Receptor Protein Kinase FERONIA Controls Leaf Starch Accumulation by Interacting with Glyceraldehyde-3-Phosphate Dehydrogenase

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Abstract: Cell expansion is coordinated by several cues, but available energy is the major factor determining growth. Receptor protein kinase FERONIA (FER) is a master regulator of cell expansion, but the details of its control mechanisms are not clear. Here we show that FER interacts with cytosolic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GAPC1 and GAPC2), that catalyzes a key reaction in glycolysis, which contributes to energy production. When there is an *FER* deficiency, there are corresponding decreases in the enzyme activity of GAPDH and increased amounts of starch. More importantly, *gapc1/2* mutants mimic *fer4* mutants. These data indicate that FER regulated starch content is an evolutionarily conserved function in plants that connects the cell expansion and energy metabolism pathways.

Introduction

In contrast to animals, plant cells are immobile and so plant growth and development depends on cell expansion rather than cell migration. Cell expansion is controlled by external factors such as light, temperature and internal growth regulators including brassinosteroids (BRs) and plant hormones (Lehman et al., 1996; Szekeres et al., 1996; Guo et al., 2009). FERONIA (FER), a kind of receptor-like kinase, plays an important role in regulating cell elongation. For example, FER can activate BR and auxin, which have positive roles in cell growth and development (Guo et al., 2009; Duan et al., 2010). FER can also inhibit ABA and ethylene, which

have negative roles in cell growth (Duan et al., 2010; Deslauriers and Larsen, 2010; Yu et al., 2014). Haruta et al. (2014) demonstrated that FER can sense RALF peptides to regulate H⁺-ATPase in the plasma membrane. These peptides are responsible for excreting hydrogen ions into the apoplast to increase the growth and extensibility of cells. The plasma membrane H⁺-ATPases are some of the most important end targets for auxin signals. Auxin-enhanced H⁺ pumping lowers the cell wall pH, activates pH-sensitive enzymes and proteins within the wall, and initiates cell-wall loosening and growth (Hager, 2003; Rober-Kleber et al., 2003; Takahashi et al., 2012). The transport of H⁺ ions by H⁺-ATPase is accompanied by the consumption of ATP. Thus, cell elongation needs to provide ATP to cells, which is necessarily associated with energy metabolism. However, the role of FER in energy metabolism has remained elusive.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reversibly catalyzes the oxidation of D-glyceraldehyde-3-phosphate to 1, 3-diphospho-glycerate and plays an important role in glycolysis, a fundamental process in biology that provides the energy for metabolism (Brooks, 2010). GAPDH functions as a cytoskeletal element and controls amino acid and sugar metabolism, making GAPDH a key regulator in energy metabolism (Muñoz-Bertomeu et al., 2009). There are four GAPDH genes in *Arabidopsis thaliana*; these are two cytosolic GAPC genes and two plastidial GAPCp genes. GAPC genes have previously been implicated in cell elongation, ABA and pollen development in *Arabidopsis* (Rius et al., 2006, 2008; Muñoz-Bertomeu et al., 2010; Muñoz-Bertomeu et al., 2011). However, the molecular mechanism by which

proteins regulate GAPC activity and control energy metabolism remains unknown. In the present study we show that FER interacts with GAPC to regulate energy metabolism and cell elongation in *Arabidopsis*.

Different concentrations

Materials and Methods

Plant Growth Conditions

Dark treatment of *Col-0* and *fer4* seeds were surface sterilized and germinated on 1/2 MS medium supplemented with different concentrations sucrose/mannitol and 1.0% agar (pH 5.8) at 22°C. The plant which grown in soil were 16h light/8h dark at 22°C. Seeds for the mutant lines were purchased from the ABRC (Arabidopsis Biological Resource Center, Columbus, OH) and GABI-Kat collections. We ordered the T-DNA insert mutants of *FLR1* (*flr1*: 2C-80049) in the web site <http://signal.salk.edu/cgi-bin/RiceGE>. Rice seeds were disinfected in 30% sodium hypochlorite solution and then wash the solution out five times using ddH₂O. The disinfected seeds were incubated in petri dishes in 30°C. The sprout seedlings were plant into fertilizer soil in a green-house. Two-week-old seedlings were transplanted to crop field in Taohua Village, Changsha, China.

Histochemical staining and determination of starch content

For the part of histochemical staining, 5-d old dark-grown plants were faded in 80% ethanol with the temperature at 65°C for 30 min. Subsequently, seedlings were rinsed with water, stained with 0.01% IKI solution and captured on stereo microscope with a HD camera.

For the analysis of starch content, 7-d old seedlings were frozen under liquid nitrogen, and ground to a powder, then faded in 80% ethanol with the temperature at 65°C for 30 min, rinsed with water. The starch was extracted with 80% calcium nitrate in boiling water for 10 min. Subsequently, supernatant stained with 0.01% IKI solution and starch were assayed spectrophotometrically at 620 nm at 25°C.

RNA Extraction and Real-Time PCR Analyses

Arabidopsis seedlings and adult plants were harvested, powdered in liquid nitrogen, total RNA was isolated by Trizol (Takara) and digested by DNase I (Takara) for reverse transcript to cDNA (Fermentas). RT-PCR was performed using CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with SYBR Premix ExTaqII (Takara) according to the manufacturer's protocol, mRNA level of ACTIN2 was used for data normalization as the internal control. Each reaction was performed in triplicate with 1 µl of the first-strand cDNA in a total volume of 20 µl. Data are means of three biological samples. Primers used for PCRs are shown in Supplemental Table.

Yeast Two-Hybrid

Full-length coding sequences of *FER* was cloned into pGBKT7 vector, *GAPC1* and *GAPC2* were cloned into pGADT7, by the primers in Table S1. Successfully constructed vectors were co-transformed into yeast cells AH109, and plated on Synthetic dropout minimal (SD/-Trp-Leu-His) medium supplemented with 20 mM 3-aminotriazole for 1 week (Yu et al., 2010).

BiFC Assay

For BiFC assays, the coding sequences of *FER* and *GAPC1/GAPC2* were

amplified and cloned into plasmids pE3308 and pE3449. Mesophyll protoplasts were isolated from 5-week-old *Arabidopsis* rosette leaves. FER-nVenus, GAPC1-cCFP and GAPC2-cCFP were introduced into Mesophyll protoplasts using PEG-mediated transfection (Yoo et al., 2007). The assay was repeated three times.

GAPDH Enzyme Activity Assay

GAPDH activity assay was done using colorimetric GAPDH assay kit. Total protein extracted from 4-weeks old *Arabidopsis* leaves using 100 μ l ice-cold cell lysis buffer, and incubate at 2-8°C for 20 min with gentle agitation to lyse tissue. GAPDH assay mixture contained 121.25 μ l GAPDH assay buffer, 10 μ l 3-PGA, 5 μ l L-Cysteine, 2.5 μ l β -NADH, 5 μ l ATP, 1.25 μ l 3-PGK for each sample. GAPDH activity was measured spectrophotometrically at 25 °C by monitoring the decrease in absorbance of NADH for 10 min at 340 nm.

Results and Discussion

FER Transcript and Protein levels are activated by Sucrose

It has been shown that ABA signaling pathways are associated with sugar signaling pathways (Rook et al., 2001; Arroyo et al., 2003) and our previous work showed that FER inhibited the ABA response by activating ABI2 phosphates through the GEF1/4/10-ROP11 pathway (Yu et al., 2012). This prompted us to analyze the relationship between FER and sugar (energy) response. We tested this relationship with seven-day-old *Arabidopsis* seedlings treated with different concentrations of sucrose. A qPCR analysis showed that *FER* transcript levels in the seedlings

correlates with an increase in sucrose concentration (Fig. 1A). To address whether FER protein levels are also altered by sucrose, total protein was extracted from 7 day old Col-0 seedlings and FER protein levels were assessed using immunoblotting with an anti-FER antibody. High sucrose levels correlated with high FER protein levels, in agreement with the effect of sucrose on *FER* gene expression (Fig.1B). These results indicated that sucrose leads to FER expression at the transcript and protein levels.

FER mutants accumulate starch and inhibit cell elongation

Sucrose influences starch metabolism in plants (Rook et al., 2001; Gupta and Kaur, 2005), and we found it can induce the expression of FER. Thus we decided to analyze *fer* mutant phenotypes under sucrose treatments. To characterize the sensitivity of *fer-4* mutants to sucrose levels, we treated wildtype (Col-0) and *fer-4* mutants with different concentrations of sucrose under dark conditions and observed the starch accumulation of seedlings using IKI staining. The wild type and *fer-4* mutants had no observable starch accumulation without sucrose. However, when the sucrose treatment increased, starch increased more quickly in *fer-4* mutants than in WT seedlings (Fig. 2A, B). We noticed the same phenotype in another *FER* null mutant, *srn*, under a C24 background (Fig. S1A). There was no difference in starch accumulation between wild type and *fer-4* mutants grown on mannitol (Fig. 2C), excluding the possibility that the phenotype of starch accumulation is caused by osmotic stress.

Sucrose-induced hypocotyl elongation was effected in fer mutants

Sucrose stimulated hypocotyl cell elongation in dark-grown

Arabidopsis seedlings (Zhang et al., 2010). We analyzed hypocotyl lengths and found that *fer-4* hypocotyls were hypersensitive to sucrose (Fig. 3A, B and Fig. S1B). Dark-grown *fer-4* and wild type hypocotyl lengths are not significantly different ($P>0.05$) without sucrose treatment. However, we found that when *fer-4* was grown with 60 mM sucrose its hypocotyl length was one-third that of the wild type, a significant effect ($P<0.01$), and that *fer-4* mutants were hypersensitive to sucrose. In order to clarify whether the change in hypocotyl length was caused by osmotic pressure, we performed the same treatment using mannitol. Our results revealed that wild type and *fer-4* hypocotyl lengths slightly decreased when mannitol increased, but there was no significant difference ($P>0.05$). Taken together, our results show that loss of function in *FER* induces sucrose hypersensitivity.

FER interacts with GAPC1 and GAPC2

Based on the evidence that starch accumulation is regulated by FER and previous reports that sucrose effects starch via a glycolytic pathway (Plaxton, 1996; Geigenberger et al., 2004), we speculated that FER may interact with specific factors that control the glycolytic pathway. Therefore, we screened proteins involved in the process of glycolysis and identified glyceraldehyde-3-phosphate dehydrogenase as potentially interacting with FER directly. Using a bimolecular fluorescence complementation (BiFC) assay, we found that GAPC1-cCFP and GAPC2-cCFP interact with FER-nVenus in *Arabidopsis* protoplasts (Fig.4 C) at the plasma membrane. We confirmed that the catalytic domain of GAPC1 and GAPC2 interact with FER using a yeast-two-hybrid (Y2H) system (Fig. 4A, B). Taken together, our

data show that FER can interact with GAPC1 and GAPC2 and in turn regulate starch metabolism in leaves through the glycolytic pathway.

GAPDH enzyme activity is suppressed in *fer-4* mutants

In *Arabidopsis thaliana*, GAPDH consists of four genes; two cytosolic GAPC genes and two plastidial GAPCp genes (Rius et al., 2008; Muñoz-Bertomeu et al., 2009; Muñoz-Bertomeu et al., 2010; Guo et al., 2012). *GAPC* mutants have reduced GAPC enzyme activity and decreased glycolysis rates (Rius et al., 2008). We have shown that FER interacts with the catalytic domain of GAPC1 and GAPC2 and we have hypothesized that FER may regulate the GAPDH enzyme activity in plants. To address this question, two null mutants of *GAPC1* and *GAPC2* were subjected to GAPDH enzyme activity analysis (Fig. S3A-C). One-month-old leaves of Col-0, *fer-4*, FER-OE (FER overexpression), *gapc1-1* and *gapc2-2* were collected to determine the GAPDH enzyme activity. GAPDH enzyme activity of *fer-4* mutants is lower than in the wild type, but GAPDH enzyme activity of FER-OE leaves was higher than in the wild type (Fig. 5A). This indicates that FER regulates GAPC enzyme activity in plants, accelerating the process of glycolysis and energy metabolism.

GAPC mutant leaf starch phenotype is mimicked in *fer* mutants

Since FER controls starch accumulation through regulating GAPC1 and GAPC2 enzyme activity, we asked whether *gapc1* and *gapc2* mutants also affected starch accumulation. To mimic normal physiological conditions, we performed these assays with normal growth conditions in soil. One-month-old leaves of Col-0, *fer-4*,

FER-OE, *gapc1-1*, and *gapc2-2* were grown under normal condition for one month, then the leaves of different genotypes were collected and starch content were measured. As expected, the starch content of *fer-4*, *gapc1-1* and *gapc2-2* mutants is higher than in wild-type plants (Fig. 5B) and starch accumulation in FER-OE plants is slightly lower than in wild-type plants (Figs. 5B, S4A).

Starch content increased in FLR1 (FER homolog) mutant rice

FER is an evolutionarily conserved gene in angiosperms (Nguyen et al., 2014), so we speculated that FER also controls starch content in rice, one of the most important crops. Therefore, we studied *FLR1* (*FERONIA-Like Receptors* kinase, *Os03g21540*) in rice which is homologous with *FER* (Nguyen et al., 2014). We identified a T-DNA insertion mutant line (2C-80049) of *FLR1*, named *flr1* and found that the T-DNA was inserted in the exon of *FLR1* (Fig. S5A, B). We confirmed that this insertion causes a null mutation of *FLR1* (Fig. S5C). Using the null mutant *flr1*, we found that the leaf starch content in *flr1* was obviously higher than in WT (Fig. 6A, B). We found that the starch content of *flr1* mutants is approximately two times higher than the starch content in wild type plants, similar to the results we found for FER in *Arabidopsis* (Fig. 6A, B, C).

Concluding Remarks

In this work we found that the receptor kinase FER controls starch accumulation in plants. Starch content increased in the leaves of *fer-4* mutants, but it decreased in FER-OE plants. Therefore, we reasoned that FER may play a key role in closely

monitoring nutrient availability while integrating environmental, developmental, and physiological cues to adjust cellular metabolism. Further, we found that FER interacts with GAPC. The enzyme activity of GAPC declined in *fer-4* mutants, but it increased in FER-OE plants. Thus, GAPC promotes the glyceraldehyde oxidation to control the rate of glycolysis, regulating the starch/energy metabolism.

Importantly, we have shown that the function of FER in starch/energy metabolism is conserved in *Arabidopsis* and *Oryza*, suggesting conservation across angiosperms. Our previous work has shown that FER plays an important role in seed size and stress response control (Yu et al., 2012; Yu et al., 2014). In addition FER has been identified as a key player in responding to hormone cues and plant development (Yu et al., 2014; Höfte et al., 2014). Therefore, it is of particular interest to further investigate the molecular mechanism underlying the hypothesis that FER functions as a kinase that coordinates the expression of important agronomic traits, such as starch accumulation.

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TABLES, LEGENDS and FIGURES

Figure 1. FER transcript and protein levels increased after sucrose treatment

(A) *Arabidopsis thaliana* was treated with different concentrations of sucrose (x axis) for 8-d and *FER* transcript levels (y axis) were analyzed as described in the Materials and Methods section. *Beta-actin* was used as the internal control to calculate the relative mRNA levels. Means and standard deviation (SD) were obtained from three independent experiments.

(B) Immunoblot analysis of total protein extracted from 8-d Col-0 seedlings grown in the presence of the sucrose concentration indicated, then analyzed with anti-FER antibodies to detect FER protein. Coomassie stained proteins served as loading controls.

Figure 2. The effect of sucrose on starch accumulation in WT and *fer-4* mutants

(A) IKI staining of 4-d dark-grown wild type and *fer-4* mutant seedlings grown with 30 and 60 mM sucrose. Image is one representative of triplicate experiments showing the same results. Bar = 0.5 mm.

(B) Magnification of the upper hypocotyl in seedlings after treatment with 60 mM sucrose (as in Fig. 2A). Bar = 0.2 mm.

(C) IKI staining of 4-d dark-grown wild type and *fer-4* seedlings grown with 0, 30 and 60 mM mannitol. Image is one representative of triplicate experiments showing the same results. Bar = 0.5 mm.

Figure 3. Effect of sucrose on hypocotyl elongation in *fer-4* mutants

(A) Hypocotyl length of 4-d dark-grown wild type and *fer-4* seedlings grown with 0, 30, 60, 120 and 180 mM sucrose or mannitol. Three independent experiments were conducted showing the same results. Bar = 0.5 cm.

(B) Hypocotyl lengths of 4-d wild type and *fer-4* dark-grown seedlings grown with increasing concentrations of sucrose and mannitol. Each data point represents means $\pm$ SD (n = 24). Wild type and *fer-4* 0 mM sugar data points in (B) were the same treatment because the experiments were performed at the same time.

Figure 4. GAPC1 and GAPC2 interact with FER

(A) Diagram of the full-length and truncated GAPC1 and GAPC2 constructs with specific deletions. Both GAPC1 and GAPC2 exist as a tetramer of identical subunits, each contains two conserved functional domains: an NAD-binding domain at the N-terminal and a highly conserved catalytic domain at the C-terminal.

(B) Yeast two hybrid analysis shows that the catalytic domain of GAPC1 and GAPC2 interact with FER based on the observations that they grow on synthetic dropout selection medium that lacked Trp, Leu, and His supplemented with 20mM 3-AT for 4 days.

(C) DIC and BiFC fluorescence images for the transfected mesophyll protoplasts expressing FER-nVenus and GAPC1-cCFP or GAPC2-cCFP. The interaction between FER-nVenus and GAPC1-cCFP or GAPC2-cCFP was observed with a GFP filter and chlorophyll was observed as red fluorescence. Protoplasts expressing FER-nVenus and cCFP are the negative controls and failed to yield detectable green fluorescence. Three independent experiments were conducted showing the same results. Bar = 20 μm .

Figure 5. FER regulates enzyme activity and starch content

(A) GAPDH enzyme activity of wild type, *fer-4*, FER-OE, *gapc1*, and *gapc2*. * indicates significant differences ($P < 0.05$).

(B) Starch content of wild type, *fer-4*, FER-OE, *gapc1-1*, and *gapc2-2*. Seedlings were grown for five weeks in soil in a 16 h light/8 h dark photoperiod. The experiment was repeated three times with similar results. Values are means $\pm$ SE ($n = 3$). ** indicates a significant difference at $P < 0.01$. * indicates a significant difference at $P < 0.05$.

Figure 6. Starch phenotype of *flr1* mutant

(A) IKI staining of one-month old leaves of wild type and *flr1* plants. Staining was performed at the end of the dark cycle. Image is one representative of triplicate experiments showing the same results. Bar = 0.5 cm.

(B) Magnified images show IKI staining of one-month old leaves of wild type and *flr1* plants. Image is one representative of triplicate experiments showing the same results. Bar = 500 μ m

(C) Starch content of wild type and *flr1* plants. Data bar represents the means $\pm$ SD (n=3). ** indicates a significant difference at $P<0.01$.