and AtGLK2/GPRI2) interact with proline-rich regions of G-box-binding bZIP factors, and both the N-terminal and the C-terminal regions of AtGLKs can transactivate transcription in Arabidopsis cells (Tamai et al. 2002). Double mutants of *AtGLK1* and *AtGLK2* are pale green in all photosynthetic tissues and show reduced granal thylakoids in chloroplasts (Fitter et al. 2002). Overexpression of either *AtGLK1* or *AtGLK2* rescued the pale green phenotype of the double mutant in a cell-autonomous manner (Waters et al. 2008). Yasumura et al. (2005) isolated a pair of *GLK* genes from a moss, *Physcomitrella patens*, and showed that they regulate chloroplast development in the moss. These findings indicate that transcription factors encoded by *GLK* genes positively regulate chloroplast development by a mechanism conserved widely in the plant kingdom.

In this study, overexpression of *OsGLK1* promoted transcription of a set of nucleus-encoded genes related to chloroplast functions and plastid-encoded genes. In the green calli of *OsGLK1*-FOX rice, well-developed thylakoid membranes and grana stacks were observed. The overexpression of *OsGLK1* also induced chloroplast development in vascular bundle (VB) and vascular bundle sheath (VBS) cells of young leaves. *OsGLK1*-FOX calli had much higher chlorophyll (Chl) content and photosynthetic activity than the yellowish-white calli of wild-type rice, and showed photoautotrophic growth. Based on these data, we suggest that GLKs are the main regulators of chloroplast development.

Results

Screening of green calli in FOX-rice lines

In a previous paper (Nakamura et al. 2007), we reported the production of ~12 000 independent FOX-rice lines. To obtain these FOX-rice lines, primary calli derived from >25 000 rice seeds were treated with *Agrobacterium* containing the rice-FOX library, and the treated calli were selected on 2,4-D-containing selection (N6D) medium supplemented with hygromycin B (Hyg) at 30 mg l^{-1}. During selection of *Agrobacterium*-treated calli, we noticed that two lines (AQ190 and BW244) exhibited a pale green color on N6D

medium. Sequence analysis of cDNA-containing fragments obtained by genomic PCR demonstrated that an identical rice FL-cDNA (accession No. AK098909) was integrated into both these lines. Searches in the Rice Annotation Project Database (RAP-DB, http://rapdb.dna.affrc.go.jp/; Ohyanagi et al. 2006, Rice Annotation Project 2007) and the Knowledge-based Oryza Molecular biological Encyclopedia (KOME, http://cdna01.dna.affrc.go.jp/cDNA/; Rice Full-Length cDNA Consortium 2003) revealed that *AK098909* encoded the OsGLK1 protein. We also obtained two additional lines (AI109 and AX290), in which *AK098909* cDNA was integrated, although we could not observe greening of calli in these two lines during the screening of antibiotic-resistant primary calli.

Regenerated plants (T_0 generation) from calli of the two independent lines (AI109 and AQ190) were grown in a greenhouse and their progeny seeds were obtained. Then, calli induced from the seeds of the two lines and a control line transformed with the empty pRiceFOX vector were induced and selected on N6D medium with 30 mg l^{-1} Hyg. All Hyg-resistant (Hyg^R) calli derived from the seeds of both AI109 and AQ190 lines were a pale green color, while those from the control line were ivory yellow (**Fig. 1A**). Furthermore, there was good correlation between the level of greening, Chl content and *OsGLK1* expression in these calli (**Fig. 1A–C**). Since the transcript levels of *OsGLK1* were greatly enhanced in the calli from these two lines (**Fig. 1C**), we designated these lines as *OsGLK1*-FOX lines.

When T_1 seeds of the *OsGLK1*-FOX lines were grown on hormone-free medium with 30 mg l^{-1} Hyg for 4 d, imbibed embryos and germinated shoots turned dark green (**Fig. 1D**). The dark green color, however, did not continue to be significant when compared with the color of control plants as they grew (data not shown).

To confirm whether the greening phenotype of the four lines could be ascribed to overexpression of the *GLK1* gene, an expression vector pRiceFOX-*OsGLK1* was newly constructed by inserting *OsGLK1* FL-cDNA into the pRiceFOX vector and then introduced into rice cells. Subsequently, calli treated with *Agrobacterium* were grown and selected on N6D medium with 30 mg l^{-1} Hyg. As expected, some portions of calli (31 out of 70 Hyg^R blocks) showed a pale green color when treated with pRiceFOX-*OsGLK1*, whereas none of the 70 blocks of Hyg^R calli treated with pRiceFOX exhibited a pale green color. These results confirmed that the green phenotype of the calli was the result of ectopic *OsGLK1* overexpression. Transgenic plants harboring T-DNA from pRiceFOX-OsGLK1 were regenerated and designated as *OsGLK1*-FOX-RE.

Seeds from T_1 progenies of *OsGLK1*-FOX-RE #1, #5, and #7 were sown and grown on N6D medium without Hyg and produced both green and ivory-yellow calli. Green calli were distinguishable from ivory-yellow calli within 5 weeks of sowing. Subsequently, both green and ivory-yellow calli were transferred to N6D medium with 30 mg l^{-1} Hyg. All green calli were resistant to Hyg, whereas ivory-yellow calli were not.

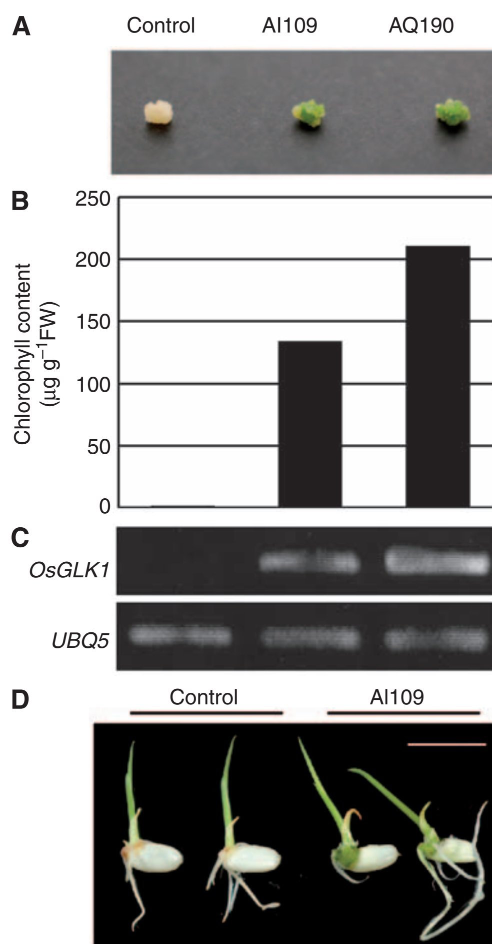


Fig. 1 Phenotypes of *OsGLK1*-FOX rice. (A) Color of calli from a vector control (T_1 generation) and *OsGLK1*-FOX lines, AI109 (T_3) and AQ190 (T_1). Progeny seeds from the transgenic lines were sown and grown on callus-induction medium (N6D) containing 2,4-D at 2 mg l^{-1} (Toki et al. 2006) for 6 weeks. (B) Chl content of the control and the *OsGLK1*-FOX calli shown in (A). (C) Semi-quantitative RT–PCR analysis of calli from the control (T1) and the *OsGLK1*-FOX lines as in (A). Upper panel shows the transcript levels of *OsGLK1* cDNA. Lower panel represents those of *UBQ5* used for loading adjustment. (D) Color of shoots from the control and the *OsGLK1*-FOX (AI109) lines. T_1 seeds were sown and grown on hormone-free medium for 4 d. Scale bar = 1 cm.

Expression of endogenous *OsGLK1* is synchronized with greening of regenerating calli

Wild-type rice calli normally show a green color 7–10 d after transfer to regeneration medium. To test whether the expression level of endogenous *OsGLK1* changes during this period, we performed quantitative real-time RT–PCR analysis using *OsGLK1*-specific primers. The wild-type calli

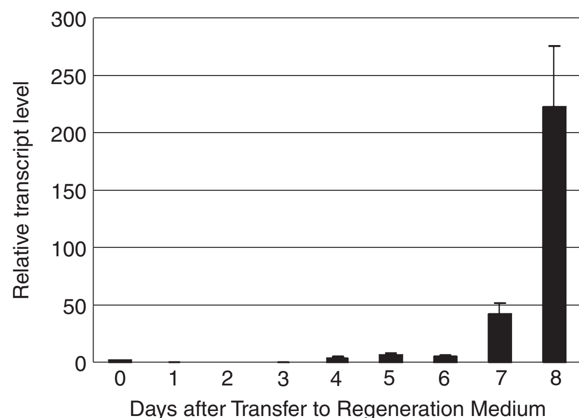


Fig. 2 Induction of *OsGLK1* expression during regeneration of rice calli. Wild-type rice seeds were sown and cultured on N6D medium for 2 weeks and calli were transferred to regeneration medium. Total RNA was extracted on the indicated day. Quantitative real-time PCR was used to assess *OsGLK1* mRNA levels. Data are the mean ± SD of three independent measurements. *OsGLK1* mRNA levels were calculated relative to the abundance of *Actin1* mRNA, with the *OsGLK1* mRNA level of calli at day 0 set to 1.

grown on N6D medium for 14 d were transferred to the regeneration medium, and total RNA was periodically extracted from them. *Actin1* transcript was employed as an internal normalization standard. The *OsGLK1* transcript level began to increase markedly 7 d after transfer to the regeneration medium (**Fig. 2**). Concurrent with the increase in *OsGLK1* mRNA level, the calli began to exhibit a green color 7–8 d after transfer to the regeneration medium (data not shown).

Overexpression of *OsGLK1* induces expression of genes related to chloroplast function

To confirm whether overexpression of *OsGLK1* induces expression of chloroplast-related genes, we conducted an expression microarray analysis of ~44 000 rice genes. Total RNA was extracted from calli derived from T_1 seeds of the AI109 and control lines grown on N6D medium with 30 mg l^{-1} Hyg for 13 d at 30°C, and subjected to 44k oligo-DNA microarray analysis with four biological replicates.

Expression levels of 294 genes were upregulated >5-fold in *OsGLK1*-FOX rice calli compared with the control (**Supplementary Table S1**). Rice genes homologous to the previously reported Arabidopsis genes related to chloroplast function (Leister and Schneider 2003, Eckhardt et al. 2004, Lysenko 2007) were selected by BLAST search. Among them, genes with transcript levels that were markedly increased by overexpression of *OsGLK1* are listed in **Table 1**. The transcript levels for a large number of genes involved in photosystem I (PSI), photosystem II (PSII), intersystemic electron transport, CO_2 assimilation, Chl biosynthesis and the transcriptional machinery required for plastid-encoded gene expression were found to be markedly elevated in AI109 *OsGLK1*-FOX rice calli.

Interestingly, genes involved in the late steps of Chl biosynthesis pathway, *CHLH*, *CHLM*, *CHL27*, *PORA*, *PORB* and *CHLP* (Eckhardt et al. 2004), were highly expressed in the *OsGLK1*-FOX calli, while increases in the expression levels of genes involved in the early steps, which lead to protoporphyrin synthesis, were not obvious in the *OsGLK1*-FOX line (**Table 2**).

The transcript levels of sigma factors, *OsSIG1*, *OsSIG2B*, *OsSIG5*, and *OsSIG6*, were also enhanced in the *OsGLK1*-FOX calli. In various plant species, it is known that the photosystem genes encoded by the plastid genome are transcribed by plastid-encoded plastid RNA polymerase (PEP), which is composed of core enzyme subunits encoded by the plastid genome and a sigma factor encoded by the nuclear genome. Sigma factors play a role in donating specific binding of −35 to −10 promoter motifs to the core PEP complex (Weihe and Börner 1999, Allison 2000). In addition, it has been reported that OsSIG1 and OsSIG5 recognize the *psaA* operon and *psbA* promoter, respectively, in rice (Tozawa et al. 2007, Kubota et al. 2007). In Arabidopsis, transcription of *psbA* and *psaA* is known to be regulated by Sig5 (Tsunoyama et al. 2004), while that of *psbA* and *rbcL* is regulated by Sig6 (Ishizaki et al. 2005, Loschelder et al. 2006). Based on these findings, it was expected that an increase in the transcript levels of sigma factors would lead to an upregulation of the expression of plastid genome-encoded photosystem genes in the *OsGLK1*-FOX calli. Therefore, we analyzed the expression levels of three chloroplast-encoded genes (*psbA*, *psaA* and *rbcL*) by Northern blot hybridization. Predictably, the transcript levels of all three genes were elevated in calli of the *OsGLK1*-FOX lines (**Fig. 3**). In contrast to the increased expression levels of sigma factor genes, transcript levels for the core subunits of PEP (*rpoA*, *rpoB*, *rpoC1* and *rpoC2*) in the *OsGLK1*-FOX calli were almost the same as those in wild-type calli (**Supplementary Fig. S1**).

Ectopic development of chloroplasts in *OsGLK1*-FOX lines

To determine whether chloroplasts developed in the green calli of the *OsGLK1*-FOX lines or not, intracellular structures of calli from *OsGLK1*-FOX lines were observed using a transmission electron microscope. In cells of *OsGLK1*-FOX calli grown under white light, well-developed thylakoid membranes and grana structures were observed in the plastids (**Fig. 4D**), which can be observed in chloroplasts of leaves (**Fig. 4A, B**), but not in the cells of the control callus (**Fig. 4C**). Furthermore, such structures were not observed in cells of *OsGLK1*-FOX calli when grown in the dark (**Fig. 4E, F**). Transcript levels of *RbcS* (accession No. AK068266) and

Table 1 Chloroplast-related genes upregulated in green calli from *OsGLK1*-FOX rice compared with calli from control rice transformed with the empty pRiceFOX vector

Accession No.	Gene product	Mean fold change (OsGLK1-FOX/control)
AK098909	OsGLK1	193.26
(i) PSI subunits		
AK120372	PSI reaction center subunit II (PSI-D)	38.34
AK120598	PSI reaction center subunit IV (PSI-E)	35.96
AK060493	PSI reaction center subunit III (PSI-F)	16.52
AK098847	PSI reaction center subunit V (PSI-G)	27.23
AK060254	PSI reaction center subunit VI (PSI-H)	18.46
AK058788	PSI reaction center subunit X (PSI-K)	122.64
AK058207	PSI reaction center subunit XI (PSI-L)	38.56
AK059037	PSI reaction center subunit N (PSI-N)	97.43
Os05g0242400	PSI reaction center subunit N (PSI-N)	10.49
AK058848	Conserved hypothetical protein (PSI-O)	36.00
AK060904	Chl a/b-binding protein family protein (Lhca1)	80.21
AK104283	Lhca2 protein (Lhca2)	18.31
AK106085	Chl a/b-binding protein type III (Lhca3)	169.12
AK060222	Lhca4 protein (Lhca4)	147.84
AK066291	Lhca5 protein (Lhca5)	12.00
AK067780	Chl a/b-binding protein family protein (Lhca6)	21.74
(ii) PSII subunits		
AK103937	33 kDa subunit of oxygen evolving system of PSII (PsbO)	130.98
AK065248	23 kDa polypeptide of PSII (PsbP)	37.98
CI426428	PSII oxygen evolving complex protein (PsbQ)	5.70
AK121083	PSII 10 kDa polypeptide (PsbR)	31.68
AK058284	PSII subunit PsbS (PsbS)	59.45
AK119161	PSII reaction center W protein (PsbW2)	20.93
AK105813	PSII protein PsbX family protein (PsbX)	105.94
AK060602	PSII core complex proteins psbY (PsbY)	6.04
AK061619	Chl a/b-binding protein 2 (Cab2/Lhcb1)	126.82
AK060851	Chl a/b-binding protein 1 (Cab1/Lhcb1)	53.08
AK058289	Chl a/b-binding protein 1 (Cab1/Lhcb1)	18.61
AK066762	PSII type II Chl a/b-binding protein (Lhcb2)	255.52
AK109399	Type III Chl a/b-binding protein (Lhcb3)	47.46
AK119534	Chl a/b-binding protein CP29 precursor (Lhcb4)	63.38
AK098872	Chl a/b-binding protein family protein (Lhcb5)	22.57
AK066070	Chl a/b-binding protein CP24 (Lhcb6)	73.99
(iii) Intersystemic electron transport		
AK071634	Rieske iron–sulfur protein	6.30
CI439180	Rieske [2Fe–2S] region domain containing protein	4.33
AK120704	Rieske iron–sulfur protein	4.15

continued

Table 1 Continued

Accession No.	Gene product	Mean fold change (OsGLK1-FOX/control)
AK067025	Rieske iron–sulfur protein	3.84
AF093636	Plastocyanin	164.42
AK120393	Ferredoxin I	108.76
AK120232	Ferredoxin I	2.20
AK059896	Ferredoxin	2.58
D17790	Ferredoxin-NADP reductase	9.93
AK106213	Ferredoxin-NADP reductase	126.56
(iv) ATPase		
AK072104	ATP synthase γ-subunit	5.12
Os02g0750100	H^+-transporting ATP synthase δ-subunit	2.11
AK066019	H^+-transporting ATP synthase chain 9-like protein	4.23
(v) CO_2 assimilation		
AK068266	Ribulose 1,5-bisphosphate carboxylase small subunit	217.61
AK121444	Ribulose 1,5-bisphosphate carboxylase small subunit	16.13
AK068555	Ribulose 1,5-bisphosphate carboxylase small subunit	22.68
AK099574	Ribulose 1,5-bisphosphate carboxylase small subunit	14.01
Os12g0291200	Ribulose 1,5-bisphosphate carboxylase small subunit	10.58
AK104332	Ribulose bisphosphate carboxylase activase	62.26
AK066594	3-phosphoglycerate kinase	2.85
AK067755	GAPDH	168.48
AK071685	GAPDH	140.74
AK102013	CP12 protein-like protein	94.00
AK103722	CP12	50.81
AK073758	Fructose 1,6-bisphosphate aldolase	77.34
AK070516	Fructose 1,6-bisphosphatase	31.79
AK065201	Fructose 1,6-bisphosphatase	16.53
AK119209	Sedoheptulose 1,7-bisphosphatase	33.94
AK066306	Ribulose-phosphate 3-epimerase	2.23
AF529237	Phosphoribulokinase	107.57
(vi) Plastid genome expression		
AK065997	Sigma factor SIG1	3.92
AK067693	Sigma factor SIG2B	2.11
AK105697	Sigma factor SIG5	4.00
AK068874	Sigma factor SIG6	50.17

A data set for all the upregulated genes by overexpression of *OsGLK1* cDNA with mean fold change values of >5.0 can be found in **Supplementary Table SI** on line. A complete set of microarray data was deposited to the Gene Expression Omnibus (GEO) repository under accession number GSE11451.

Table 2 Changes in transcript level of rice genes involved in Chl biosynthesis in the microarray analysis

Accession No.	Gene product	Mean fold change	P value
AK099931	Glutamyl-tRNA synthetase (GltX)	1.07	0.452
AK099393	Glutamyl-tRNA reductase (HemA)	1.13	0.451
AK064826	Glutamate-1-semialdehyde aminotransferase (HemL)	1.60	<0.001
AK101836	Delta-aminolevylinic acid dehydratase (Alad)	1.44	<0.001
AK060914	Porphobilinogen deaminase	1.08	0.793
AK107127	Uroporphyrinogen III (HemD)	1.28	0.003
AK070859	Uroporphyrinogen decarboxylase (HemE)	1.88	<0.001
AK106203	Uroporphyrinogen decarboxylase (HemE)	1.15	0.298
AK070391	Coproporphyrinogen III oxidase (Lin2)	1.31	0.084
AK108365	Protoporphyrinogen (PpxI)	1.19	0.406
AK060389	Magnesium-chelatase subunit chlI (ChlI)	1.62	0.007
AK072463	Magnesium-chelatase subunit chlD (ChlD)	1.51	0.003
AK067323	Magnesium-chelatase subunit chlH (ChlH/Gun5)	7.18	<0.001
AK059151	Magnesium-protoporphyrin *O*-methyltransferase (ChlM)	5.42	<0.001
AK069333	Magnesium-protoporphyrin IX monomethyl ester aerobic oxidative cyclase (Chl27)	26.81	<0.001
AK103940	Protochlorophyllide reductase (PorA)	219.73	<0.001
AK068143	NADPH:protochlorophyllide oxidoreductase (PorB)	24.88	<0.001
AK061968	Geranylgeranyl reductase (ChlP)	5.10	<0.001
AK068855	Chl synthase (ChlG)	1.12	0.107
AF284781	Chlorophyllide a oxygenase 1 (Cao1)	1.92	0.002
AK063367	Chlorophyllide a oxygenase 2 (Cao2)	0.38	0.024

Results of differential expression of individual genes for Chl biosynthesis between the *OsGLK1*-FOX callus and the control callus are shown with mean fold changes and *P* values (*t*-test).

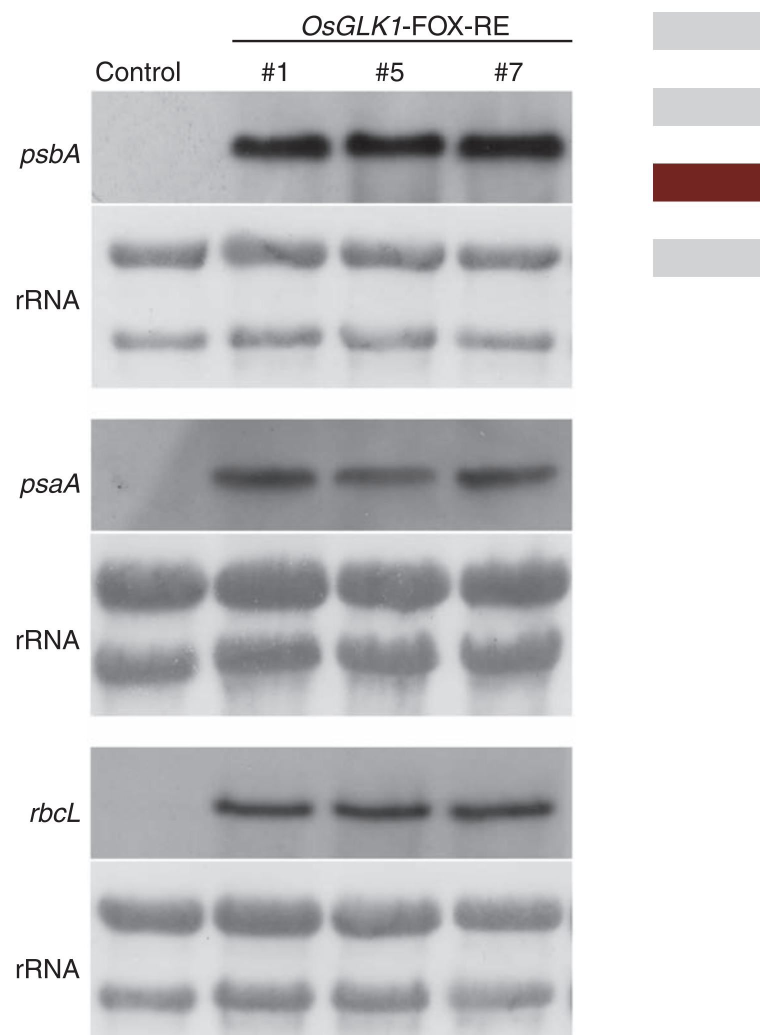


Fig. 3 Expression of three plastid-genome-encoded genes, *psbA*, *psaA* and *rbcL*, in the calli of *OsGLK1*-FOX and control lines. Total RNA was isolated from calli of the *OsGLK1*-FOX (RE #1, #5 and #7) and control lines grown on N6D medium with 30 mg l^{-1} hygromycin B (Hyg) for 7 d after sowing. Northern blot hybridization was performed as described in Materials and Methods.

a photosynthetic glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*: AK067755) genes, which were markedly increased in light-grown *OsGLK1*-FOX calli (**Supplementary Table S1**), were not enhanced in dark-grown *OsGLK1*-FOX calli (**Fig. 5**). These observations indicate that overexpression of *OsGLK1* ectopically induces light-dependent development of chloroplasts.

As described above, the green color in the germinated shoots from *OsGLK1*-FOX seeds was darker than that in control shoots during the early growth stage (**Fig. 1D**). We prepared cross-sections of leaves from AQ190 *OsGLK1*-FOX and control lines, and observed them using an optical microscope. The darker green color was observed in the coleoptiles and young leaves of 4-day-old *OsGLK1*-FOX rice seedlings (**Fig. 6A, B**). In addition, chloroplast development was prominent in the VBs of *OsGLK1*-FOX coleoptiles (**Fig. 6D**), while few chloroplasts were observed in the

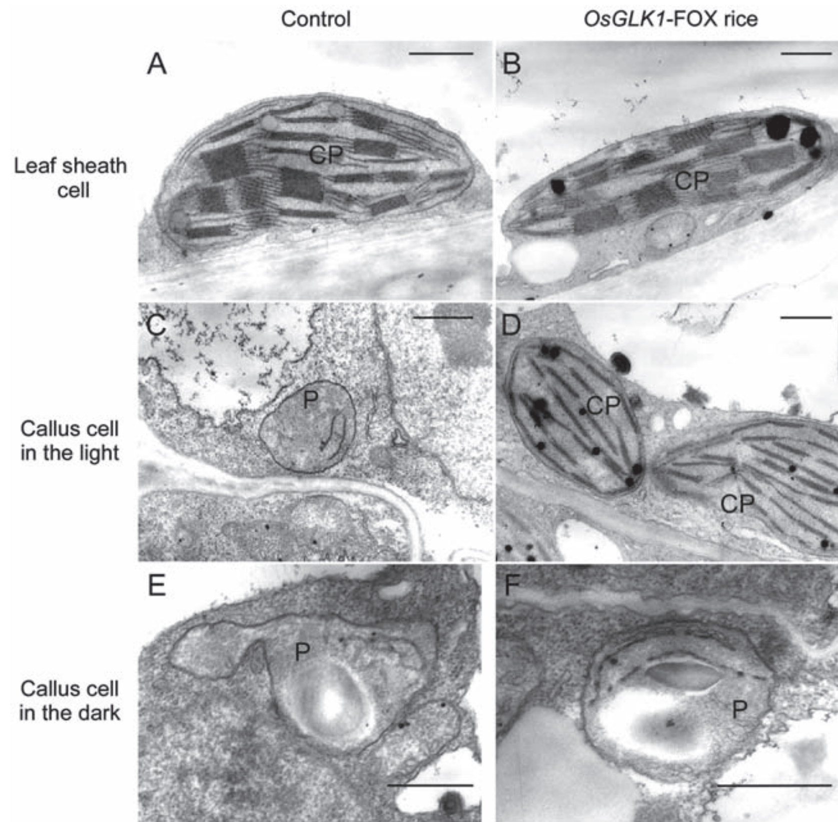


Fig. 4 Chloroplast and proplastid ultrastructures in control rice (A, C, E) and *OsGLK1*-FOX rice (B, D, F). Leaf sheath cells of 4-day-old T_1 seedlings grown on hormone-free medium with 30 mg l^{-1} Hyg (A, B). T_1 calli grown on N6D + Hyg medium for 14 d under light (C, D) and dark (E, F) conditions. Scale bars = 1 μm. CP, chloroplast; P, proplastid.

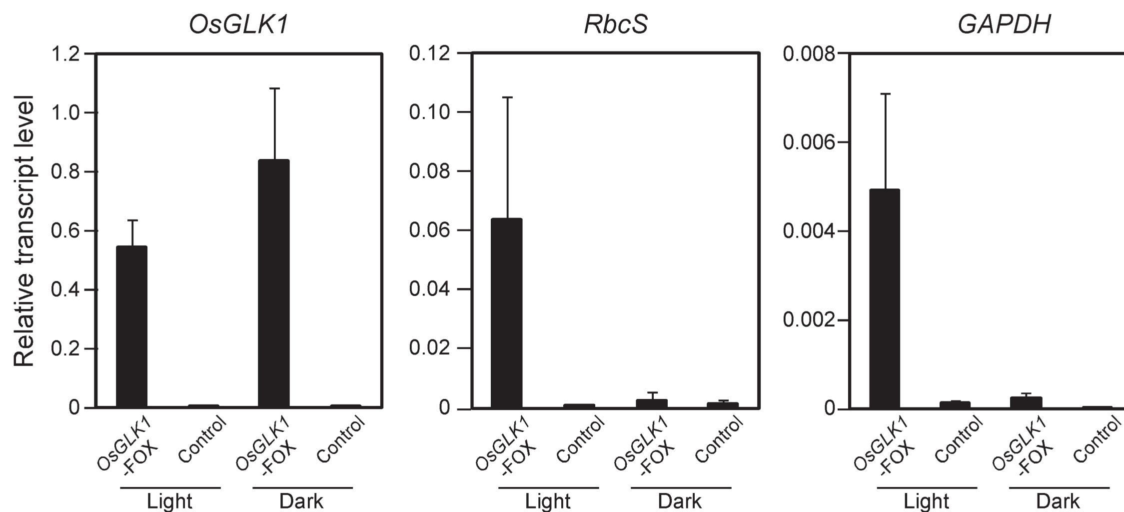


Fig. 5 Expression levels of *OsGLK1*-upregulated genes in *OsGLK1*-FOX rice calli under light and dark conditions. Total RNA was isolated from calli of the *OsGLK1*-FOX (AI109) and control lines grown on N6D medium with 30 mg l^{-1} Hyg for 14 d after sowing under continuous light and dark conditions, and subjected to real-time PCR analysis using primers that specifically amplify *OsGLK1*, *RbcS* (accession No. AK068266) and plastid-type *GAPDH* (AK067755) cDNAs. RNA levels were quantified and normalized to the levels of corresponding *Actin1* mRNA, which was assigned a value of 1. Error bars indicate SD for three biological replicates.

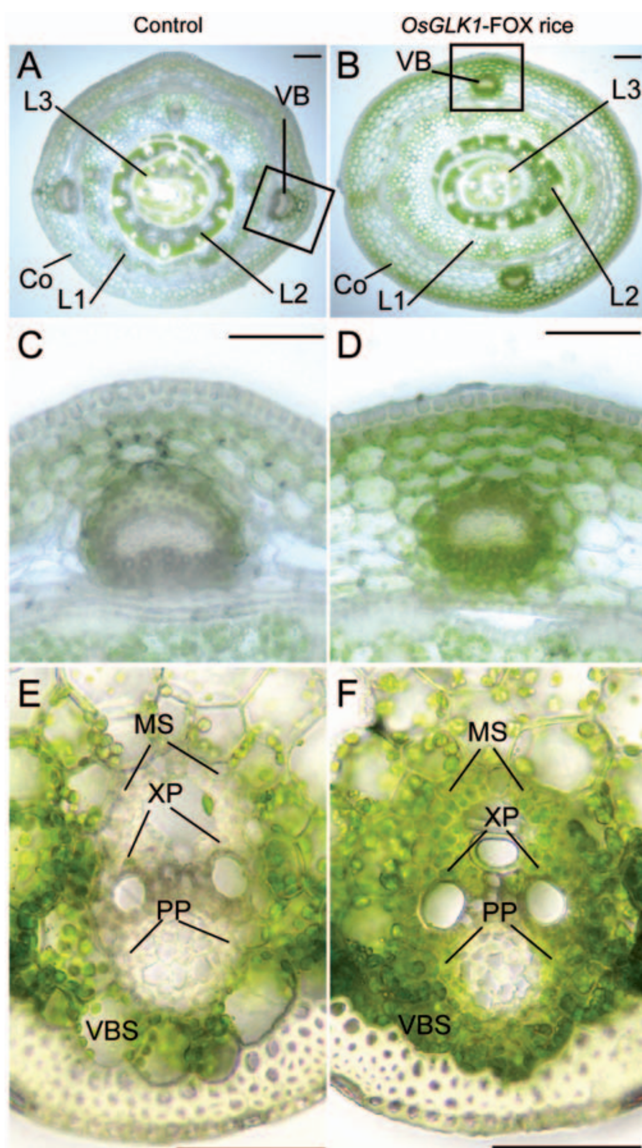


Fig. 6 Cross-sections of leaves from control (A, C, E) and *OsGLK1*-FOX (B, D, F) rice. (A, B) Cross-sections of leaves of 4-day-old T_1 seedlings grown on hormone-free medium with 30 mg l^{-1} Hyg. (C, D) Magnified views of VB in coleoptiles surrounded by rectangular boxes in (A) and (B), respectively. (E, F) VB and VBS cells of leaf sheaths of 14-day-old T_1 shoots. Scale bars = 100 µm (A–D) and 50 µm (E, F). VB, vascular bundle; VBS, vascular bundle sheath; MS, mestome sheath; XP, xylem parenchyma cells; PP, phloem parenchyma cells; Co, coleoptile; L1, first leaf; L2, second leaf; L3, third leaf.

control coleoptiles (**Fig. 6C**). In 14-day-old *OsGLK1*-FOX rice plants, xylem and phloem parenchyma cells of VB, and VBS and mestome sheath cells in leaf sheaths exhibited a green color (**Fig. 6F**), while the corresponding cells in wild-type plants exhibited little green coloration (**Fig. 6E**). In addition, it is noteworthy that the *OsGLK1*-FOX rice accumulated

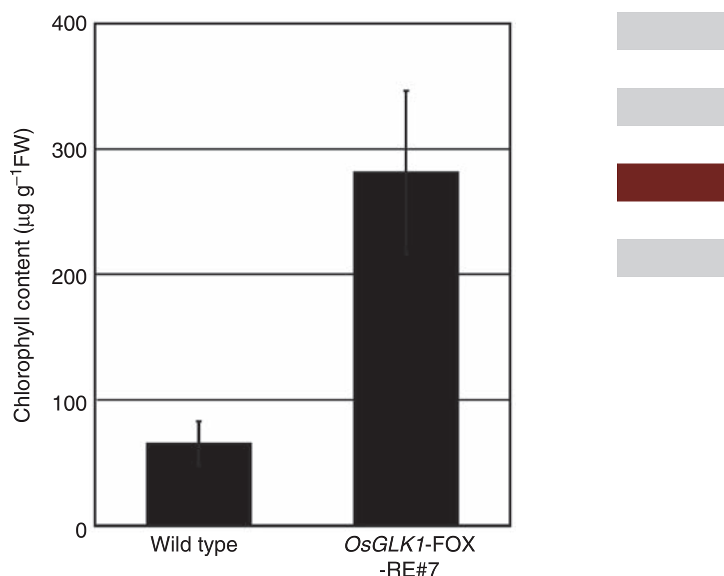


Fig. 7 Chl content in roots of control and *OsGLK1*-FOX lines. Seedlings were grown on solid hormone-free medium for 15 d under continuous illumination, before roots were collected for determination of the Chl content. The data are mean ± standard error (n = 3).

significant levels of Chl in roots (**Fig. 7**). However, we did not detect obvious differences in the Chl content of the uppermost leaf blades between the *OsGLK1*-FOX rice and control rice at this age (data not shown). Furthermore, developed chloroplasts observed in the VB and VBS cells of 14-day-old *OsGLK1*-FOX rice plants could not be detected in those cells of 2-month-old plants (data not shown). These observations demonstrate that overexpression of the *OsGLK1* gene induces the development of chloroplasts at least in coleoptiles and leaf sheaths of young plants both ectopically and orthotopically.

Chl content and photosynthetic activity of *OsGLK1*-FOX calli

Substantial amounts of Chl were present in the green calli of *OsGLK1*-FOX lines, whereas the Chl content in the control calli was extremely low (**Fig. 8A**). Photosynthetic activity, measured as light-dependent O_2 evolution, was detected in the *OsGLK1*-FOX calli, but not in the control calli (**Fig. 8B**).

Although no obvious difference in the growth of cultured cells was observed between *OsGLK1*-FOX and control lines when cultivated on N6D agar medium containing a standard concentration of 3% sucrose, the *OsGLK1*-FOX calli grew better than the control calli on N6D medium containing only 0.1% sucrose (one-thirtieth of the standard). Moreover, the fresh weight of *OsGLK1*-FOX calli was significantly higher than that of the control calli, even on N6D medium without sucrose after 2 weeks of tissue culture (**Fig. 9A, B**).

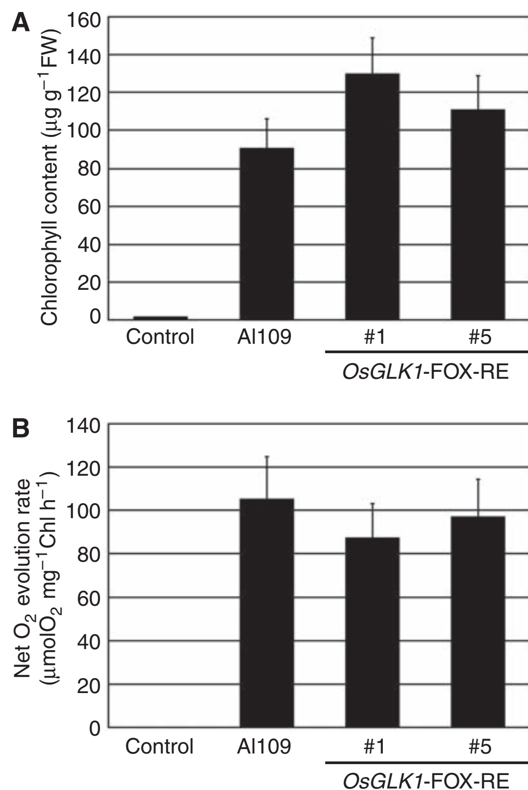


Fig. 8 Chl content and photosynthetic activity in calli of the *OsGLK1*-FOX and control lines. (A) Chl content of calli of wild-type, *OsGLK1*-FOX lines (AI109, RE #1 and #5) lines. The data are mean ± SD of at least three independent measurements. (B) The net O_2 evolution rates under saturated light and saturated CO_2 concentrations in calli of the control and *OsGLK1*-FOX lines. The data are mean ± SD of at least three independent experiments.

These results indicate that the *OsGLK1*-FOX cultured cells have the ability to proliferate photoautotrophically, and that their developed chloroplasts induced by the overexpression of *OsGLK1* are both functional and active.

Discussion

OsGLK1 positively regulates chloroplast development in rice

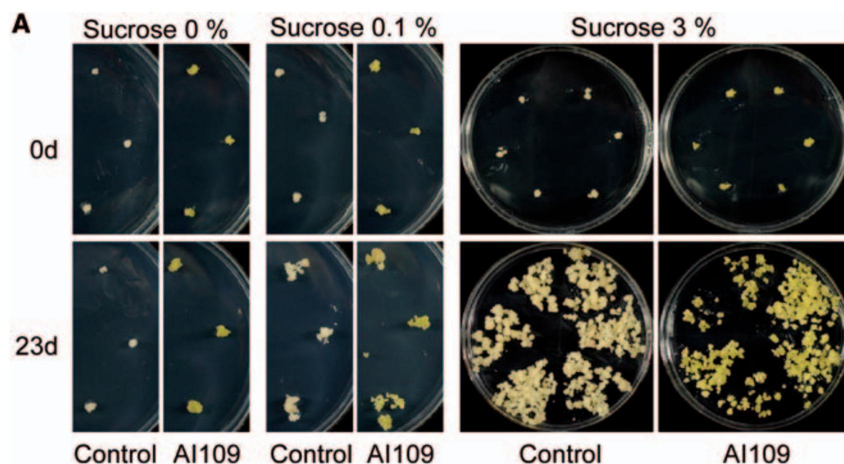
Calli of the *OsGLK1*-FOX line grown under white light on medium containing 2,4-D exhibited a pale green color and had developed chloroplasts, while the control calli did not (**Figs. 1**, **4**). Similar results were obtained in VB and VBS cells in coleoptiles and leaf sheaths of the *OsGLK1*-FOX and control lines (**Fig. 6**). The *OsGLK1*-FOX calli had a high Chl content, high photosynthetic activity level (**Fig. 8**), and were able to grow on a sucrose-depleted medium (**Fig. 9**), suggesting that the developed chloroplasts were functional and active. Rossini et al. (2001) showed that *OsGLK1* transcripts do not accumulate in roots, but accumulate in leaf sheaths and leaf blades, speculating that OsGLK1 is involved in photosynthetic development. Consistent with their speculation, we showed that the expression of *OsGLK1* was synchronized with greening of calli (**Fig. 2**). Considering that chloroplast development was severely impaired in Arabidopsis double mutants of *Atglk1* and *Atglk2* and in the moss *Physcomitrella patens* double mutants of *Ppglk1* and *Ppglk2* (Fitter et al. 2002, Yasumura et al. 2005), we strongly suggest that GLKs are positive regulators of chloroplast development in the plant kingdom.

OsGLK1 upregulates plastid-encoded genes possibly through enhanced expression of nucleus-encoded plastid genes

Microarray analysis showed that transcript levels of a large number of genes related to chloroplast functions were elevated in calli from the *OsGLK1*-FOX line compared with those from the control (**Tables 1**, **2** and **Supplementary Table S1**). It is notable that the expression levels of genes encoding sigma factors, which regulate the expression of plastid-encoded genes, were higher in the microarray data (**Table 1**). In addition, Northern blot analysis revealed that the transcript levels of three plastid-encoded genes, *psaA*, *psbA* and *rbcL*, were also higher in *OsGLK1*-FOX calli than in control calli (**Fig. 3**). These data indicate that OsGLK1 regulates chloroplast development by elevating transcript levels of nuclear-encoded genes for chloroplast functions, and also plastid-encoded genes for photosynthetic machinery by activating expression of nuclear-encoded sigma factor genes (summarized in **Fig. 10**).

In particular, overexpression of *OsGLK1* caused a significant increase in the expression level of *OsSIG6* among sigma factor genes (**Table 1**). In Arabidopsis, SIG6 plays a key role in chloroplast development in young seedlings, and transcript levels of PEP-dependent genes, including *rbcL*, *psbA* and *psaA*, are significantly decreased in 4-day-old seedlings of Arabidopsis *sig6* mutant (Ishizaki et al. 2005, Loschelder et al. 2006). We observed a dark-green color in young shoots of *OsGLK1*-FOX rice (**Fig. 1D**). Moreover, PEP-dependent genes (*rbcL*, *psbA* and *psaA*) were upregulated in green calli from *OsGLK1*-FOX rice (**Fig. 3**). These observations may be mediated by the upregulation of *OsSIG6* expression, though it is not known whether OsSIG6 functions in young seedlings similarly to Arabidopsis SIG6.

Contrary to the enhanced expression of sigma factor genes, expression levels of genes for PEP core subunits were not increased in the *OsGLK1*-FOX calli (**Supplementary Fig. S1**), indicating that transcription of the PEP core subunit genes is independent of OsGLK1. The result further suggested that the amounts of PEP core subunits in rice calli are sufficient to generate photosynthetically active plastids (**Fig. 8B**) in combination with *OsGLK1* overexpression, and that transcript levels of the PEP-dependent genes are largely



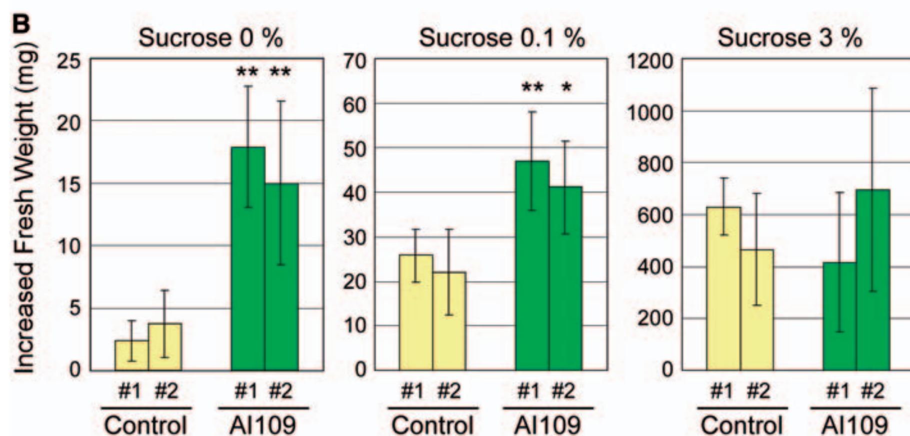


Fig. 9 Autotrophic growth of *OsGLK1*-FOX calli. (A) Rice calli of the AI109 and control lines were grown on N6D medium with 30 mg l^{-1} Hyg for 2 weeks at 28°C and then transferred to fresh N6D medium with or without sucrose at concentrations indicated in the figure. Photographs are representative of the calli grown in fresh N6D medium for the indicated period. (B) Fresh weights of calli were measured at 0 and 23 d after transfer to fresh medium, and the difference between the two weights was calculated. The bars are the mean ± SD of six blocks of calli of indicated lines. Each asterisk indicates that the increase in fresh weight was significantly different from that of the Control #1 line (Student's *t*-test; $*P < 0.05$, $**P < 0.005$).

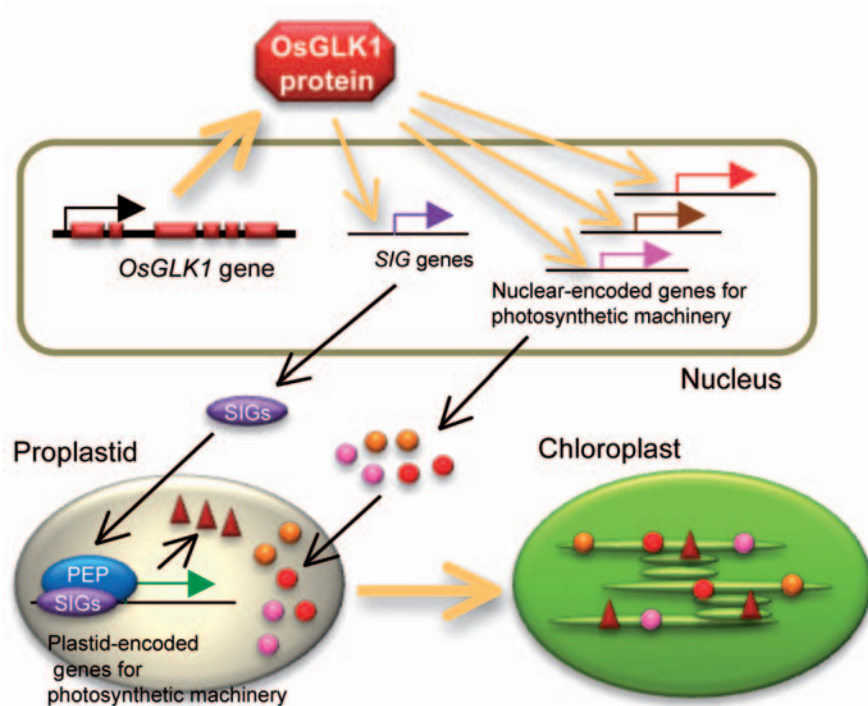


Fig. 10 A schematic model illustrating the role of OsGLK1 in expression of nuclear- and plastid-encoded genes for photosynthetic machinery and chloroplast development. OsGLK1 upregulates expression of sigma factor (*SIG*) genes and other nuclear-encoded genes for photosynthetic machinery. Then SIGs, together with PEP core subunits, induce expression of plastid-encoded genes for photosynthetic machinery. Consequently, proplastids develop into chloroplasts and acquire photosynthetic function.

affected by the activity of sigma factors. However, comparing the grana stacks in plastids between leaf-sheath cells (**Fig. 4A, C**) and light-grown *OsGLK1*-FOX cultured cells (**Fig. 4D**), chloroplasts in the *OsGLK1*-FOX green calli were not fully developed. Accordingly, it is likely that the amounts of PEP core subunits in the plastids are still insufficient to induce fully developed chloroplasts. Expression of *rpoB*, encoding a PEP core subunit, is shown to be under the control of a nuclear-encoded plastid RNA polymerase (NEP) (Silhavy and Maliga 1998, Liere and Maliga, 1999, Shiina et al. 2005). In our microarray analysis, the expression level of *OsRpoTp* gene (accession No. AK069977) encoding an NEP enzyme (Kusumi et al. 2004) was not enhanced by the overexpression of *OsGLK1* (data not shown). This inability of OsGLK1 to enhance the NEP transcription system could be an explanation of why the chloroplasts were not fully developed in the *OsGLK1*-FOX calli. Our findings also implied a role of transcription factor(s) other than OsGLK1 in the accumulation of *OsRpoTp* transcripts.

Upregulation by OsGLK1 of genes encoding the late steps of Chl biosynthesis

We found that calli from *OsGLK1*-FOX lines synthesized substantial levels of Chl (**Fig. 8A**). In plants and algae, it is widely accepted that the rate-limiting step in the Chl biosynthesis pathway is the initial step, i.e. the synthesis of 5-aminolevulinic acid (ALA) (Papenbrock and Grimm 2001, Tanaka and Tanaka 2007). ALA is synthesized from glutamate in three steps of catalysis, by glutamyl-tRNA synthetase (GltX), glutamyl-tRNA reductase (HemA) and glutamate-1-semialdehyde aminotransferase (HemL). It has been reported that transcription of *HEMA1* is upregulated by light during de-etiolation in Arabidopsis (McCormac et al. 2001). However, the expression levels of rice *GLTX* (accession No. AK099931) and *HEMA* (AK099393) in greening *OsGLK1*-FOX calli were almost the same as those in wild-type calli (mean difference 1.13:1 and 1.07:1, respectively), while the expression level of *HEML* (AK064826) was only slightly higher (mean difference 1.60:1) in greening *OsGLK1*-FOX calli (**Table 2**). In addition, the expression levels of genes related to the late steps of Chl biosynthesis, such as the reduction of protochlorophyllide to chlorophyllide, were significantly elevated in *OsGLK1*-FOX rice (**Table 2**). These results suggest that expression of genes involved in the early steps of Chl biosynthesis are regulated by factor(s) other than OsGLK1, whereas the genes involved in the late steps of Chl biosynthesis are under the control of OsGLK1.

Possible involvement of GLK1 in the defense response

Savitch et al. (2007) showed a subset of Arabidopsis genes that were highly upregulated by overexpression of *AtGLK1*, and designated them the 'GLK1 regulon'. However, almost all known chloroplast-related genes were not included in the GLK1 regulon. The *AtGLK1* gene was expressed at a low level in leaves of wild-type Arabidopsis (Savitch et al. 2007). We assume that the level of endogenous *AtGLK1* expression was sufficient for the induction of the expression of chloroplast-related genes, and further expression of *AtGLK1* did not affect their expression.

Savitch et al. (2007) also reported that overexpression of *AtGLK1* upregulated the expression of a subset of genes encoding disease defense-related genes and conferred resistance against *Fusarium graminearum*, a broad host pathogen. The expression of only one rice homolog among them, which encodes isochorismate synthase (ICS), a key enzyme in salicylic acid biosynthesis and required for systemic acquired resistance (SAR) (Wildermuth et al. 2001), was markedly upregulated by the overexpression of *OsGLK1* in our microarray analysis (mean fold change = 24.7, **Supplementary Table S1**), while others were not (data not shown). Meanwhile, despite the upregulation of the *ICS*-homolog gene, no transcript accumulations of *PR-1a* or *PR-1b*, both of which are molecular markers of SAR (Agrawal et al. 2000a, Agrawal et al. 2000b), were observed. Regardless, we cannot exclude the possibility that OsGLK1 regulates gene expression of disease defense-related genes in leaves or other rice organs because we only used calli during our microarray analysis. It would be interesting to investigate whether *OsGLK1*-FOX rice is resistant to pathogen invasion, as observed in *AtGLK*-overexpressing Arabidopsis plants.

Overlapped functions of OsGLK1 and OsGLK2 in plastid differentiation

Although we have shown that ectopic overexpression of *OsGLK1* can induce chloroplast development in calli as well as in VB and VBS cells in young shoots, it remains unclear where and when the native OsGLK1 acts in the rice plant. Our current suggestion is that OsGLK1 facilitates chloroplast development during the early stages of shoot growth in a light-dependent manner because shoots and embryos were dark green in seedlings of the *OsGLK1*-FOX lines and reverted to a normal color as they grew. This suggestion is supported by the observation that the transcript level of *OsGLK1* is higher in leaves of young seedlings than in mature leaves (Rossini et al. 2001).

As mentioned above, Fitter et al. (2002) showed that *Atglk1 Atglk2* double mutant plants were pale green throughout development and had reduced granal thylakoids in chloroplasts, whereas no phenotype specific to *Atglk1* single mutant plants was observed, suggesting that AtGLK1 and AtGLK2 are functionally redundant. Furthermore, overexpression of either *AtGLK1* or *AtGLK2* suppressed various phenotypes of *Atglk1 Atglk2* double mutants, such as pale green leaves and siliques, low levels of *LHCB6* transcript and early flowering under long-day conditions, confirming

that AtGLK1 and AtGLK2 are functionally equivalent (Waters et al. 2008). Rice also has two GLK genes, *OsGLK1* and *OsGLK2*, and these *OsGLK*s show overlapping expression patterns, suggesting that the roles of OsGLK1 and OsGLK2 may be similar (Rossini et al. 2001). Consistent with these results, we observed that calli of *OsGLK2*-FOX lines show a green color on N6D medium, and *OsGLK2* exhibits similar expression patterns during regeneration of the wild-type callus (data not shown). To assess the roles of OsGLKs further, we are producing single and double knockout/knockdown lines of *OsGLK* genes.

Post-transcriptional regulation of OsGLK1 function under different light conditions

The transcript levels of *ZmG2*, *ZmGLK1*, *OsGLK1*, *OsGLK2*, *AtGLK1* and *AtGLK2* are regulated by light (Rossini et al. 2001, Fitter et al. 2002), while the transcript levels of *Brassica GLK1* and *GLK2* are induced by cold stress (Savitch et al. 2005). In rice, development of membrane structures in chloroplasts of *OsGLK1*-FOX calli was not observed in the dark (**Fig. 4**), even though *OsGLK1* was highly expressed. In addition, expression of a subset of chloroplast-related genes was not markedly affected by overexpression of *OsGLK1* in darkness (**Fig. 5**). These results strongly suggest that the function of OsGLK1 might be post-transcriptionally regulated by light.

The mechanism by which light enables OsGLK1 to function is unclear; however, we propose the following hypotheses: (i) signaling from light activates OsGLK1 by modifications such as phosphorylation; (ii) OsGLK1 requires a light-regulated accompanying factor(s); (iii) in darkness, the function of OsGLK1 is downregulated by signals, e.g. COP1 signals like HY5, LAF1 and HFR in Arabidopsis (Osterlund et al. 2000, Seo et al. 2003, Jang et al. 2005); (iv) the function of GLK1 is suppressed by a factor(s) that is/are downregulated by light (e.g. phytochrome interacting factors; Castillon et al. 2007). To investigate these hypotheses, we are producing an anti-OsGLK1 antibody and a yeast two-hybrid system to analyze factors that accompany OsGLK1.

Niwa et al. (2006) produced Arabidopsis green callus by overexpressing *CES101*, which encodes a receptor-like kinase. Furthermore, AtGLK1 may possibly regulate or be regulated by CES101 in Arabidopsis. Although we did not find rice cDNA significantly similar to CES101 in our BLAST search, it would be interesting to investigate whether OsGLK1 and OsCES101 facilitate the same signaling pathway in the induction of chloroplast development.

OsGLK1 upregulates a set of genes for chloroplast function similarly to AtGLK1

During preparation of this paper, Waters et al. (2009) reported that GLKs coregulate and synchronize expression of a suite of nuclear photosynthetic genes in Arabidopsis. AtGLK1- and AtGLK2-regulated genes in their microarray data are similar to the OsGLK1-regulated genes in our microarray data, suggesting the existence of common roles of GLKs in chloroplast development between these two distantly related species. Furthermore, they showed that GLKs directly bind to promoters of several photosynthesis genes, implying that GLKs directly upregulate expression of a set of photosynthesis-related genes. It is likely that OsGLKs also elevate transcript levels of chloroplast-related genes by binding directly to their promoters. However, there are differences between the results in these two studies. In Arabidopsis, the transcript levels of *RbcS1* (a nucleus-encoded small subunit of RuBisCO) were not affected by *GLK* overexpression or *glk1 glk2* mutants, whereas expression of nucleus-encoded *RbcS* genes were highly elevated by *OsGLK1* overexpression (**Table 1**). GLK-upregulation profiles of the Chl biosynthesis pathway were similar, with the exception that *GLK1* overexpression markedly promoted the transcript level of *CAO*, encoding the chlorophyllide a oxygenase, with a 13.6-fold increase in Arabidopsis (Waters et al. 2009). *OsGLK1* overexpression, however, promoted expression of *CAO1*, transcription of which is positively regulated by light (Lee et al. 2005), only by 1.92-fold. Interestingly, expression of *CAO2*, the second *CAO* gene in rice whose expression is repressed under light and higher in darkness (Lee et al. 2005), was downregulated by *OsGLK1* overexpression (**Table 2**).

Among the progenies of our *OsGLK1*-FOX lines, we are currently selecting lines with a single copy of the transgene insertion per haploid genome and homozygous for the insertion. We also have been producing transgenic rice plants bearing the antisense *OsGLK1* construct and those with the chimeric *OsGLK1* repressor (Hiratsu et al. 2003) to further characterize the unique roles of rice GLK transcription factors in chloroplast differentiation. We expect that these lines may contribute not only to the basic aspects (e.g. uncovering mechanisms of chloroplast development), but also to applied aspects (e.g. genetic engineering for crop improvement), since rice is one of the most important crops and is recognized as the best model plant among the monocot crops (Khush 2005).

During the preparation of this paper, Kakizaki et al. (2009) reported that GENOMES UNCOUPLED 1 (GUN1), a chloroplastic pentatricopeptide repeat protein, downregulates expression of photosynthesis-related nuclear genes through suppression of *AtGLK1* expression. Then, the authors proposed that AtGLK1 acts as a positive regulator not only for the expression of photosynthesis-related nuclear genes, but in a plastid-to-nucleus signaling pathway that coordinates plastid protein import and nuclear photosynthetic gene expression. Since a putative GUN1 ortholog has been found in rice (Koussevitzky et al. 2007), it may be interesting to know whether the provisional GUN1 could play a similar

role in plastid-to-nucleus signaling by regulating *OsGLK1* gene expression in rice.

Materials and Methods

Plant materials, growth conditions and rice transformation

Cultured cells, regenerated plants and seedlings of rice (*Oryza sativa* L., cultivar 'Nipponbare') were grown aseptically in growth chambers under constant white fluorescent light (70 µmol photons $m^{-2}s^{-1}$) at 30°C. When transgenic rice plants were further grown in soil, the plants were transferred to growth chambers with irradiation of white light (~300 µmol $m^{-2}s^{-1}$) at a cycle of 14 h light (28°C)/10 h dark (25°C). Rice transformation was performed and growth media were prepared as previously described (Toki et al. 2006).

Plasmid construction

To prepare pRiceFOX-*OsGLK1*, a fragment of full-length *OsGLK1* cDNA was excised with *Sfi*I from pFLC1B-OsGLK1, the *OsGLK1* FL-cDNA (AK098909) clone in λ-FLC-1-B vector (Rice Full-Length cDNA Consortium 2003), and then inserted into compatible *Sfi*I sites of pRiceFOX (Nakamura et al. 2007) in the forward orientation.

The pRiceFOX and the pRiceFOX-*OsGLK1* plasmids were transferred into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by electroporation.

RNA preparation and RT–PCR

An RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions to extract total RNA from the leaf blades of each transgenic plant grown for 2 weeks after their transfer to soil. First-strand cDNAs were synthesized from each RNA preparation (1 µg/reaction) with an oligo(dT) primer using an ExScript RT reagent kit (Takara Bio Inc., Otsu, Japan) in a total volume of 20 µl, in accordance with the manufacturer's instructions.

The specific sequences of the primers for amplifying cDNA for *OsGLK1* (5′-AGCTGCGAGATTTCCTGCTC-3′ and 5′-ATAGCTGCGTCGATGCTCTC-3′), for *RbcS* (5′-CCC GGATACTATGACGGTAGG-3′ and 5′-AACGAAGGCATCA GGGTATG-3′), and for *GAPDH* (5′-GTGGCCAACATTAT CAGCAA-3′ and 5′-GGTCATGGTTCCCTTTACGA-3′) were used in semi-quantitative and quantitative RT–PCR. The primer pairs for amplifying *Actin1* cDNA (AK100267; 5′-CTTCATAGGAATGGAAGCTGCGGGTA-3′ and 5′-TTCCT GTGCACAATGGATGG-3′) or *UBQ5* cDNA (AK061988; 5′-ACCACTTCGACCGCCACTACT-3′ and 5′-ACGCCTAAGCC TGCTGGTT-3′) were used as loading controls for semi-quantitative RT–PCR. The *Actin1* primer pair was also used as an internal control for normalization of quantitative RT–PCR (Jain et al. 2006).

Semi-quantitative RT–PCR was performed with 1 µl of cDNA template per 50 µl reaction with TaKaRa Ex *Taq* for 30 s at 94°C, followed by 28 cycles of 10 s at 98°C, 30 s at 60°C and 30 s at 72°C. Quantitative RT–PCR was performed with a Thermal Cycler Dice Real Time system (Takara Bio) using SYBR Premix Ex *Taq* (Takara Bio) and 1 µl of cDNA template per 25 µl reaction, in accordance with the manufacturer's instructions. The threshold cycle (Ct) was auto-calculated by the analysis software that accompanied the system. The expression level, normalized to that of the endogenous control gene (*Actin1*), was calculated by relating the measured Ct to a standard curve obtained by diluting PCR-amplified DNA for which the exact DNA concentration was known.

Microarray analysis

T_1 seeds of the AI109 and vector control lines were sown and grown for 13 d on callus-induction medium containing 2,4-D at 2 mg l^{-1}. Total RNA was isolated from each callus using an RNeasy plant mini kit (Qiagen). The RNAs (400 ng aliquots) were labeled with a Low RNA Input Linear Amplification/Labeling Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Aliquots of Cy5-labeled cRNA (1 µg each) of the AI109 sample and Cy3-labeled cRNA (1 µg each) of the vector control samples were used for hybridization in a Rice Gene Expression Microarray (4×44k; Agilent Technologies). Four biological replicate sample sets were analyzed. After hybridization, microarray slides were scanned (scanner model G2505B with G2565BA software; Agilent Technologies) and the data were analyzed using Feature Extraction software (version 9.1; Agilent Technologies) at the default settings. All microarray procedures and data analyses were performed according to the manufacturer's manual.

Northern blot analysis

Northern blot hybridization was performed according to standard protocols (Sambrook et al. 1989). Total RNA was isolated from 7-day-old calli using a Total RNA Isolation Kit (Cartagen Molecular Systems Inc., San Carlos, CA, USA) according to the manufacturer's instructions. Total RNA (2–8 µg) was fractionated by electrophoresis on a 1.2% (w/v) agarose gel with 0.6 M formaldehyde and blotted onto a nylon membrane (Hybond-N+; GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK). Equal loading of total RNA in all lanes was checked by methylene blue staining. Hybridization and detection were performed according to the standard protocol for digoxigenin (DIG) hybridization (Roche Diagnostics, Rotkreuz, Switzerland).

To generate gene-specific probes, the following primers were used to obtain PCR fragments of each gene from the

genomic DNA of rice: *psaA* forward (5′-ATGATGATTCG TTCGCCGGA-3′) and reverse (5′-ACATGGATTTGGTGCC CCGC-3′), *psbA* forward (5′-ATGATCCCTACCTTATTGAC-3′) and reverse (5′-TTACCAAGGAACCATGCATA-3′), and *rbcL* forward (5′-AAGCTGGTGTTAAGGATTAT-3′) and reverse (5′-TTACCAAGGAACCATGCATA-3′). To use the PCR products as direct templates for in vitro transcription, the T7 polymerase recognition site (5′-TAATACGACTCACTA TAGGGCGA-3′) was added to the reverse primers at their 5′-termini. The conditions for PCR using 'PrimeSTAR HS DNA Polymerase with GC Buffer' (Takara Bio) were 35 cycles of 10 s at 98°C for denaturation, 15 s for annealing and 5 s at 72°C for elongation. Annealing temperatures for amplification of *psaA*, *psbA* and *rbcL* were 56°C, 52°C and 50°C, respectively. In vitro transcription reactions were carried out with a DIG RNA labeling kit (Roche) according to the manufacturer's instructions.

Anatomical and ultrastructural studies

Microscopic observation was performed as described (Ueno 2004). Calli grown for 14 d on N6D medium in the dark, or under white light, were fixed in 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 3 h. Subsequently, they were washed with phosphate buffer and post-fixed in 2% (w/v) OsO_4 in phosphate buffer. They were then dehydrated through an acetone series and embedded in Spurr's resin (Spurr 1969). Ultrathin sections were taken from the prepared calli, stained with uranyl acetate and lead citrate, and observed under an electron microscope (Model HU7000; Hitachi, Tokyo, Japan).

Determination of the photosynthetic rate in calli

Photosynthetic O_2 evolution of 1-week-old calli was measured with a Clark-type O_2 electrode (Rank Brothers Ltd., Bottisham, Cambridge, UK) supported by 2 mM $NaHCO_3$ under saturated light from a tungsten projector lamp. The net photosynthetic O_2 evolution under illumination was defined as the difference between respiration rate in the dark and gross photosynthetic rate under saturated light.

Determination of Chl content in roots and calli

Roots and calli were homogenized in 80% acetone. The suspensions were held in the dark at 4°C for 1 week, followed by centrifugation at 3000 × *g* for 3 min. Chl concentrations in the supernatant were determined spectrophotometrically (Beckman DU 640 spectrophotometer; Beckman Coulter, Fullerton, CA, USA) using the equation of Arnon (1949).

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