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Research Paper

Sugar Starvation Enhances Leaf Senescence and Genes Involved in Sugar Signaling Pathways Regulate Early Leaf Senescence in Mutant Rice

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Abstract: To clarify the complex regulatory relationship between changes in sugar content and leaf senescence during the grain-filling stage of rice, genotype-dependent differences in sugar content and the temporal transcriptional patterns of genes involved in sugar signaling pathways were determined in mutant rice exhibiting early leaf senescence and its wild type Zhefu 142. The effects of exogenous glucose or sucrose on the senescence of detached leaves under dark conditions were also investigated. Chlorophyll, soluble sugar, sucrose and fructose contents decreased, whereas electrolytic leakage and malondialdehyde levels increased in mutant leaves at the grain-filling stage. These results suggested that sugar starvation is positively correlated with the early leaf senescence of mutant plants. Detached leaf segments incubated in exogenous sugar solutions under dark conditions exhibited delayed senescence. The high expression of *Hxk1* in leaves of mutant plants at the initial grain-filling stage suggested that *Hxk1* is involved in the hexose-sensing process at the early stage of leaf senescence. The low expression levels of *Hxk2* and *Frk1* in the senescing leaves of mutant rice during the grain-filling stage are indicative of weakened hexose phosphorylation. In addition, the high expression levels of *SuSy1*, *SuSy2* and *SuSy4* in leaves of mutant plants at the initial grain-filling stage are accompanied by the high transcript levels of *SUT1*, which favor sucrose translocation and remobilization from the early senescing leaves of mutant rice. The relatively reduced transcript levels of *chFBP*, *cyFBP*, *SPS1*, *SPS2* and *SPS6* indicated that during the grain-filling stage, sucrose biosynthesis is weakened in the senescing leaves of mutant rice.

Key words: rice; sugar starvation; leaf senescence; signaling pathway; detached leaf segment; grain-filling stage

Rice (*Oryza sativa* L.) is one of the most important cereal crops worldwide given that it is the staple food of approximately half of the world's population (IRGSP, 2005). Rice yield is highly dependent on photosynthate assimilation by functional leaves during the grain-filling stage. The photosynthetic intensity and durability of rice leaves are essential for grain filling. Senescence is the final stage of plant development. In annual plants, the hydrolysates of macromolecules, such as starch, proteins and lipids, in senescing leaves are translocated to developing seeds and fruits and thus contribute to the final yield (Woo et al, 2013). However, leaf senescence often occurs prematurely under severe environmental stress during the grain-filling stage. Premature leaf senescence decreases photosynthetic rate and assimilate biosynthesis, thereby retarding nutrient translocation from source leaves to developing grains, causing incomplete grain setting and reducing the final yield (Wu et al, 2012; Distelfeld et al, 2014; Zhao et al, 2018).

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Leaf senescence corresponds to the programmed degradation of cells, tissues, organs and the entire organism (Lim et al, 2007). Senescing leaves undergo drastic coordinated changes, such as chlorophyll breakdown, photosynthesis termination, membrane deterioration, malondialdehyde (MDA) elevation, macromolecule hydrolysis, reactive oxygen species (ROS) accumulation, abscisic acid (ABA) generation and senescence-associated gene (SAG) expression. These changes cause cell structure disassembly and cell death (Takahashi et al, 2003; Lim et al, 2007). Intercellular sugars have long been thought to act as important signal molecules that regulate plant metabolism and development, as well as gene expression during leaf senescence (Gibson, 2005; Hanson and Smeekens, 2009). The roles of intercellular sugars in the induction or acceleration of leaf senescence have been debated for several decades. A decrease in photosynthesis precedes leaf yellowing and is followed by SAG transcription (Quirino et al, 2000; van Doorn, 2008). The results of research on *Arabidopsis* leaves subjected to dark conditions suggest that low carbohydrate levels induce SAG expression (Quirino et al, 2000; Lam et al, 2001). These discoveries support the hypothesis that leaf senescence is induced or enhanced by sugar starvation. Nevertheless, other evidence supports the opposing hypothesis that leaf senescence is induced or enhanced by increases in sugar levels (van Doorn, 2008). For example, sugar levels in tobacco leaves that are on the verge of senescence are higher than those in younger and older tobacco leaves (Masclaux et al, 2000). Detached barley leaves exposed to strong light exhibit accelerated yellowing and increased soluble sugar levels (Parrott et al, 2005). The interruption of phloem export in girdled wheat leaves promotes sugar accumulation and accelerates chlorophyll degradation (Feller and Fischer, 1994). Although the above mentioned results confirm that sugar level is a signal that regulates leaf senescence, direct evidence that proves whether leaf senescence is affected by sugar starvation or accumulation has not yet been found.

Sugar signaling regulates several aspects of plant growth and development (Biswal and Pandey, 2018). Hexoses, including glucose and fructose, are potent plant signaling molecules. Hexokinase (Hxk) and fructokinase (Frk) play crucial regulatory roles in the perception and transduction of hexose signals in the hexose-mediated signaling pathway (Rolland et al, 2002). Sucrose, a disaccharide, also participates in the regulation of tissue development and gene expression. In leaves, sucrose phosphate synthase (SPS) catalyzes the rate-limiting step of sucrose biosynthesis from UDP-glucose and fructose-6-phosphate. Subsequently, cytosolic and chloroplastic fructose-1,6-bisphosphatases (FBPase) participate in the sucrose biosynthetic pathway in the cytosol and the formation of starch precursors in the chloroplast (Daie, 1993). Sucrose can be cleaved by either sucrose synthase (SuSy), the main sucrose-cleaving enzyme in the vascular system, into UDP-glucose and fructose, or by cell wall invertase (CIN) into glucose and fructose, thereby yielding twice as many hexoses. Sucrose cleavage connects the conversion processes of sucrose and hexoses, which are vital for the allocation of crucial carbon resources and the initiation of sugar signaling pathways (Koch, 2004). Low CIN activity is required for inducing leaf yellowing in tobacco. *CIN* overexpression results in high invertase activity and delays leaf yellowing (Balibrea Lara et al, 2004). In addition, sucrose translocation catalyzed by sucrose transporters (SUTs) plays a major role in grain filling after anthesis and contributes to the final yield (Aoki et al, 2003). Over the past several decades, a number of researchers have proposed that sugar signaling is involved in the regulation of catabolic events associated with leaf senescence and have identified some sugar sensors (Biswal and Pandey, 2018). However, the underlying molecular basis for the effect of sugar on leaf senescence is still poorly understood until now.

This study investigated the genotypic differences in sugar levels and temporal transcriptional patterns of genes involved in sugar signaling pathways in mutant rice with the early leaf senescence phenotype and its wild type cultivar Zhefu 142 (*O. sativa* L. ssp. *indica*) during the grain-filling stage. In addition, the effect of sugar treatment on leaf senescence was investigated by incubating detached leaf segments in exogenous glucose and sucrose solutions under dark conditions. The corresponding transcriptional pattern of genes associated with sugar signaling pathways in the detached leaf segments was determined through quantitative real-time PCR (qRT-PCR) to clarify the possible interplay between the transcripts of distinct genes related to sugar signaling and leaf senescence.

MATERIALS AND METHODS

Plant materials and growth conditions

Mutant rice plants with the early leaf senescence phenotype were obtained from the mature seeds of the

gramma-irradiated *indica* restored line Zhefu 142. Stable inherited mutants were selected through successive self-pollination, and phenotype selection was performed from the M_2 to M_8 generations. The seeds of M_8 generation were used in this study. The mutant plants did not display noticeable phenotypic abnormalities during the seedling and tillering stages. However, during the late-tillering stage, the leaves of the mutant plants began to display lesions and accelerated senescence beginning from the tips of the lower leaves. The brown lesions then became exacerbated and expanded to cover the whole leaf blade. However, the topmost two fully expanded leaves and central leaves of the plants retained normal green appearance. After flowering, the flag leaves of mutant rice plants displayed senescence symptoms, and the lesions gradually spread from the tip down to the whole leaf blade during the grain-filling stage until the leaf blade completely withered at approximately 25 d after flowering. By contrast, wild type plants retained their green appearance during the same period (Li et al, 2014). Our previous studies have revealed that a cytosine deletion of gene encoding vacuolar H^+-ATPase subunit A1 results in the phenotypic abnormalities in the mutant plant (Yang et al, 2016).

Rice seeds were sown in the seedling nursery at the experimental field of the Zijingang Campus (120°04′ E, 30°18′ N) of Zhejiang University, Hangzhou, China. A completely randomized field plot was designed with three replications for each genotype. Each replication was planted in 10 × 12 rows spaced at 18 cm × 18 cm with one rice seedling for each hill. Field management was performed in accordance with the local cultivation mode. The soil type was periodically waterlogged paddy soil with 1.69 g/kg total N, 24.5 mg/kg available P, and 103.7 mg/kg exchangeable K. At the full heading stage, 60–80 rice plants with uniform flowering days were randomly selected and tagged. From the beginning of the grain-filling stage, the flag leaves of the tagged plants were sampled over 7-day intervals at 9:00 am with three independent biological replications. Some fresh samples were used for the determination of physiological parameters, and the other leaf samples were immediately frozen in liquid nitrogen and maintained at -80 ℃ for gene expression analysis.

To investigate the effect of sugar on leaf senescence, an additional experiment on detached leaf segments incubated with exogenous sugar solutions was conducted in accordance with the procedure described by Dian et al (2003). After three weeks of transplanting, the four-leaf stage seedlings were chosen for exogenous sugar treatments. The topmost second leaves of seedlings were fully extended and thrived at that stage. Prior to exogenous sugar treatments, seedlings were cultured for 2 d under dark conditions to deplete endogenous sugars. After 2 d, the second leaves at the topmost position were carefully excised from wild type plants. The detached leaf segments were cut into about 20 mm in length and transferred to solutions containing 175 mmol/L sucrose or glucose and incubated in the darkness at 28 ℃. According to Dian et al (2003), 175 mmol/L of sugar solution was pretty suitable for the growth of the detached leaf segment. A group of detached leaf segments was incubated in 175 mmol/L mannitol solution as the osmosis control group, and another group of leaf segment was incubated in normal nutrient solution as the control group. Previous studies confirmed that 3, 6 and 12 h were the right time for tested genes to respond to conduct exogenous sugars in detached leaf segments. Thus, to conduct gene expression analysis, the leaf segments were collected after 3, 6 and 12 h incubation, respectively. Another incubation experiment was implemented with gradient concentrations of sucrose set as 20, 40, 80, 160, 320, 480 and 600 mmol/L. Detached leaf segments were separately immersed in the gradient concentrations of sucrose and incubated in the dark at 28 ℃. Leaf samples were collected after 6 h incubation. Several of the leaf segments were incubated in Hoagland nutrient solution as a control. For chlorophyll content measurement and phenotypic observation, the detached leaf samples were harvested after 6 d of sucrose treatments, with three replications for each treatment.

Determination of total chlorophyll content, electrolytic leakage and MDA content

Leaf samples were ground and soaked in 95% ethanol (Molecular Biology Grade, Sangon Biotech Co. Ltd., Shanghai, China) for 24 h. After centrifugation, total chlorophyll was extracted from the supernatant. Chlorophyll contents were determined as described by Lichtenthaler (1987). The electrolytic leakage of leaf tissues was measured in accordance with Lu et al (2008). MDA content of fresh leaves was determined through the 5% thiobarbituric acid reaction (Saher, 2004).

Measurement of soluble total sugar, sucrose, glucose and fructose contents

Fresh leaf samples were dehydrated at 105 ℃ for

nearly 30 min to deactivate enzymes and were subsequently baked at 60 ℃ for 72 h. Dried leaf samples were ground into powder using a pulverizer, and 0.5 g powder sample was extracted with 80% ethanol at 80 ℃ for 30 min with three repetitions. After centrifugation at 5000 × *g* for 15 min, the supernatant was collected for the determination of soluble total sugar, sucrose, glucose and fructose contents as described by Luo and Huang (2011). To measure sucrose content, 0.4 mL supernatant was mixed with 200 μL of 2.0 mol/L NaOH and then incubated in boiling water for 5 min. In one tube, 2.8 mL of 30% HCl and 0.8 mL of 0.1% dioxybenzene were added to the cooled solution and mixed. The mixed solution was maintained at 80 ℃ for 10 min. The absorbance of the solution was measured at 480 nm, and sucrose content was calculated in reference to a standard sucrose curve. To quantify fructose content, 1 mL supernatant was mixed with 2 mL of 0.1% dioxybenzene and 1 mL water and then incubated at 80 ℃ for 10 min. When the sample had cooled down, absorbance was measured at 480 nm, and fructose content was calculated in reference to a standard fructose curve. Glucose content was determined on the basis of the oxidation of glucose catalyzed by glucose oxidase. A total of 2 mL supernatant and 4 mL glucose oxidase reagent (0.1 mg/mL *o*-dianisidine, 0.1 mg/mL horseradish peroxidase and 1 U/mL glucose oxidase) were coincubated at 30 ℃ for 10 min, and then, mixed with 8 mL of 10 mol/L H_2SO_4 solution for reaction termination. The absorbance of the mixture solution was determined at 460 nm within 1 h. The glucose content of the supernatant was calculated in reference to a standard glucose curve. Total soluble sugar was measured in accordance with the anthrone-H_2SO_4 colorimetry method (Li et al, 2017).

RNA extraction and cDNA synthesis

Frozen leaf tissues (100 mg) were crushed into fine powder in liquid nitrogen by using a mortar and pestle. Total RNA was extracted with Trizol reagent (Invitrogen, USA) in accordance with the manufacturer's protocol. RNA quality was evaluated by using a spectrophotometer (NanoDropTM 1000, Thermo Fisher Scientific, USA), and genomic DNA was removed through incubation with RNase-free DNase I at 37 ℃. Subsequently, exactly 1 μg RNA was reverse transcribed into cDNA through the addition of an oligo (dT) primer in 50 μL reaction buffer. The mixture was incubated at 37 ℃ for 15 min and then terminated through heating at 95 ℃ for 5 min.

Quantitative real-time PCR (qRT-PCR)

Aliquots of the cDNA mixture were used as templates for qRT-PCR analysis with SYBR Green Real-time PCR Master Mix Reagent Kit (Toyobo, Osaka, Japan). qRT-PCR was performed on a Bio-Rad CFX96 System (Bio-Rad, USA) in accordance with the manufacturers' protocol under the following cycling conditions: 95 ℃ for 30 s; 40 cycles at 95 ℃ for 5 s and 58 ℃ for 10 s; and 72 ℃ for 15 s. A melting curve protocol from 58 ℃ to 95 ℃ following the final PCR cycle was performed to detect a single gene-specific peak for all the tested primers. Gene-specific primer pairs were designed by using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Supplemental Table 1. *Actin-1* was used as the internal reference gene for data normalization. Relative expression levels were analyzed through the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Average values and standard errors were calculated on the basis of three independent biological repetitions.

Statistical analysis

All determinations were performed in three independent experiments. Statistical significances were estimated by analysis of variance (ANOVA) at the 0.05 level, using the SPSS statistical software package (Chicago, USA). Standard error was calculated and shown in figures.

RESULTS

Genotypic-dependent differences in senescence-related physiological parameters during grain-filling stage

To compare the senescence-associated changes in the flag leaves of mutant rice and its wild type during the grain-filling stage, chlorophyll content, membrane integrity and lipid peroxidation were evaluated as cellular indicators of leaf senescence. Membrane integrity and lipid peroxidation were estimated on the basis of electrolytic leakage and MDA content. As shown in Fig. 1, the total chlorophyll contents of mutant leaves sharply decreased throughout the entire grain-filling stage and were significantly lower than those of the wild type leaves at 7 d after flowering (DAF). Compared with those in wild type leaves, electrolytic leakage and MDA content in mutant leaves increased rapidly and peaked at 28 DAF. By

contrast, in the wild type leaves, the total chlorophyll content slightly decreased, and the electrolytic leakage and MDA levels stabilized throughout the entire grain-filling stage (Fig. 1). These results suggested that the flag leaves of the mutant plant exhibit the physiological characteristics of early senescence during the grain-filling stage.

Genotypic-dependent differences in sugar levels during the grain-filling stage

As shown in Fig. 2, in the mutant leaves, soluble sugar and sucrose contents drastically decreased to approximately 26 and 15 mg/g, respectively, at 14 DAF. At 14 DAF, fructose levels in the mutant leaves decreased to levels lower than those in the wild type leaves. These results suggested that sugar insufficiency or starvation occur in the functional leaves of the mutant plant during the grain-filling stage. By contrast, the soluble sugar, sucrose and fructose levels were relatively stable in the wild type leaves throughout the entire grain-filling stage (Fig. 2). In addition, the glucose levels of the two rice genotypes changed negligibly during the grain-filling stage.

Exogenous sugar treatment delayed darkness-induced leaf senescence

Detached leaf segments were incubated in 175 mmol/L glucose, sucrose and mannitol solutions in the dark. Normal nutrient solution treatment was set as a control. As shown in Fig. 3-A, the detached leaf segments incubated in glucose and sucrose solutions retained their green color. By contrast, the detached leaf segments under the mannitol and control treatments gradually yellowed as incubation progressed. Moreover, the chlorophyll contents of leaf segments incubated in sucrose and glucose solutions were significantly higher than those incubated in mannitol and normal nutrient solutions (Fig. 3-B). These results suggested that exogenous glucose and sucrose treatments delay the darkness-induced senescence of detached leaf segments.

The detached leaf segments were incubated in a series of gradient concentrations of sucrose (20, 40, 80, 160, 320, 480 and 600 mmol/L). As shown in Fig. 3-C, the senescence of the detached leaf segments under dark conditions was affected by incubation in exogenous sucrose. Specifically, incubation in sucrose solutions with high concentrations prevented the darkness-

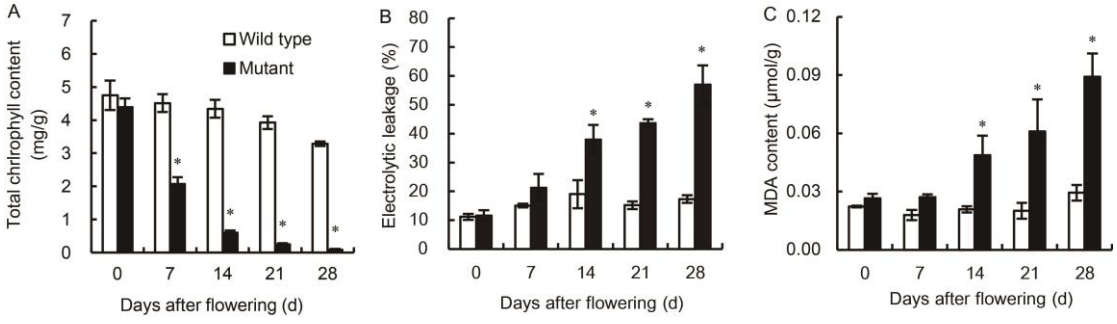

Fig. 1. Genotypic-dependent differences in total chlorophyll content (A), electrolytic leakage (B) and malondialdehyde (MDA) content (C) in flag leaves of wild type and mutant genotypes during grain-filling stage.
Vertical bars represent standard errors (n = 3). Asterisks represent significant differences between the mutant and the wild type at the 0.05 level.

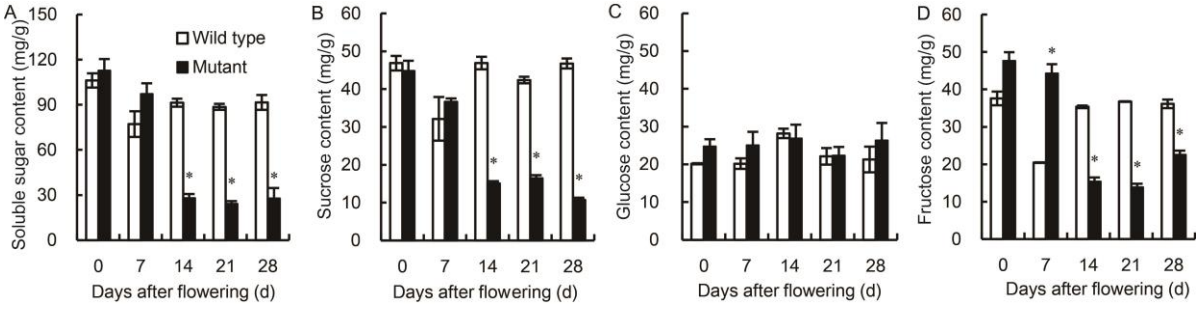

Fig. 2. Genotypic-dependent differences in soluble sugar (A), sucrose (B), glucose (C) and fructose (D) contents in flag leaves of wild type and mutant genotypes during grain-filling stage.
Vertical bars represent standard errors (n = 3). Asterisks represent significant differences between the mutant and the wild type at the 0.05 level.

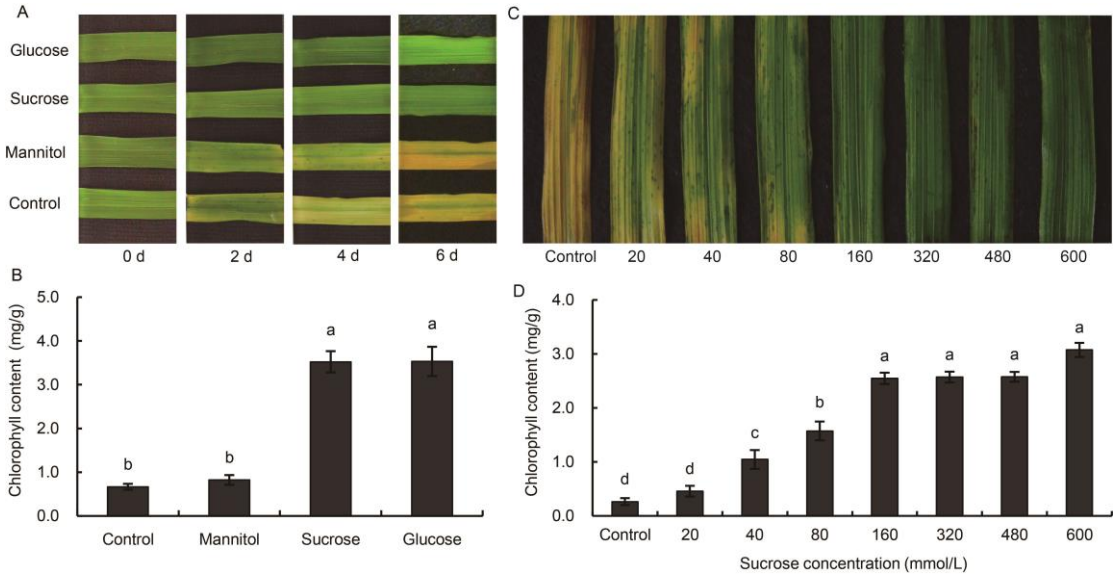

Fig. 3. Temporal analyses of the darkness-induced senescence of detached leaf segments incubated in 175 mmol/L glucose and sucrose, mannitol (osmosis control), hoagland nutrient solutions (control), and a gradient series of sucrose solutions for 6 d.

A, Morphological changes exhibited by detached leaf segments after 0, 2, 4 and 6 d of incubation. **B**, Chlorophyll contents of detached leaf segments after 6 d of incubation in glucose, sucrose, mannitol and control. **C**, Morphological changes exhibited by detached leaf segments incubated in 20, 40, 80, 160, 320, 480 and 600 mmol/L sucrose solutions. **D**, Chlorophyll contents of detached leaf segments incubated in 20, 40, 80, 160, 320, 480 and 600 mmol/L sucrose solutions.

Vertical bars represent standard errors (n = 3). Values with the same lowercase letters are not significantly different between different sugar treatments at the 0.05 level.

induced leaf senescence (Fig. 3-C). The chlorophyll contents of the detached leaf segments incubated in sucrose solutions still retained high levels with the increment of sucrose concentrations (Fig. 3-D). These results suggested that the exogenous sugar treatments postpone the degradation of chlorophyll pigment, and the extent of the delay of darkness-induced senescence by exogenous sugar in detached leaf segments is dependent on sucrose concentration.

Expression patterns of genes related to response of hexose signaling pathway to sugar levels

Hxk and Frk mainly phosphorylate glucose and fructose, respectively, and are important catalytic enzymes in the hexose signaling pathway. As shown in Fig. 4-A, the *Hxk1* expression levels in the mutant rice were higher than those in the wild type rice at the initial grain-filling stage and gradually decreased as grain filling progressed to levels lower than those in the wild type at the end of the grain-filling stage. The *Hxk2* expression levels in the mutant leaves began to decrease from the initial grain-filling stage and were significantly lower than those in the wild type leaves during the whole grain-filling stage (Fig. 4-B). Moreover, the expression levels of *Hxk1* and *Hxk2* were depressed by exogenous 175 mmol/L sucrose or

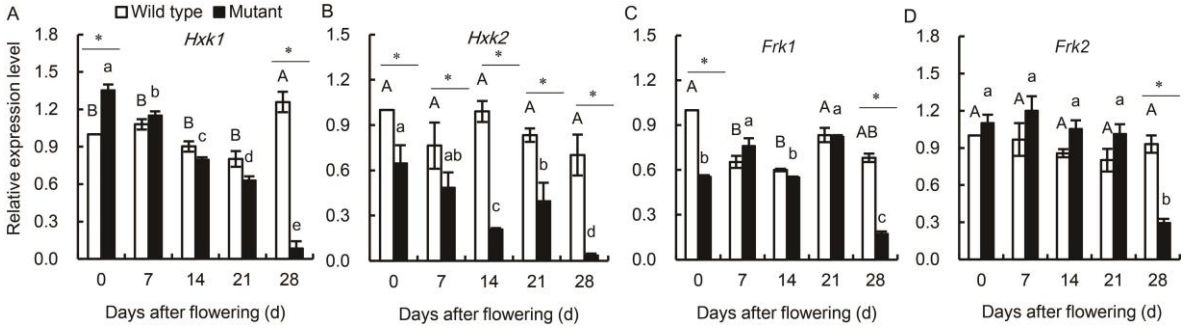

Fig. 4. Genotype-specific differences in the expression patterns of *Hxk1* (A), *Hxk2* (B), *Frk1* (C) and *Frk2* (D) in flag leaves of wild type and mutant genotypes during grain-filling stage.

Vertical bars represent standard errors (n = 3). Asterisks represent significant differences between the wild type and the mutant at the 0.05 level. Values with the same uppercase and lowercase letters are not significantly different among wild type and mutants at the 0.05 level, respectively.

glucose treatments (Fig. 5-A and -B). However, the expression levels of *Hxk1* and *Hxk2* were dependent on exogenous sucrose concentration. The *Hxk1* transcript levels gradually decreased as exogenous sucrose concentration increased and dropped to the minimum expression level under 320 mmol/L sucrose treatment (Fig. 5-E). By contrast, the *Hxk2* transcription was enhanced under low sucrose concentrations (20 and 40 mmol/L) and was depressed under high sucrose concentration (80 mmol/L) (Fig. 5-F).

The *Frk1* expression levels in the mutant leaves were lower than those in the wild type leaves at the initial and final stages of grain filling (Fig. 4-C). The transcription of *Frk1* in detached leaf segments was depressed by treatment with 175 mmol/L sucrose or glucose and was especially depressed after 3 h of sucrose and glucose treatments (Fig. 5-C). The transcription of *Frk1* was depressed by either extremely low concentration of sucrose (20 mmol/L) or high concentrations of sucrose (from 80 to 320 mmol/L) (Fig. 5-G). These results suggested that *Frk1* participates in the response to sucrose starvation or accumulation. By contrast, the genotypic- dependent expression of *Frk2* was not significantly different between the two rice genotypes until the final grain-filling stage (Fig. 4-D). Treatment with exogenous 175 mmol/L glucose slightly changed the transcript levels of *Frk2* in detached leaf segments, and exogenous 175 mmol/L sucrose failed to alter the *Frk2* expression (Fig. 5-D), only treatment with extremely high sucrose (600 mmol/L) concentration depressed the expression of *Frk2* in detached leaf segments (Fig. 5-H).

Transcriptional regulation of sucrose conversion to monosaccharides mediated by *CIN* and *SuSy*

CIN and SuSy play important roles in regulating sucrose conversion in plants. Throughout the entire grain-filling stage, the *CIN1* and *CIN4* expression levels rapidly decreased and were lower in the mutant rice than those in the wild type rice, which raised at 28 DAF for the wild type rice (Fig. 6-A and -B). Moreover, the transcriptions of *CIN1* and *CIN4* were severely suppressed by treatment with 175 mmol/L sucrose or glucose (Fig. 6-C and -D). The *CIN1* transcription was severely suppressed in detached leaf segments incubated with sucrose solutions (Fig. 6-E). By contrast, the *CIN4* transcription gradually decreased with the increment in sucrose concentrations and then began to plateau when sugar concentrations raised to more than 80 mmol/L (Fig. 6-F). These results suggested that the regulatory transcription of *CIN1* and *CIN4* in rice tissues is dependent on sucrose

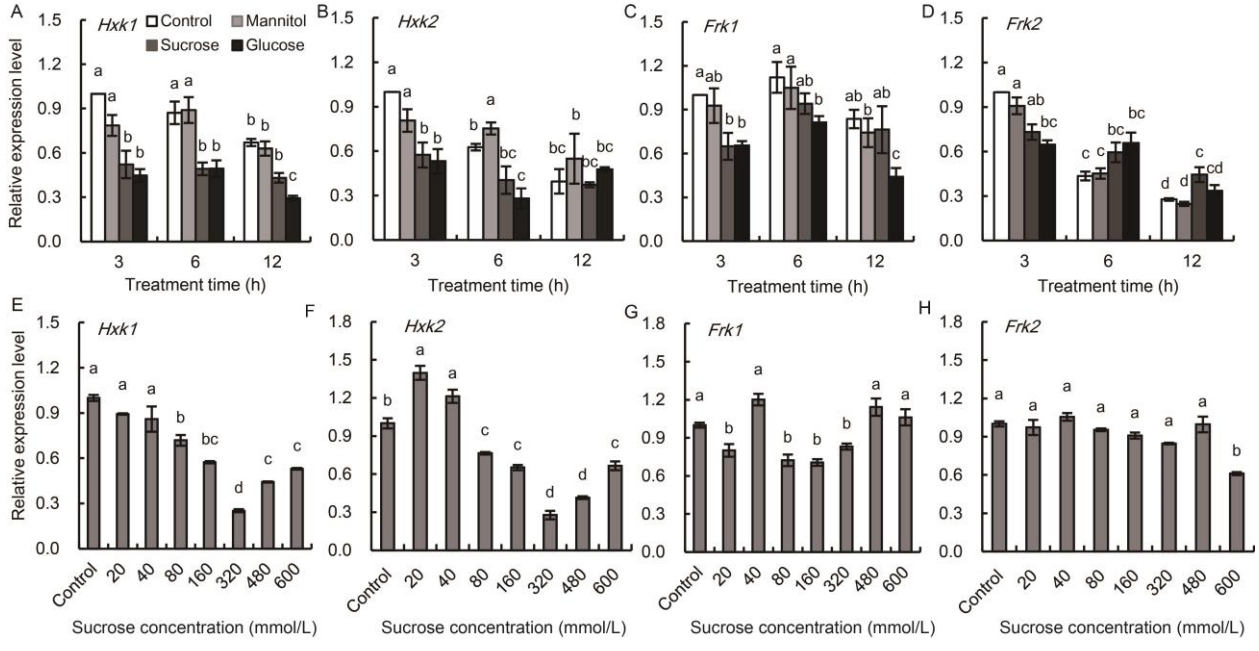

Fig. 5. Temporal analyses of *Hxk1* (A), *Hxk2* (B), *Frk1* (C) and *Frk2* (D) in detached wild type leaf segments incubated in 175 mmol/L sucrose, glucose and mannitol (osmosis control), and transcriptional analyses of *Hxk1* (E), *Hxk2* (F), *Frk1* (G) and *Frk2* (H) in the detached wild type leaf segments incubated in a gradient series of sucrose concentrations.

For temporal analyses, the leaf segments were collected after 3, 6 and 12 h incubation, respectively, and treatment with Hoagland nutrient solution was set as the control. For transcriptional analyses, the leaf segments were collected after 6 h incubation. Vertical bars represent standard errors (n = 3). Values with the same lowercase letters are not significantly different between different sugar treatments at the 0.05 level.

content.

As shown in Fig. 7, at the initial stage of grain filling, the mutant rice displayed higher *SuSy1*, *SuSy2* and *SuSy4* transcript levels than the wild type. At 14 DAF, the transcript levels of *SuSy1*, *SuSy2* and *SuSy4* decreased in the mutants to levels lower than those in the wild type plants. In addition, *SuSy1*, *SuSy2* and *SuSy4* levels in the detached leaf segments were significantly suppressed by treatment with exogenous 175 mmol/L sucrose or glucose (Fig. 7-D to -F). *SuSy1* and *SuSy2* transcript levels were significantly depressed by treatment with 20 and 80–480 mmol/L sucrose and were elevated by treatment with 40 mmol/L sucrose (Fig. 7-G and -H). These results indicated that *SuSy1* and *SuSy2* are sensitive to extremely low and high sucrose levels. By contrast, the transcription of *SuSy4* in detached leaf segments was slightly induced by treatment with low sucrose concentrations and severely suppressed by treatment with high sucrose concentrations (Fig. 7-I).

Transcriptional regulation of sucrose translocation and biosynthesis in senescing leaves of mutant rice

SUT1 and *SUT4* expression levels were quantified to investigate the transcriptional regulation of sucrose translocation in senescing leaves. As shown in Fig. 8-A, at the initial grain-filling stage (from 0 to 7 DAF), the *SUT1* expression in the mutant rice was significantly higher than that in the wild type. Subsequently, at 21 DAF, the *SUT1* expression in the mutant gradually decreased to levels lower than that in the wild type. This result suggested that *SUT1* is involved in sucrose translocation during the early stage of leaf senescence in mutant rice. By contrast, no significant difference in *SUT4* expression was found between the two rice genotypes until 21 DAF, when *SUT4* expression in the mutant became significantly lower than that in the wild type (Fig. 8-B).

During sucrose metabolism, FBPase and SPS are responsible for irreversible sucrose synthesis from UDP-glucose and fructose-6-phosphate. In this study, the expression levels of the two *FBP* and the four *SPS* isoforms in the two rice genotypes were determined through qRT-PCR. As shown in Fig. 8-C, during the grain-filling stage, the *chFBP* expression in the mutant rice decreased to levels that are lower than those in the wild type. In the wild type plants, *chFBP* transcription increased during the early- and mid-stage of grain filling and subsequently decreased. The

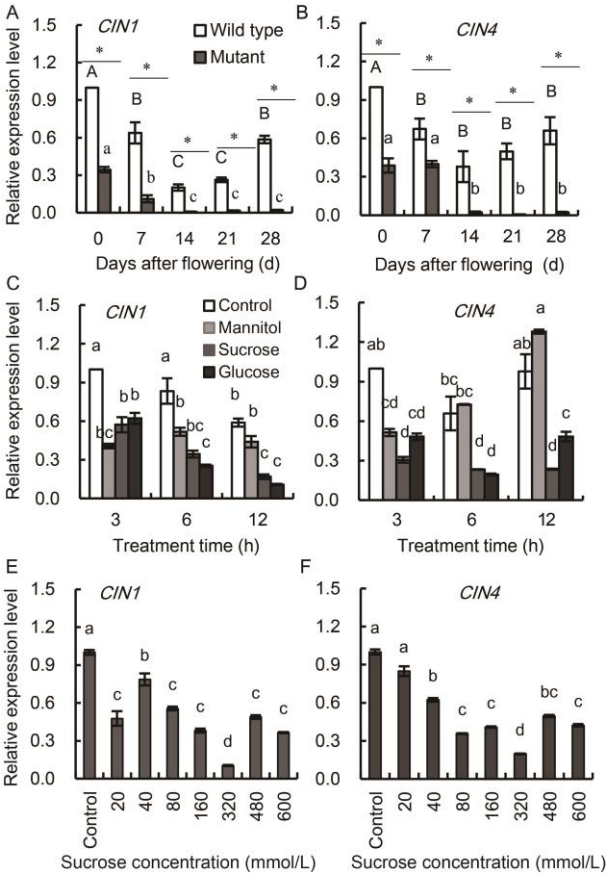

Fig. 6. Relative expression levels of *CIN1* and *CIN4* genes in the mutant and wild type leaves under different treatments.

A and **B**, Genotypic-dependent differences in the expression patterns of *CIN1* (**A**) and *CIN4* (**B**) in the flag leaves of wild type and mutant genotypes during the grain-filling stage. Asterisks represent significant differences between the wild type and the mutant at the 0.05 level. Values with the same uppercase and lowercase letters are not significantly different among the wild type and the mutants at the 0.05 level, respectively. **C** and **D**, Temporal analyses of *CIN1* (**C**) and *CIN4* (**D**) in detached wild type leaf segments incubated in 175 mmol/L sucrose, glucose and mannitol (osmosis control). Leaf segments were collected after 3, 6 and 12 h incubation, respectively. Values with the same lowercase letters are not significantly different among different sugar treatments at the 0.05 level. **E** and **F**, Transcriptional analyses of *CIN1* (**E**) and *CIN4* (**F**) in the detached wild type leaf segments incubated in a gradient series of sucrose concentrations. Leaf segments were collected after 6 h incubation. Treatment with Hoagland nutrient solution was set as the control. Values with the same lowercase letters are not significantly different among different sugar concentration treatments at the 0.05 level.

Vertical bars represent standard errors (n = 3).

cyFBP expression in the mutant plants decreased to levels lower than those in the wild type plants (Fig. 8-D). The expression levels of *SPS1*, *SPS2* and *SPS6* in the mutant gradually decreased by different degrees and were significantly lower than those in the wild type (Fig. 8-E to -G). In the wild type, in addition to the marked increase in the transcription level of *SPS1*

during the mid-stage of grain filling, *SPS2* and *SPS6* expression levels remained relatively stable throughout the entire grain-filling stage. These results suggested that sucrose synthesis mediated by *chFBP*, *cyFBP*, *SPS1*, *SPS2* and *SPS6* is weakened in the senescing leaves of mutant rice during the grain-filling stage. By contrast, the expression level of *SPS8* in the mutant rice was significantly higher than that in the wild type rice at 0 and 14 DAF and was significantly lower than that in the wild type at 7 and 28 DAF (Fig. 8-H). The *SPS8* expression in the wild type rice remained relatively stable throughout the entire grain-filling stage.

DISCUSSION

The hypothesis that leaf senescence is induced by increases in sugar levels has been a point of contention for several decades (Yoshida, 2003). Some previous studies have found that leaf sugar content increases during the senescing period of several plant species, such as tobacco, wheat, maize and castor bean (Noodén et al, 1997; Jongebloed et al, 2004). *Arabidopsis* and tomato plants that overexpress *Hxk* exhibit higher sugar status, inhibited growth, and rapid leaf senescence (Yoshida, 2003; Pourtau et al, 2006). However, sugar

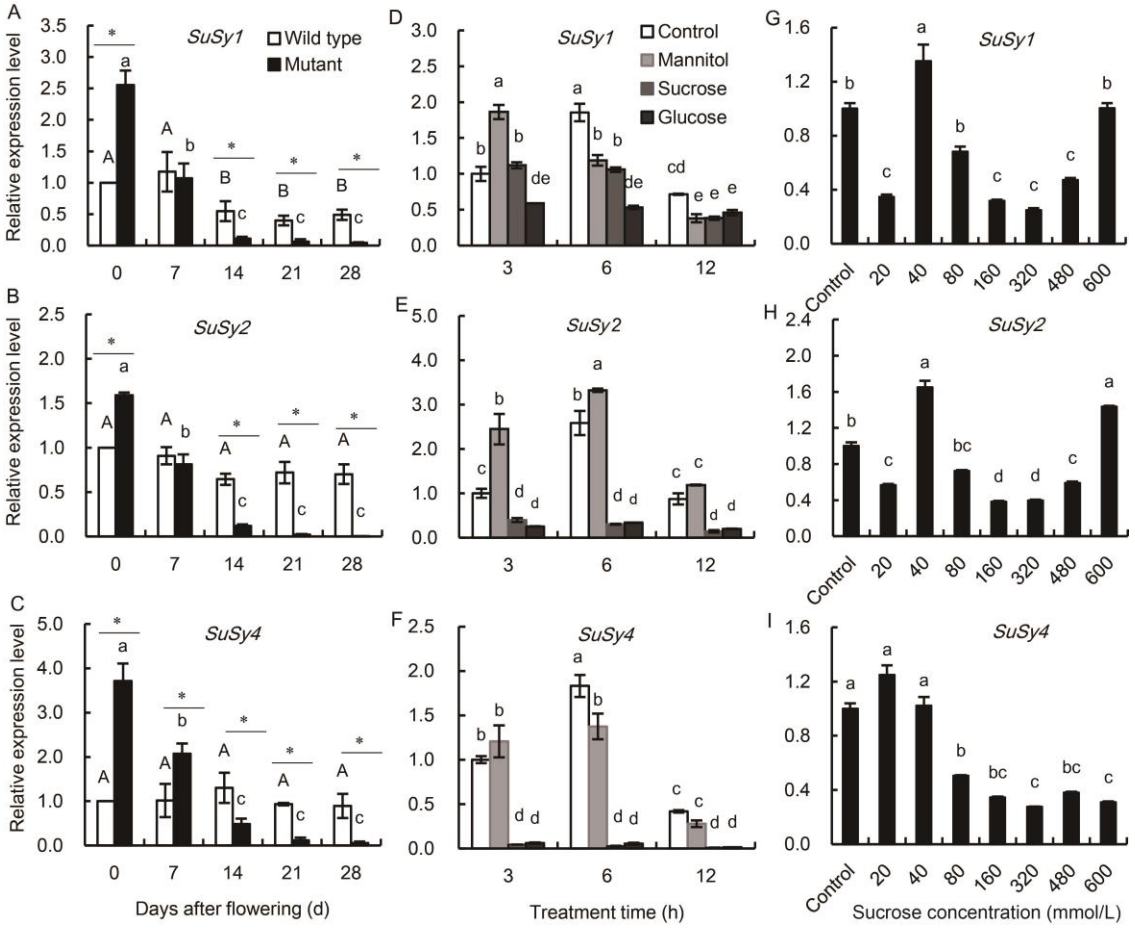

Fig. 7. Relative expression levels of *SuSy1*, *SuSy2* and *SuSy4* genes in the mutant and wild type leaves under different treatments.

A–C, Genotypic-dependent differences in the expression patterns of *SuSy1* (**A**), *SuSy2* (**B**) and *SuSy4* (**C**) in the flag leaves of wild type and mutant genotypes during the grain-filling stage. Asterisks represent significant differences between the wild type and the mutant at the 0.05 level. Values with the same uppercase and lowercase letters are not significantly different among the wild type and the mutants at the 0.05 level, respectively. **D–F**, Temporal analyses of *SuSy1* (**D**), *SuSy2* (**E**) and *SuSy4* (**F**) in detached wild type leaf segments incubated in 175 mmol/L sucrose, glucose and mannitol (osmosis control). Leaf segments were collected after 3, 6 and 12 h incubation, respectively. Values with the same lowercase letters are not significantly different among different sugar treatments at the 0.05 level. **G–I**, The transcriptional analyses of *SuSy1* (**G**), *SuSy2* (**H**) and *SuSy4* (**I**) in the detached wild-type leaf segments incubated in a gradient series of sucrose concentrations. Leaf segments were collected after 6 h incubation. Treatment with Hoagland nutrient solution was set as the control. Values with the same lowercase letters are not significantly different among different sugar concentration treatments at the 0.05 level.

Vertical bars represent standard errors (n = 3).

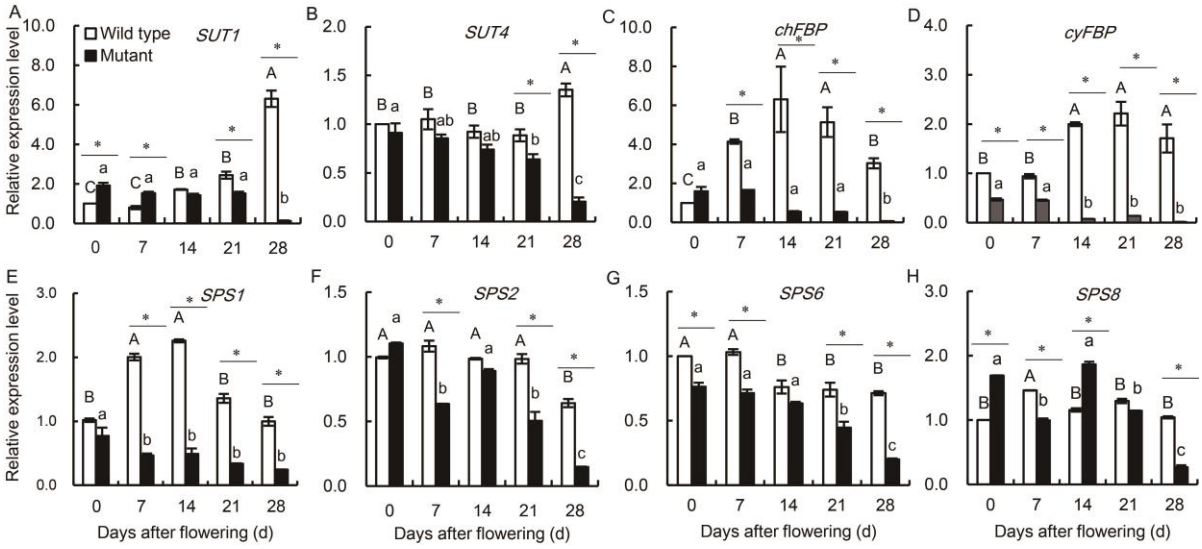

Fig. 8. Genotypic-dependent differences in the expression patterns of *SUT1* (A) , *SUT4* (B), *chFBP* (C), *cyFBP* (D), *SPS1* (E), *SPS2* (F), *SPS6* (G) and *SPS8* (H) in the flag leaves of wild type and mutant genotypes during the grain-filling stage.

Vertical bars represent standard errors (n = 3). Asterisks represent significant differences between the wild type and the mutant at the 0.05 level. Values with the same uppercase and lowercase letters are not significantly different among wild type and mutants at the 0.05 level.

accumulation in senescing leaves probably has been attributed to the conversion of starch into hexoses and the export of reduced sugars caused by sieve tube occlusion or excess carbon that has resulted from reductions in amino acid synthesis (Jongebloed et al, 2004; Wingler et al, 2006). Therefore, sugar accumulation in senescing leaves is likely a consequence of age-dependent changes in carbohydrate metabolism (Wingler and Roitsch, 2008).

However, arguments that favor the opposing hypothesis that leaf senescence is induced or enhanced by sugar starvation (van Doorn, 2008). For example, sugar depletion under dark conditions results in leaf yellowing and increases electrolyte leakage (van Doorn, 2008). Glucose starvation in the root tips of maize plants results in rapid sugar and lipid depletion followed by cell death, which can be prevented by sugar application during the first 80 h of sugar starvation (Brouquisse et al, 1998). In the present study, the analysis of physiological parameters has revealed that the changes associated with the leaf senescence of mutant rice at 14 DAF include a marked decline in total chlorophyll content and rapid increases in electrolytic leakage and MDA content (Fig. 1). Progressive chlorophyll loss and massive increases in electrolytic leakage and MDA content are general features of leaf senescence in plants (Hensel et al, 1993). Our previous studies have also found that maximal fluorescence yield, variable fluorescence, maximal quantum yield of PSII photochemistry, and net photosynthetic rate in mutant rice considerably decrease during the grain-filling stage (Lin et al, 2018). Moreover, the expression levels of light-harvesting Chl a/b binding protein in mutant rice are significantly lower than those in wild type during the grain-filling stage (Lin et al, 2018). These results clearly suggested early senescent phenotype for the mutant rice in comparison with the wild type. At 7 DAF, the soluble sugar, sucrose and fructose levels in the mutant rice significantly decreased compared with those in the wild type (Fig. 2), suggesting that sugar starvation is positively correlated with the process of leaf senescence. These results are in agreement with the findings reported by Masclaux et al (2000) for tobacco and therefore demonstrate that sugar starvation enhances leaf senescence. According to Rose et al (2006), leaf cells subjected to sugar starvation first adapt to the lack of carbohydrates by gradually replacing carbohydrate metabolism with protein and lipid consumption, and this metabolic reorganization likely results in cell autophagy through the induced expression of autophagy-related genes. Interestingly, for the wild type, the contents of sucrose and fructose at 7 DAF were significantly lower than those at the other stages of grain filling (Fig. 2-B and -D), which probably is contributed to the initiation of fast carbohydrate mobilization from source leaves to sink organs after pollen pollination (Ma et al, 2017). Moreover, in this study, treatments with exogenous glucose or sucrose prevented the yellowing of

detached leaf segments under dark conditions (Fig. 3-A and -C) and significantly inhibited the degradation of chlorophyll (Fig. 3-B and -D), whereas sugar deficiency (control and mannitol treatments) resulted in severe yellowing and chlorophyll degradation (Fig. 3). These results distinctly suggested that sugar starvation enhances leaf senescence, whereas exogenous sugar treatment delays leaf senescence. In addition, the onset of the visible senescence symptoms of cut flowers is delayed by exogenous sugar application (van Doorn, 2008), and the expression of *SAG12* in *Arabidopsis* leaves, a well-characterized senescence marker, is repressed by high sugar level (Noh and Amasino, 1999). These results provide further support for the hypothesis that supply exogenous sugar delays leaf senescence.

Over the past few decades, several sugar-signaling pathways, including the hexose-dependent signaling pathway, have been found to be involved in the regulation of leaf senescence (Jang et al, 1997). Hxk and Frk have central roles as conserved glucose and fructose sensors in the hexose signaling pathway. When UDP-glucose is available for cellulose synthesis, Hxk or Frk immediately phosphorylates free fructose for the next metabolic step (Wingler et al, 2006; Smeekens et al, 2010). Hxk and Frk are differentiated by their substrate specificity and affinity. Hxk phosphorylates glucose and fructose but has lower affinity for fructose than for glucose (Granot, 2007). The overexpression of *Arabidopsis Hxk1* in tomato results in early leaf senescence, indicating that an Hxk1-dependent signaling pathway is involved in the regulation of leaf senescence (Xiao et al, 2000; Jongebloed et al, 2004). In the present study, *Hxk1* expression levels in the mutant leaves were higher than those in the wild type at the initial grain-filling stage and then gradually and significantly decreased by the end stage of grain-filling (Fig. 4-A). This result suggested that *Hxk1* participates as a signal molecule in hexose sensing during the initial stage of leaf senescence. Meanwhile, a significant higher *Hxk1* expression level in the wild type than the mutant at 28 DAF (Fig. 4-A) also suggested its involvement in the senescence process of wild type at the final grain-filling stage. In addition, *Hxk2* effectively phosphorylates glucose but fails to provide signaling functions in the plant cells (Xiao et al, 2000). The significantly lower expression of *Hxk2* in the mutant than in the wild type (Fig. 4-B) may indicate that glucose phosphorylating has been weakened in the senescing leaves of mutant rice during the grain-filling stage. Additionally, the depressed transcription levels of *Hxk1* and *Hxk2* in detached leaf segments incubated with high concentrations of exogenous sugars (Fig. 5-E and -F) suggested that high sugar levels exert a deactivating effect. By contrast, the enhanced expression of *Hxk2* in detached leaf segments incubated with low concentrations of sucrose (20 and 40 mmol/L) indicated its sensitivity to low sugar levels (Fig. 5-F).

Frk has a considerably higher affinity for fructose than Hxk and catalyzes the key metabolic step of fructose phosphorylation. Frk activity is strongly and negatively correlated with SuSy activity because fructose, the substrate of Frk, may reduce sucrose cleavage through the feedback inhibition of SuSy activity (Stein and Granot, 2018). Frk activity affects the rate of carbon redistribution from sucrose. This effect suggests that Frk favors the maintenance of balance between hexose and sucrose together with SuSy (Davies et al, 2005). In this study, the low expression levels of *Frk1* in leaves of mutant plants at the initial grain-filling stage were indicative of the low levels of fructose phosphorylation at the early stage of leaf senescence. Moreover, *SuSy1*, *SuSy2* and *SuSy4* transcript levels were high in mutant leaves at the initial grain-filling stage (Fig. 7), perhaps suggesting that SuSy-catalyzed sucrose cleavage is promoted. This effect likely decreased sucrose content (Fig. 2-B), stabilized glucose levels (Fig. 2-C), and slightly increased fructose levels (Fig. 2-D) in the mutant rice by compensating for hexose consumption during the initial grain-filling stage. During the mid-stage of grain-filling, the continuously stable transcription of *Frk1* and *Frk2* in the mutant leaves (Fig. 4-C and -D) contributed to phosphate-free fructose levels and decreased fructose levels at the mid-stage of grain-filling (Fig. 2-D). In addition, plant *Frk* is sensitive to sugar level. This sensitivity may participate in directing carbon to different metabolic pathways in the cytoplasm or organelles (Stein et al, 2017). In this study, 3 h of incubation with exogenous 175 mmol/L sucrose or glucose depressed *Frk1* transcription in detached leaf segments (Fig. 5-C). However, *Frk2* is not as sensitive as *Frk1* to exogenous sugar treatments (Fig. 5-C and -D). This result may be attributed to the differential intracellular localization of various *Frk* isoforms in the cytoplasm or plastids.

Sucrose transduction and translocation also have important regulatory roles in sugar signaling pathways during leaf senescence (Rolland et al, 2002). In

addition to SuSy, CIN hydrolyzes sucrose into glucose and fructose, which in turn serve as intermediates for plant development and defensive chemistry through the Shikimate pathway that is associated with hormone metabolism (Essmann et al, 2008). A growing body of evidence suggests that the CIN enzyme is an essential component that participates in the hormone-mediated delay of leaf senescence (Balibrea Lara et al, 2004). Jin et al (2009) demonstrated that the elevation of CIN activity prolongs leaf lifespan through the blockage of abscisic acid-induced senescence and the increment in hexose level owing to enhanced sucrose hydrolysis. By contrast, the suppression of *CIN* expression shortens leaf lifespan and taproot growth (Tang et al, 1999). In this study, the significantly lower expression levels of *CIN1* and *CIN4* in the mutant leaves than those in wild type leaves (Fig. 6-A and -B) likely destabilized endogenous sugar levels and prevented the maintenance of leaf normal development by mutant rice. In addition, exogenous sugar treatments depressed the expression levels of *CIN1* and *CIN4* (Fig. 6-C and -D). These expression patterns reflected the responses of *CIN1* and *CIN4* to changes in exogenous sugar levels. *CIN1* and *CIN4* showed reduced expressions at 0 and 7 DAF in the mutant (Fig. 6), and the decrease in sucrose and fructose levels at 14 DAF (Fig. 2-B and -D), which probably be attributed to the high sucrose translocation and low sucrose synthesis in term of high *SUT1* expression (Fig. 8-A) and decreased *FBP* expressions (Fig. 8-C and -D). The high sucrose translocation at the initial grain-filling stage resulted in a decrease in sucrose of leaves during the mid-stage of grain filling. During the process of sucrose reduction in the senescing leaves, the expression levels of *CIN1* and *CIN4* were weakened by the reduced sucrose. Meanwhile, the high *Hxk1* expression and relatively stable *Frk1* and *Frk2* expressions at the initial grain-filling stage contributed to the transduction of fructose into phosphorylated glucose, which led to a decrease in fructose level and relative stable glucose level. Additionally, SUT-catalyzed sucrose translocation is necessary to initiate leaf senescence (Aoki et al, 2003). In this study, the significantly higher expression of *SUT1* in the mutant rice than in the wild type rice at the initial grain-filling stage (from 0 to 7 DAF) (Fig. 8-A) favors sucrose translocation and remobilization from early senescent leaves. In addition, the expression levels of genes involved in sucrose biosynthesis markedly decreased in the mutant leaves (Fig. 8). This expression pattern suggests that sucrose synthesis mediated by *chFBP*, *cyFBP*, *SPS1*, *SPS2* and *SPS6* have weakened in the senescing leaves of mutant rice during the grain-filling stage. This result may account for the gradual decrease in the sucrose content of mutant rice leaves during the grain-filling stage.

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SUPPLEMENTAL DATA

The following material is available in the online version of this article at http://www.sciencedirect.com/science/journal/16726308; http://www.ricescience.org.

Supplemental Table 1. Primers used in quantitative real-time PCR.

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