Physical and functional interaction of FgFtr1–FgFet1 and FgFtr2–FgFet2 is required for iron uptake in *Fusarium graminearum*

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FgFtr1 and FgFtr2 are putative iron permeases, and FgFet1 and FgFet2 are putative ferroxidases of *Fusarium graminearum*. They have high homologies with iron permease ScFtr1 and ferroxidase ScFet3 of *Saccharomyces cerevisiae* at the amino acid level. The genes encoding iron permease and ferroxidase were localized to the same chromosome in the manner of FgFtr1/FgFet1 and FgFtr2/FgFet2. The GFP (green fluorescent protein)-fused versions of FgFtr1 and FgFtr2 showed normal functions when compared with FgFtr1 and FgFtr2 in an *S. cerevisiae* system, and the cellular localizations of FgFtr1 and FgFtr2 in *S. cerevisiae* depended on the expression of their putative ferroxidase partners FgFet1 and FgFet2 respectively. Although FgFtr1 was found on the plasma membrane when FgFet1 and FgFtr1 were co-transformed in *S. cerevisiae*, most of the FgFtr1 was found in the endoplasmic reticulum compartment when co-expressed with FgFet2. Furthermore, FgFtr2 was found on the vacuolar

membrane when FgFet2 was co-expressed. From the two-hybrid analysis, we confirmed the interaction of FgFtr1 and FgFet1, and the same result was found between FgFtr2 and FgFet2. Iron-uptake activity also depended on the existence of the respective partner. Finally, the FgFtr1 and FgFtr2 were found on the plasma and vacuolar membrane respectively, in *F. graminearum*. Taken together, these results strongly suggest that FgFtr1 and FgFtr2 from *F. graminearum* encode the iron permeases of the plasma membrane and vacuolar membrane respectively, and require their specific ferroxidases to carry out normal function. Furthermore, the present study suggests that the reductive iron-uptake system is conserved from yeast to filamentous fungi.

Key words: *Fusarium graminearum*, iron, iron transporter, *Saccharomyces cerevisiae*, vacuole.

INTRODUCTION

Virtually all living organisms require iron as a biological cofactor in the process of respiration and for enzyme activity. Iron has been recognized as an important nutrient in microorganisms, and most microorganisms acquire iron by reductive [1] and siderophore-mediated iron-uptake systems [2]. Iron depletion results in the loss of pathogenic ability and growth defects in most microorganisms [3]. The well-defined model system of the single-cellular eukaryotic organism *Saccharomyces cerevisiae* has been studied to identify iron metabolism and those results have elucidated many aspects of iron metabolism in higher organisms. Although *S. cerevisiae* depends on a reductive iron-uptake system to obtain iron [4–6], it also adopts the siderophore uptake system. *S. cerevisiae* can not synthesize siderophore by itself [7]; however, it utilizes its well-organized siderophore transporters to monopolize on siderophores produced by other microorganisms [8–10]. The deletion of the gene that encodes the high-affinity iron transporter protein causes growth defects under iron-depleted conditions [4,5], but deletion of the genes that encode siderophore transporters do not have the same effects [10]. In *S. cerevisiae*, the high-affinity iron transporter ScFtr1p is found on the plasma membrane [1], and its homologue ScFth1p is found on the vacuolar membrane [11]. Interestingly, the ScFtr1p on the plasma membrane is coupled with surface ferroxidase ScFet3p [1], and the ScFtr1p–ScFet3p complex cannot carry out its function

without the respective partner protein [12]. The expression of these two proteins is regulated by iron, and the transcriptional-activating factor Aft1p activates the expression of both genes [13,14]. Interestingly, copper has an important function in the intracellular targeting of ScFtr1p–ScFet3p to the plasma membrane [1]. Without copper the ScFtr1p–ScFet3p complex remains on the ER (endoplasmic reticulum), and this mis-localization results in cellular iron deficiency. The same phenomenon is observed in the vacuole. ScFth1p, which has high homology with ScFtr1p, is coupled with the ScFet3p homologue ScFet5p on the vacuolar membrane. The ScFth1p–ScFet5p complex functions as an iron transporter, and its expression is up-regulated by iron deficiency [11]. Double deletion of *ScFTH1* and *ScFET5* results in a respiratory defect that causes a growth defect when the cells are transferred from glucose medium to ethanol or glycerol medium. This phenotype is recovered by incorporation of *ScFET5* or *ScFTH1* [11].

In the filamentous fungi, iron is an important nutrient that affects their pathogenicity, especially in the case of animal pathogens [15]. Siderophore production by many kinds of filamentous fungi has been studied, and its synthesis pathway has been identified [16,17]. However, little is known about the reductive iron transporter system of filamentous fungi. Recently, *Fusarium graminearum* protein FgFtr1, a homologue of ScFtr1p, was shown to function as a putative iron transporter on the plasma membrane [18]. FgFtr2 was further identified as another ScFtr1p homologue,

Abbreviations used: BPS, bathophenanthroline disulfonate; CMC, carboxymethyl cellulose; DAPI, 4',6-diamidino-2-phenylindole; ER, endoplasmic reticulum; FAS, ferrous ammonium sulfate; GFP, green fluorescent protein; ICP-AES, Inductively Coupled Plasma Atomic Emission Spectrophotometer; LY, Lucifer Yellow; PDB, Potato Dextrose Broth; RT, reverse transcriptase; WMM, 'watch' minimal medium.

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and it was predicted to be an iron transporter for intracellular organelles. Single-deletion mutants of *FgFtr1* or *FgFtr2* do not differ from the wild-type [18]. However, double-deletion strains lacking *FgFtr1* and *FgFtr2* exhibit iron deficiencies in the fungal cells. In addition, *ScFet3p* homologues *FgFet1* and *FgFet2* are found in the *F. graminearum* genome localized next to the *FgFtr1* and *FgFtr2* genes respectively. These data suggest that *FgFet1*–*FgFtr1* or *FgFet2*–*FgFtr2* may work in the same physiological pathway as their *S. cerevisiae* counterparts. In the present study, we show that *F. graminearum* has two *ScFtr1p* and *ScFet3* homologues that form iron transportation complexes with evolutionarily conserved functions.

MATERIALS AND METHODS

Strains, media and culture conditions

The yeast strains used in the present study were BY4741 (Mata, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0) and YPH499 (Mata, *ura3*-52, *lys2*-80, *ade2*-101, *trp1*-D 63, *his3*-D 200, *leu2*-D 1); derivatives were obtained from the haploid gene deletion collections in the BY4741 background. Yeast cells were cultured in YPD [1% (w/v) yeast extract/2% (w/v) peptone/2% (w/v) dextrose]-rich medium, minimal medium (0.67% yeast nitrogen base without amino acid, 2% dextrose and 0.12% amino acid drop-out mixture), and defined iron medium [0.67% yeast nitrogen base without copper and iron, 2% dextrose, 25 mM Mes buffer (pH 6.1), 0.5 μM copper and amino acid drop-out mixture]. Iron-limited conditions were prepared by the addition of 100 μM BPS (bathophenanthroline disulfonate) and FAS (ferrous ammonium sulfate) at various concentrations. *Escherichia coli* strain DH-5α was used for DNA cloning and plasmid propagation. *F. graminearum* (lineage 7) wild-type strain Z03643 and its mutant strains [18] were cultivated in PDB (Potato Dextrose Broth) at 25 °C. For fungal transformation [18], the fungi were grown in CMC liquid medium [15 g of CMC (carboxymethyl cellulose), 1 g of yeast extract, 0.5 g of MgSO₄, 1 g of NH₄NO₃ and 1 g of KH₂PO₄ per litre] and YPG liquid medium (3 g of yeast extract, 10 g of peptone and 20 g of glucose per litre).

Plasmid construction

To clone putative iron transportation genes, *F. graminearum* was cultivated in PDB medium with 100 μM BPS for 5 days and total RNA was extracted using a modified TRIzol® (Invitrogen) method. Using total RNA as a template, RT (reverse transcriptase)-PCR was performed to amplify the 5'-terminal fragment of *FgFet1* with the *FgFet1*-A and *FgFet1*-B primers; the 3'-terminal fragment of *FgFet1* was amplified with *FgFet1*-C and *FgFet1*-D; the 5'-terminal fragment of *FgFet2* was amplified with *FgFet2*-A and *FgFet2*-B; and the 3'-terminal fragment of *FgFet2* was amplified with *FgFet2*-C and *FgFet2*-D (see Supplementary Table 1 at <http://www.BiochemJ.org/bj/407/bj407ppppadd.htm>). Each fragment was subcloned into pGEMT-easy vector (Promega) and confirmed by DNA sequencing. The construction of plasmids *pFgFtr1* and *pFgFtr2* containing full-length *FgFet1* and *FgFet2* respectively, have been described [18]. Basically, the plasmid *pFgFet1* was constructed by ligating the 5'- and 3'-fragments of *FgFet1* into pBlueScript II SK, digested with *SpeI* and *XhoI*, and then ligating them into *SpeI*/*XhoI*-digested *pFTR1-P*, in which the promoter region of *ScFtr1* is inserted into pRS415 vector. For *pFgFet2*, the *SpeI*/*Sall*-digested *FgFet2* was ligated into *SpeI*/*Sall*-digested *pFTR1-P*. To attach GFP (green fluorescence protein) to *pFgFtrs*, the *XbaI* and *NheI* restriction enzyme sites were introduced into the 3'-termini of *FgFtr1* and *FgFtr2* using

the following primers: for *FgFtr1*, the primers *FgFtr1*-GFP-up and *FgFtr1*-GFP-dw; for *FgFtr2*, the primers *FgFtr2*-GFP-up and *FgFtr2*-GFP-dw. Mutagenesis was then performed as described previously [19] to produce *pFgFtr1/XbaI* and *pFgFtr2/NheI*. The *NheI*/*ApaI* double-digested DNA fragment of the GFP gene was inserted into *XbaI*/*ApaI*-digested *pFgFtr1/XbaI* and *NheI*/*ApaI*-digested *pFgFtr2/NheI* respectively, to produce *pFgFtr1-GFP-LEU* and *pFgFtr2-GFP-LEU*. To make histidine and uracil markers, *pFgFtrs-GFP-LEU* was digested with *SacI* and *Sall* and ligated into *SacI*/*Sall*-digested pRS413 and pRS316 vectors and *pFgFtr1-GH*, *pFgFtr2-GH*, *pFgFtr1-GU* and *pFgFtr2-GU* were constructed respectively.

Northern blot analysis

For Northern blot analysis, total RNA was extracted from *F. graminearum* grown in PDB for 3 days at 25 °C. Total RNA (10 μg) was separated on 1% formaldehyde agarose gel electrophoresis, and Northern blot analysis was performed as described [18]. Probes were prepared by PCR on the internal region of the reading frame of interest: for *FgSit1*, the primers *FgSit15* and *FgSit13*; for *FgSit2*, the primers *FgSit25* and *FgSit23*; and for *FgAct*, the primers *FgAct-5* and *FgAct-3* were used.

Plate assay, uptake assay and fluorescence microscopic analysis

For plate assays, modified synthetic complete medium were used in which copper and iron were omitted, and 10 μM FAS or 500 μM FAS, 0.5 μM copper and 100 μM BPS were added. Uptake assays in *S. cerevisiae* were performed as described previously [8] using multiscreen filtration plates (Millipore) with 1 μM ⁵⁵Fe(III). For uptake assays in *F. graminearum*, fungal cells were cultured at 25 °C for 3 days. The mycelia were filtered twice by Swinnex Filter Holder (Millipore) and washed with citrate buffer prior to incubation with 1 μM ⁵⁵Fe(III) at 25 °C for 1 h. The activated cells were then applied to filtration plate wells that had been equilibrated with distilled water for 30 min. After washing five times with 400 μl of distilled water, the multiscreen filtration plate was dried at 60 °C for 20 min. The dried mycelia were weighed, and the incorporated radioactivity was detected with a liquid scintillation counter. For fluorescence microscopy, each yeast strain was grown overnight in defined low iron medium with 20 μM FAS and 100 μM BPS. The imaging was performed with a fluorescence microscope (AXIO Imager.A1/M1; Carl Zeiss). For FM4-64 and LY (Lucifer Yellow) staining, cells were cultured in CMC medium for 3 days at 25 °C. Cultured cells were transferred to basal medium [WMM ('watch' minimal medium)] for culture overnight. Cultured cells were resuspended with the same volume in WMM containing 10 μM FM4-64 or 4 mg/ml LY and incubated for 45 min. Cells were washed with PBS (pH 7.5) containing 15 mM NaN₃ and 15 mM NaF, and then the image analysis was performed using a fluorescence microscope.

Vacuole extraction from *F. graminearum* and iron measurement

Cells were cultured in CMC medium for 3 days at 25 °C and then transferred to overnight cultures of PDB medium and PDB medium containing 100 μM BPS. To make protoplasts, cells were suspended in driselase solution for 6 h at 30 °C with gentle shaking. The protoplasts were filtered and then collected by centrifugation at 1160 g for 10 min at 4 °C and resuspended with 5 ml of buffer A [10 mM Mes/Tris (pH 6.9), 0.1 mM MgCl₂ and 12% ficoll]. Protoplasts were homogenized with 30 strokes on ice and centrifuged at 60 000 g for 30 min. The floating white wafers were transferred to a new tube containing 2.5 ml of buffer A and homogenized again. Samples were transferred to an ultracentrifuge tube and overlaid with an equal volume of

Figure 1 *FgFtr1* and *FgFtr2* are localized as neighbouring genes with *FgFet1* and *FgFet2* respectively on *F. graminearum* chromosomes

A *F. graminearum* genome database [25] search identified genes homologous with *ScFTR1* and *ScFET3*. The *FgFtr1* and *FgFtr2* genes were adjacent and displayed divergent orientation with *FgFet1* and *FgFet2* respectively on the same chromosome. As described in the text, the G + C content of *FgFtr2* and *FgFet2* genes was lower than in neighbouring genes.

buffer B [10 mM Mes/Tris (pH 6.9), 0.5 mM MgCl_2 and 8 % ficoll] and centrifuged at 60000 *g* for 30 min. White wafers were collected and dissolved in 50 μl of buffer C [10 mM Mes/Tris (pH 6.9), 5 mM MgCl_2 and 25 mM KCl]. To analyse the iron content, the prepared white wafers were analysed with the ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrophotometer) at KBSI (Korea Basic Science Institute). The α -mannosidase assay was performed as follows. The extracted vacuoles were mixed with 0.6 ml of buffer [0.1 M Mes/NaOH (pH 6.5) and 0.2 % (v/v) Triton X-100] and 0.2 ml of substrate (4 mM *p*-nitrophenyl- α -mannopyranoside). After 1 h at 37 °C an equal volume of stop solution (0.5 M glycine/ Na_2CO_3 , pH 10.0) was added to the reaction mixture and activity was determined using a spectrophotometer at 400 nm.

Plasmid construction for two-hybrid analysis and GFP fusion of *FgFtr1* and *FgFtr2*

The cytosolic fragment of the *ScFTR* and *FgFTR* genes were amplified by PCR, digested with *EcoRI* and *XhoI*, and inserted into pGADT7 vector. Primers used for these genes were as follows: for pAD-*ScFTR1*, *ScFTR1*-F and *ScFTR1*-R; for pAD-*ScFTH1*, *ScFTH1*-F and *ScFTH1*-R; for pAD-*FgFtr1*, *FgFtr1*-F and *FgFtr1*-R; and for pAD-*FgFtr2*, *FgFtr2*-F and *FgFtr2*-R. The cytosolic fragment of the *ScFET3* and *ScFET5* were amplified by PCR, digested with *EcoRI* and *PstI*, and inserted into the pGBKT7 vector. These genes were amplified with *ScFET3*-F and *ScFET3*-R for pBD-*ScFET3*, and with *ScFET5*-F and *ScFET5*-R for pBD-*ScFET5*. *FgFet1* was digested with *NdeI* and *EcoRI* and then inserted into the pGBKT7 vector. *FgFet2* was digested with *NcoI* and *SalI* and then inserted into the pGBKT7 vector. The constructed pAD-FTRs and pBD-FETs were co-transformed into strain AH109. Transformants were replica-spotted on both SD LW– and SD AHLW– plates. To fuse GFP to the *FgFtr* genes, PCR-amplified gene products were inserted into the pGEMT-Easy vector and digested out with *XbaI*/*ApaI* (*FgFtr1*–GFP) and *NheI*/*ApaI* (*FgFtr2*–GFP). The digested fragments of *FgFtr1*–GFP and *FgFtr2*–GFP were transformed into *F. graminearum* and the GFP-tagged genes were confirmed by Southern blot analysis.

RESULTS AND DISCUSSION

Previously, we reported that *FgFtr1* and *FgFtr2* may function as iron transporters in *F. graminearum* [18]. The formation of the

S. cerevisiae *ScFtr1p*–*ScFth1p* and *ScFet3p*–*ScFet5p* complexes on the plasma and vacuolar membranes respectively is a critical step in the physiological functions of these proteins [11]. In *F. graminearum*, we found two *ScFtr1p* homologues with high homology with each other at the amino acid level [18]. The putative amino acid sequences of *FgFtr1* and *FgFtr2* have a high degree of similarity to *ScFtr1p* and *ScFth1p*, suggesting the potential for differential localization of these proteins similar to that in *S. cerevisiae* [1]. *F. graminearum* genome sequence analysis revealed that *FgFtr1* is localized adjacent to the *ScFet3* homologue *FgFet1*, which has putative ferroxidase function (Figure 1). Furthermore, *FgFtr2* is localized in the same way with *FgFet2*, another putative *ScFet3* homologue (Figure 1). To identify the possibility that these genes were acquired through lateral gene transfer, the G + C content of *FgFtr2* and *FgFet2* was calculated to be 48 % and 51 % respectively, which was lower than the 55 % G + C content of the neighbouring genes. This difference suggests that these genes were incorporated recently into the *F. graminearum* genome from other organisms [20]. In addition, the arrangement of the genes may indicate that the *F. graminearum* gene products have similar physiological functions as their *S. cerevisiae* homologues.

To identify the cellular localization of each gene product, *FgFtr1* and *FgFtr2* were tagged with GFP at their C-terminus and introduced into an *S. cerevisiae* system utilizing the *scftr1* deletion strain. *ScFtr1p* is responsible for iron transport in *S. cerevisiae* and the *scftr1* strain has a growth defect phenotype under iron-deficient conditions (Figure 2). The introduction of *FgFtr1*–GFP complemented the growth defect phenotype of *scftr1* on iron-deficient medium when compared with an empty vector control (Figure 2, right-hand panel). Similarly, the *FgFtr2*–GFP partially complemented the growth defect of the *scftr1* deletion strain (Figure 2, right-hand panel). From the plate assays, we confirmed that the GFP-tagged *FgFtr1* and *FgFtr2* were functional even though there were differences in the complementation activity.

Next, to identify the cellular localization of *FgFtr1* and *FgFtr2* and their dependence on *FgFet1* and *FgFet2* respectively, *FgFtr1*–GFP along with *FgFet1*, and *FgFtr2*–GFP along with *FgFet2*, were co-transformed into the *S. cerevisiae* *scftr1/scfet3* double-deletion strain. As a positive control, *ScFet3*–GFP was transformed into the same strain and subsequently found on the plasma membrane (Figure 3A). Interestingly, *FgFtr1*–GFP was found on the plasma membrane only when it was co-expressed with

Figure 2 The FgFtr1–GFP and FgFtr2–GFP complement the growth defect of the *S. cerevisiae* *scftr1* strain in iron-deficient conditions

Translational fusions of *FgFtr1* and *FgFtr2* with GFP were constructed and transformed into the *scftr1* deletion strain to confirm their relevant physiological functions. The serially diluted transformants were spotted on iron-rich medium (500 μ M FAS and 100 μ M BPS, left-hand panel) and iron-deficient medium (10 μ M FAS and 100 μ M BPS, right-hand panel) and incubated for 3 days. EV, empty vector.

FgFet1 (Figure 3A). When FgFtr1–GFP was co-expressed with FgFet2–GFP, FgFtr1 was found in the ER membrane and the GFP signal co-localized with DAPI (4',6-diamidino-2-phenylindole) staining (Figure 3A). This mis-localization is probably caused by the failure of FgFtr1 to form a complex with FgFet1 [1]. Similarly, FgFtr2–GFP was found in the periphery of DAPI-stained nuclei [21] when co-expressed with FgFet1 (Figure 3A) compatible with ER localization. Similar to the ScFth1p–ScFet5p complex, FgFtr2–GFP was found on the vacuolar membrane when co-expressed with FgFet2 (Figure 3A). These results imply the possible formation of complexes between FgFtr1 and FgFet1 and between FgFtr2 and FgFet2 that are involved in proper cellular localization. It also seems that the generation of the mature proteins and formation of the appropriate complexes are important factors in the physiological functions of FgFtr1 and FgFtr2. The cellular localization of FgFtr1 and FgFtr2 in *F. graminearum* was then analysed using the GFP-tagged version of *FgFtr1* and *FgFtr2* on the chromosome. As shown in Figure 3(C), the FgFtr1 was found on the plasma membrane and some vesicles (Figure 3B, panels b and c). However, most of the signal was found on the plasma membrane. The plasma membrane of filamentous fungi is impermeable to the LY dye, which makes it a useful membrane marker [22]. We found that FgFtr1

Figure 3 The cellular localization of FgFtr1 and FgFtr2 depends on the co-expression of FgFet1 and FgFet2 respectively

(A) The *fgftr1* and *fgfet2* genes were cloned from *F. graminearum* mRNA as described in the Materials and methods section, and the cloned genes were transformed into the *S. cerevisiae* *scftr1/scfet3* double-deletion strain with constructed *FgFtr1*–GFP and *FgFtr2*–GFP in the indicated combinations. The Fet3–GFP was used as a membrane protein reference, and pRS415 was used as a negative control. The FgFtr2 localized on the vacuole membrane when co-expressed with FgFet2, and when the FgFet2 was not co-expressed, FgFtr2 was found on the ER. DAPI staining was used to identify the localization of the nuclei. The localization of FgFtr2 was changed from the ER to the vacuolar membrane when co-expressed with FgFet2. (B) Wild-type *F. graminearum*, *FgFtr1*–GFP and *FgFtr2*–GFP were grown in PDA broth with 100 μ M BPS for 3 days. Panel a, the wild-type negative control; panels b and c, FgFtr1; and panels d and e, FgFtr2. Panels f and g show the LY and FM4-64 staining respectively. The left-hand panel shows GFP and the right-hand panel shows DIC (differential interference contrast).

Figure 4 The physiological function of FgFtr1 depends on FgFet1

(A) The *FgFet1* and *FgFet2* genes were co-transformed into the yeast *scftr1/scfet3* double-deletion strain in combination with *FgFtr1* and *FgFtr2* respectively, in the indicated combinations. The serially diluted transformants were spotted on high-iron (500 μ M FAS and 100 μ M BPS, left-hand panel) and low-iron (10 μ M FAS and 100 μ M BPS, right-hand panel) medium respectively. EV, empty vector. (B) The ferrous iron-uptake assay using ⁵⁵Fe(III) was performed with the same strains as indicated in (A). The uptake activities of deletion strains, which have *FgFtr1* or *FgFtr2* with *FgFet1* or *FgFet2* respectively, were relatively low. The inset shows the values plotted using a different y axis.

co-localized with LY on the plasma membrane. Furthermore, the FgFtr2 was found on the vacuolar membrane (Figure 3B, panels d and e), and FgFtr2 co-localized with the vacuolar membrane marker FM4-64 (Figure 3B, panel g) [22]. These results were in agreement with the *S. cerevisiae* assay shown in Figure 3, and indicate that *F. graminearum* has iron-transporting systems on both the plasma membrane and vacuolar membrane as in *S. cerevisiae* [1].

To confirm the physiological role of the complex formation between FgFtr1–FgFet1 and FgFtr2–FgFet2, we performed complementation experiments in the *S. cerevisiae* *scftr1/scfet3* double-deletion strain background. As shown in Figure 4(A), the *scftr1/scfet3* double-deletion strain failed to grow on iron-deficient medium. However, the mutant phenotype was eliminated only when the double mutant was transformed with the pair of *FgFet1* and *FgFtr1*. This result was acceptable because the

vacuolar iron transporter complex could not complement the *scftr1/scfet3* deletion strain in *S. cerevisiae* [11]. To identify the iron-uptake activity of these complexes, we performed an iron-uptake assay in the same strain background. As shown in Figure 4(B), the highest iron-uptake activity was found in the wild-type strain, and very low activity was observed even when *FgFtr1*/FgFet1 was introduced into the *scftr1/scfet3* deletion strain. When comparing iron-uptake activity of only the strains that have a combination of two plasmids of *FgFtr* genes and *FgFet* genes, we found that the strain that was transformed with *FgFtr1*/FgFet1 exhibited the highest iron-uptake activity, even though the difference was quite small. Generally, this level of iron-uptake activity is enough to rescue the growth of the cells. For example, the very small difference in surface ferric reductase activity for siderophore uptake in *S. cerevisiae* makes it possible to rescue the growth defect in iron-depleted medium [8]. Moreover,

Figure 5 FgFtr1 and FgFtr2 interact with FgFet1 and FgFtr2 respectively

(A) The fragments of ScFtr1, ScFth1, ScFet3 and ScFet5 were constructed as described previously [22], and the fragments of FgFtr1, FgFtr2, FgFet1 and FgFet2 were constructed as shown. Open bars represent the fragments of genes from *S. cerevisiae* and *F. graminearum* used in the two-hybrid analysis. **(B)** and **(C)** Two-hybrid analysis. —AHLW indicates the SD selective medium without alanine, leucine, histidine and tryptophan. —LW indicates the SD selective medium without leucine and tryptophan.

the iron-uptake activities of the remaining strains were almost the same; these results were obtained from two independent experiments.

To identify the interaction between FgFtr1 and FgFet1 or FgFtr2 and FgFet2, we performed two-hybrid analysis with the fragments of the FgFtr genes and the full-length FgFet genes. The physical interaction between FgFtr1 and FgFet3 was identified and the cytosolic fragment of both of the genes was shown to be important in the interaction [12]. As shown in Figure 5(A) the fragmentations of corresponding proteins were constructed with the different compartments as previously reported [12], and two-hybrid analysis was performed. As shown in Figure 5(B), ScFtr1 and ScFet3 interacted very strongly, and the same results were found between ScFth1 and ScFet5. Furthermore, we found interactions between FgFtr1 and FgFet1, and similar results between FgFtr2 and FgFet2 (Figure 5C). Interestingly, the interaction between FgFet1 and FgFtr2 was confirmed, and the same result was found between ScFet3 and ScFth1 [12]. These results indicate that the interaction between iron permease and ferroxidase is an important factor in iron uptake in *F. graminearum*, and the reductive iron-uptake pathway is conserved from *S. cerevisiae* to *F. graminearum*.

To identify the physiological function of FgFtr1 and FgFtr2 in *F. graminearum*, we performed the iron-uptake assay with the *F. graminearum* strains using the *fgftr1* or *fgftr2* single-deletion strains and the *fgftr1/fgftr2* double-deletion strain. As shown in Figure 6(A), the iron-uptake activity of the double-deletion strain

was higher than that of the wild-type strain. This result was the opposite of what we had predicted. We expected the *fgftr1/fgftr2* double-deletion strain to display lower iron-uptake activity compared with the wild-type. To explain this result, we tested the expression of siderophore transporters in *F. graminearum*. In filamentous fungi, the siderophore transporter is responsible for most of the iron uptake. We therefore hypothesized that the siderophore transporter may work as an iron transporter in the double-deletion strain through up-regulation of genes encoding siderophore transporters under intracellular iron deficiency. As FgSit1 was reported to be an important protein for regulating cellular iron utilization [24], we tested the expression level of *FgSit1* in the *fgftr1/fgftr2* double-deletion strain. As shown in Figure 6(B), the expression levels of *FgSit1* and *FgSit2* increased in the *fgftr1/fgftr2* double-deletion strain. In a previous study, we reported that the expression level of the *FgSid* gene [18], which is a putative L-ornithine-N⁵-monooxygenase involved in the siderophore synthesis pathway [25], is increased in the *fgftr1/fgftr2* double-deletion strain. We obtained these same results regarding *FgSit1* and *FgSit2*. From the present study, we suggest that FgFtr1 and FgFtr2 also work as regulators of iron utilization in *F. graminearum*, and are localized to the plasma membrane and the vacuolar membrane respectively, with their specific partners, FgFet1 and FgFet2. We further investigated the vacuolar iron contents when *fgftr1* and *fgftr2* were deleted. As shown in Figure 6(C), the iron contents were measured with ICP-AES and were not changed in the cells cultured in PDA

Figure 6 Ferrous iron uptake increased, and expression of *FgSit1* and *FgSit2* was up-regulated, in the *fgfr1/fgfr2* double-deletion strain

(A) The ferrous iron-uptake assay was performed as described with *fgfr1*, *fgfr2*, *fgfr1/fgfr2* and wild-type *F. graminearum*. (B) Total RNA was extracted from the indicated strains and Northern blot analysis was performed. The internal region of *FgSit1* and *FgSit2* were used as probes. *FgAct* was used as a loading control. (C) The wild-type and deletion strains of *F. graminearum* were grown in PDA (open bars) or PDA plus 100 μ M BPS medium (closed bars), and the iron content of vacuoles from corresponding strains was measured using ICP-AES. The iron content of the *fgfr2* deletion strain was higher than wild-type, especially in the cells grown in PDA plus 100 μ M BPS.

medium. However, large differences in the iron content were seen when BPS was added to the medium. The *fgfr2* deletion strain especially showed a higher iron content in the vacuole when compared with the wild-type strain. This may result from the inhibition of iron export from the vacuole to the cytosol, as in the case of *ScFth1* [11]. These results indicate that *F. graminearum* has a reductive iron-uptake mechanism in the plasma membrane as well as the vacuolar membrane, which have high homology with *S. cerevisiae*.

As stated above, most filamentous fungi depend on the siderophore-mediated iron transport system, even though little is known about the uptake mechanism of siderophores. However, the reductive iron transport system presumably works by taking up iron from the environment. For example, in *Ustilago maydis*, the permease and ferroxidase double-deletion strain is no longer virulent, which may allude to the involvement of the reductive iron-transport system in pathogenicity [26]. Taken together, several issues remain that must be further explored in order to understand the detailed mechanism of iron transport in filamentous fungi. First, we considered the regulatory mechanism of the expression of the genes involved in reductive iron transport and the siderophore iron-transport pathway. In *Aspergillus nidulans*, the transcription factor *sreA* [27] is known to be a transcriptional activator of genes that are involved in the siderophore biosynthetic pathway. However, the regulatory mechanism of the genes that are involved in the reductive iron-transport system is still unknown. Secondly, in the reductive iron-transport system, environmental ferric iron must be reduced to the

ferrous form to dissolve iron from the iron salt and then be taken up by the specific iron permease [28]. We have found another ferroxidase that is involved in oxidation of ferrous to the ferric form of iron in this report. We searched for the surface ferric reductase homologues from the *F. graminearum* genome database (*Fusarium graminearum* sequencing project. Broad Institute of MIT and Harvard; <http://www.broad.mit.edu/>) and found a few candidate genes that exist in the genome of *F. graminearum*. The ferric reductase is also involved in siderophore transport in *S. cerevisiae*, and we think this may be the case in *F. graminearum*. Identifying the regulatory mechanism of the genes involved in reductive iron transport, and the ferric reductase, will give us a detailed iron-uptake pathway for filamentous fungi, and may allow us to establish the evolutionary relationships of iron transport systems among diverse microorganisms.

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