Analyses of transgenic fibroblast growth factor 21 mature rice seeds

Mingfang Feng¹, Hua Cai¹, Ying Guan¹, Jian Sun², Liguo Zhang³ and Jing Cang*¹

¹ College of Life Science, Northeast Agricultural University, Harbin 150030, P.R. China
² College of Agriculture, Northeast Agricultural University, Harbin 150030, P.R. China
³ Heilongjiang Academy of Agricultural Sciences, Harbin 150086, P.R. China

Although some studies have been conducted on the effects of foreign protein expression on rice, the results vary with foreign gene types and protein expression. This study reveals the effects of fibroblast growth factor 21 (FGF21) expression on mature rice seeds in various aspects. Results revealed that the grain weight of the transgene rice was lower than that of non-transgenic wild-type. The sucrose content and ADP-glucose pyrophosphorylase (AGPase) activity in transgenic FGF21 rice were higher than that in non-transgenic wild-type rice, while changes in the starch content, starch branching enzyme (SBE), sucrose synthase (SuS), superoxide dismutase (SOD) and peroxidase (POD) activity were lower in transgenic FGF21 rice compared to non-transgenic wild-type. The scanning electron microscope results revealed that mature seeds of the transgenic FGF21 rice contained fewer vascular bundles with irregular arrangement compared to the wild-type. The mature seeds of CK and T1 rice lines were collected for proteome analysis, and 167 differentially expressed proteins (DEPs) were found. In addition, the most enriched pathways in both rice lines were determined to be amino sugar and nucleotide sugar metabolism and starch and sucrose metabolism, etc. This study laid the foundation for revealing the effects of exogenous protein expression on rice bioreactors.

Key Words: transgenic FGF21 rice, agronomic traits analysis, physiological analysis, SEM, proteome analysis.

Introduction

Rice seeds provide an ideal production platform for high-value recombinant biopharmaceutical proteins, such as vaccines, cytokines, antibodies, and bioactive peptides, which can be highly and stably accumulated and stored in seeds (Takaiwa et al. 2017). The production system of rice seeds has many advantages compared with the conventional fermenting systems or other cereal seed platforms, including high biomass yield, low-cost production, convenience of large scale production, low risk of gene flow (self-pollination), no contamination with mammalian pathogens, high stability for several years at ambient temperature, and easy oral administration without purification (Entesari et al. 2018, Wakasa et al. 2013).

Fibroblast growth factor 21 (FGF21) is a stress response factor that is induced by various metabolic and cellular damages in various types of cells and tissues. It acts as a potent regulator of both local and systemic lipid and energy metabolic processes. In addition to its therapeutic effects on obesity and type 2 diabetes, FGF21 has a significant protective effect against fatty liver disease, islet hyperplasia, cardiovascular disease, cardiac hypertrophy, pancreatitis, and diabetic nephropathy (Luo et al. 2017).

Although Agrobacterium-mediated methods are commonly used for rice transformation, they can alter genomic DNA and subsequent protein expression (Kohli et al. 2003, Latham et al. 2006). The safety of genetically modified crops has been a research focus for several decades. Despite published reports on the effect of Agrobacterium-mediated transformation on the host (Vamvaka et al. 2016), there are inconsistencies in the final safety assessments due to different methods. In addition, the impact on the host also varies when the foreign gene types and protein expression are different in rice bioreactors (Fang et al. 2016, Kawakatsu et al. 2013).

Abbreviations: FGF21: fibroblast growth factor 21, DEGs: differentially expressed genes, DEPs: differentially expressed proteins, GO: Gene Ontology, KEGG: Kyoto Encyclopedia of Genes and Genomes, qRT-PCR: quantitative real-time RT-PCR, AGPase: ADP-glucose pyrophosphorylase, SBE: starch branching enzyme, SPS: sucrose phosphate synthase, SuS: sucrose synthase, SEM: scanning electron microscope, HK: hexokinase, GME: GDP-D-mannose, GPI: glucose-6-phosphate isomerase, BER: base excision repair, PCNA: proliferating cell nuclear antigen.
Transgenic rice expressing FGF21 gene have several therapeutic effects, such as obesity and type 2 diabetes, etc. However, a detailed understanding of the unintended effects of genetically modified organisms and their causes is needed to develop strategies to reduce the unexpected changes in transgenic plants (Gayen et al. 2016, Wang et al. 2012). In the context of public concern about the health and environmental safety of genetically modified organisms, this study aims to more accurately and comprehensively reveal the unexpected differences between transgenic and non-transgenic plants from multiple perspectives. Herein, we investigated the effect of FGF21 transformation on rice, including analysis of agronomic traits, physiology, and mature seed microstructure and proteome. The impact of FGF21 transformation is also discussed. This study attempts to reveal the effects of exogenous protein expression on rice bioreactor itself from different perspectives.

Materials and Methods

Plant materials
The non-transgenic wild type rice (Oryza sativa L. ssp. japonica cv. Dongnong 427) and transgenic FGF21 rice of the same variety were used. Both varieties were planted in pots in an outdoor isolation space under identical climatic conditions. The FGF21 gene was transferred into the callus induced by mature embryos of rice seed using an Agrobacterium-mediated method. The vector information induced by mature embryos of rice seed using an

Agronomic traits analysis
The same cultivating conditions were applied to transgenic FGF21 rice and the non-transgenic wild-type rice, and the agronomic traits, such as tiller number, plant height, and grain weight, were examined after the rice seeds were ripe. The appearances of plants and mature seeds of transgenic and non-transgenic rice were observed simultaneously.

Physiological analysis
The sucrose and starch content, sucrose synthase (EC 2.4.1.13, SuS), sucrose phosphate synthase (EC 2.4.1.14, SPs), ADP-glucose pyrophosphorylase (EC 2.7.7.27, AGPase), starch branching enzyme (EC2.4.1.18, SBE) SOD and POD activity in non-transgenic wild-type and transgenic FGF21 (T1, T2 and T3 plant lines) mature rice seeds were measured according to the kit instructions (Suzhou Keming Biotechnology Co., Ltd.).

Proteome analysis
Different materials were made using transgenic FGF21 mature seed (T1 strain) of T3 generation and non-transgenic wild-type rice mature seed (CK strain), where each material had three biological replicates and repeated 10 seeds. The specific steps for preparation and analysis of proteome samples are as follows.

Total protein extraction and detection
Total proteins were extracted using the cold acetone method. Samples were ground into powder in liquid nitrogen, dissolved in 1.5 mL pre-chilled 90% acetone solution (containing 10% TCA, 0.07% DTT), shaken well to mix, then precipitated at −20°C for 2 h. Centrifugation at 12,000 rpm was performed for 15 min at 4°C to collect the precipitate. The pellet form the precipitate was constituted with 100 μL urea lysate, centrifuged at 12,000 rpm at 4°C for 15 min, then the supernatant was collected for quantification.

Protein identification and quantification
Digestion and reductive alkylation of disulfide bridges with DTT and iodoacetamide were performed to extract and examine acceptable proteins for subsequent enzymatic digestion of the protein. The protein was then digested with Trypsin. Finally, nano-HPLC-MS/MS (Thermo Q Exactive) analysis was used, and data dependent acquisition (DDA) was set as the data acquisition mode. A list of mass spectral peaks was prepared after identifying peaks from mass spectra, then a reference database was established to identify peptides and proteins. The reference genome is Oryza sativa IRGSP-1.0 from Ensembl database. Finally, relative quantification of the identified proteins was carried out, and the protein content relationship between CK and T1 was compared. The protein quantification software used in this experiment was MaxQuant 1.5.3.30, which implemented the search parameters presented in Table 1.

iBAQ (Schwanhäusser et al. 2011) was used for peptide and protein abundance calculations. Proteins with a fold change in a comparison >2 and unadjusted significance
level $p < 0.05$ were considered as differentially expressed proteins (DEPs).

**Protein functional annotation and enrichment analysis**

Proteins were annotated against Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to obtain their functions. Significant GO functions and pathways were examined within differentially expressed proteins with $p \leq 0.05$.

**Quantitative real-time RT-PCR (qRT-PCR) analysis**

A total of 9 candidate DEGs between transgenic and non-transgenic wild-type rice lines were selected for qRT-PCR analysis. Total RNAs of immature seeds were isolated using TRIzol reagent, and reverse transcription was carried out using a Prime-Script 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) following the manufacturer’s protocol. Real-time quantitative PCR was performed using GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) according to the manufacturer’s protocol for a BIOER Line Gene 9620 Real-time PCR Detection System (Hangzhou Bioer Technology Co., Ltd., China). *ACT1*, the gene for Actin-1, was used as the control housekeeping gene to normalize the amounts of cDNA among samples (Siahpoosh et al. 2012). The primer sequences used for gene expression analyses are shown in [Supplemental Table 1](#). The thermal cycling conditions were as follows: 1 cycle at 94°C for 30 s, followed by 40 cycles at 94°C for 5 s, then another 40 cycles at 60°C for 30 s. Melting curve analysis was added at the completion of reactions to evaluate primer specificity. Three biological replicates were performed for each gene in order to exclude sampling errors. Relative expression levels of the selected genes, normalized to the reference gene, were calculated using the $2^{-\Delta\Delta CT}$ method.

**Western blot analyses**

Western blotting was performed to validate the protein abundance in mature seeds of non-transgenic wild-type (CK) and transgenic *FGF21* rice lines (T1, T2, T3). Two DEPs, chitinase 5 (Cht5) and glucose-1-phosphate adenylyltransferase small subunit (AGPS1), were selected to confirm the results by western blotting. Total protein extraction was performed according to the instructions of the Minute™ plant tissue total protein extraction kit (Invent Biotechnologies, Inc.). Protein quantification was performed using the BCA method. The protein extracts (32 μg) were separated on 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 5% non-fat milk for 2 h at room temperature, washed with TBST (25 mM Tris, 140 mM NaCl, 3 mM KCl, pH = 7.4 with 0.1% Tween-20) three times, then incubated with the primary antibody at 4°C overnight on a shaker. The primary antibodies contain rabbit anti-AGPS1 (diluted 1:500, Beijing Protein Innovation) and rabbit anti-Cht5 (diluted 1:1000, Beijing Protein Innovation). Goat anti-rabbit IgG (diluted 1:8000, Abbkine, China) was used as the secondary antibody, and BIP-2 antibody (rabbit, Agrisera) was used as the internal reference. After washing samples, antigen-antibody complexes were detected with the secondary antibody in blocking buffer for 1 h, followed by color imaging via the United States Li-COR Odyssey two-color infrared laser imaging system after another series of washes and addition of dyes.

**Results**

**Agronic traits analysis**

The tiller number, plant height, and grain weight of transgenic *FGF21* rice and non-transgenic wild-type rice are shown in [Table 2](#). The appearances of the plants and seeds were observed simultaneously ([Fig. 1](#)), which reveal no significant difference. It was suggested that the non-transgenic wild-type and transgenic *FGF21* rice had no significant difference in tiller number and plant height ($p > 0.05$), but the grain weight of transgenic rice seeds was significantly lower than that of non-transgenic wild-type ($p < 0.05$).

**Physiological analysis**

The results of starch and sucrose content, ADP-glucose pyrophosphorylase (EC 2.7.7.27, AGPase), starch branching enzyme (EC2.4.1.18, SBE), sucrose synthase (EC 2.4.1.13, SuS), and sucrose phosphate synthase (EC 2.4.1.14, SPS), SOD, and POD activity in non-transgenic wild-type (CK) and transgenic *FGF21* rice (T1, T2 and T3 plant lines) mature seeds are shown in [Fig. 2](#). As illustrated, the sucrose content and AGPase activity in non-transgenic wild-type rice were lower than those in transgenic *FGF21* rice, while changes in the starch content, SBE, SuS, SOD, and POD activity were higher in non-transgenic wild-type rice than transgenic *FGF21* rice. For SPS activity, the T1 line was lower than the non-transgenic wild-type, whereas the T2 and T3 lines were higher than the non-transgenic wild-type.

**Table 2.** Comparison of agronomic characters between transgenic *FGF21* rice and wild-type rice

|               | Tiller number | Plant height (cm) | Grain weight (mg) |
|---------------|---------------|-------------------|-------------------|
| Wild-type rice| 11.0 ± 1.2    | 81.0 ± 4.0        | 31.7 ± 1.5*       |
| Transgenic *FGF21* rice | 10.0 ± 1.0    | 79.0 ± 2.3        | 27.0 ± 0.7*       |

* represent significant at $p < 0.05$. 

**Table 1.** The search parameters in MaxQuant

| Item                | Value                                      |
|---------------------|--------------------------------------------|
| Type of search      | MS/MS ion search                           |
| Enzyme              | Trypsin/P                                  |
| Fixed modifications | Carbamidomethyl (C)                        |
| Variable modifications | Oxidation (M), Acetyl (Protein-N-term)     |
| Database            | bta.pep.fa (22118 sequences)               |
| Instrument type     | Q Exactive                                 |
| Peptide spectrum match level FDR | 0.01                                   |
| Protein level FDR   | 0.01                                       |
| Quantification      | iBAQ                                       |
| Others              | Default                                    |
Feng, Cai, Guan, Sun, Zhang and Cang

BS
Breeding Science
Preview

Mature vascular bundles in the mature seeds of the transgenic FGF21 rice and with an irregular arrangement compared to the non-transgenic wild-type (Fig. 3E, 3F).

Identification of differentially expressed proteins

The statistical results of the DEPs screened after comparing CK with T1 are shown in Fig. 4 and Supplemental Table 2. Of the 167 DEPs found, 49 were up-regulated and 118 were down-regulated.

Fig. 1. Comparison of the appearance of wild-type and transgenic FGF21 rice. (A) Plants; (B, C) Mature seeds.

Fig. 2. Physiological analysis of wild-type and transgenic FGF21 rice mature seeds. (A) Starch content; (B) Sucrose content; (C) AGPase activity; (D) SBE activity; (E) SPS activity; (F) SuS activity; (G) SOD activity; (H) POD activity.

Scanning electron microscope analysis

The mature seeds of non-transgenic wild-type and transgenic FGF21 rice were observed under a scanning electron microscope, and the results demonstrate no significant difference in starch grains (Fig. 3A, 3B) and proteins (Fig. 3C, 3D) between the non-transgenic wild-type and transgenic FGF21 rice mature seeds. However, differences were found in the glume structures of rice, where there were fewer vascular bundles in the mature seeds of the transgenic FGF21 rice and with an irregular arrangement compared to the non-transgenic wild-type (Fig. 3E, 3F).

Identification of differentially expressed proteins

The statistical results of the DEPs screened after comparing CK with T1 are shown in Fig. 4 and Supplemental Table 2. Of the 167 DEPs found, 49 were up-regulated and 118 were down-regulated.

Fig. 3. Scanning electron microscopy analysis of mature seeds of wild-type and transgenic FGF21 rice. (A, B) Starch grains; (C, D) Proteins; (E, F) glume structure of wild-type and transgenic FGF21 rice.

Fig. 4. DEPs of CK vs T1.
Analyses of transgenic FGF21 mature rice seeds

**Western blot analyses**

Western blot analyses of AGPS1 and Cht5 enzymes in CK, T1, T2, and T3 are shown in Fig. 8. The results suggest...
Discussion

Scanning electron microscope analysis

Rice grain consists of an endosperm, embryo, and glume, where the size and enrichment of the glume have a significant effect on the growth and development of rice. The promotion of glume growth, increase in glume area, promotion of glume substance accumulation, and increase in unit area glume weight are all conducive to the growth and enrichment of rice and can significantly increase rice grain weight, brown rice rate, and whole head rice rate (Wang et al. 1995, 1998). Li He et al. studied the glume structure of different rice varieties and found that there was no difference in the glume cell structure of rice, but there were differences between the varieties of vascular bundles and cell structure hierarchy (He et al. 2013). This is consistent with the results of this study. Although this study focused on the non-transgenic wild-type and transgenic type of the same variety, the change in the glumes structure indicates that FGF21 transformation had an effect on rice seed structure, which can partially explain the reason why the seed weight of the transgenic seeds is smaller than that of the non-transgenic wild-type.

Amino sugar and nucleotide sugar metabolism

The KEGG pathway enrichment analysis of the CK vs T1 proteome showed that there were 9 DEPs in the amino sugar and nucleotide sugar metabolism pathways, and only one was up-regulated, while the others were down-regulated (Fig. 9).

Chitinase (EC 3.2.1.14), which is widely distributed in plants, animals, bacteria, and fungi, can decompose chitin into N-acetylglucosamine. In plant-pathogen interactions, N-acetylglucosamine can be used as a pathogen to elicit a defense response in plants (Boller 1995). As can be seen in Fig. 9, chitinase expression is up-regulated. Studies have shown that chitinase is induced by biotic or abiotic stress in plants. Infection with non-affinity pathogens in Arabidopsis causes more rapid accumulation of chitinase (Class IV) (Le et al. 1997), and infection with Class III chitinase is enhanced by infection with an affinity pathogen (Samac and shah 1991). Similarly, beet sugar infection by affinity pathogens enhances the transcription of chitinase (Class IV) (Nielsen et al. 1994). It has also been reported that chitinase is expressed in normal growing plant tissues. For example, highly expressed chitinases are found during the formation of the tobacco flower (Neale et al. 1990), in the ripening stage of grapes (Robinson et al. 1997), and in rice leaves, leaf sheaths, roots, and meristems (Fan et al. 2014).

Hexokinase (EC 2.7.1.1, HK) has the function of catalyzing the phosphorylation of hexose and is one of the key enzymes in the respiratory metabolic process of plants. Studies over the past decade have found that HK plays an important role in sugar perception and sugar signal transduction in plants (Cho et al. 2006). At present, GenBank has registered HK homologues of 28 higher plants, which are

that the relative expression levels of the two enzymes, AGPS1 and Cht5, in the three transgenic seeds were higher than those in the non-transgenic wild-type and were consistent with the proteomic results, further confirming that the proteome data were reliable.
Analyses of transgenic FGF21 mature rice seeds

Thus measures of comparison are limited. For example, after gravitropic stimulation, twenty pathways, including aminoacyl-tRNA biosynthesis, pentose phosphate pathway, carbon fixation, and starch and sucrose metabolism, were identified in peanuts (*Arachis hypogaea* L.) from transcriptome data (Li *et al.* 2013). Base excision repair

Only 2 down-regulated DEPs (PARP3 and PCNA) were enriched in the base excision repair (BER) pathway in this study. BER is a critical pathway in cellular defense against endogenous or exogenous DNA damage (Córdoba-Cañero *et al.* 2009), while poly (ADP-ribose) polymerases (PARPs) are important components in the DNA damage response in humans. It has been reported that *Arabidopsis* contains three homologues to the human HsPARP1 protein, and only one of these three, *Arabidopsis* (Strzalka and Ziemienowicz 2011). Both PARP3 and PCNA were down-regulated indicated that FGF21 transformation does not cause BER effect.

Photosynthesis

In the photosynthesis pathway, the 3 DEPs (plastocyanin, oxygen-evolving enhancer protein 1 and photosystem II 22 kDa protein) were up-regulated. Plastocyanin, present in multiple gene families in different species. In this study, Hexokinase-6 (OsHK6) was down-regulated. Similar studies have shown that *Manihot esculenta* Crantz HK participates in response to adverse stress (Wu 2014) and that restorer-of fertility gene 6 functions with OsHK6 to restore Honglian CMS fertility (Huang *et al.* 2015). In this work, GME was down-regulated (Fig. 9). Similarly, it was revealed that GME plays important roles in the reproductive development, vegetative growth, and leaf senescence of *Arabidopsis* and also regulates plant growth and controls male gametophyte development in different manners (Qi *et al.* 2017).

Glucose-6-phosphate isomerase (EC 5.3.1.9, GPI) is a homodimeric enzyme that catalyzes the inter conversion between glucose-6-phosphate and fructose-6-phosphate (Lin *et al.* 2009). As shown in Fig. 9, GPI was down-regulated. Comparatively, a previous study reported that the mRNA expression of glucose-6-phosphate isomerase, matrix metalloproteinase, cytochrome P450 77A1, and ATPase family AAA domain-containing protein 1 was up-regulated in wild-type soybean under flooding stress conditions (Yin *et al.* 2017).

In summary, only chitinase is up-regulated in amino sugar and nucleotide sugar metabolic pathways, while the rest are down-regulated, which is not enough to cause stress responses.

**Aminoacyl-tRNA biosynthesis**

6 DEPs were identified in aminoacyl-tRNA biosynthesis pathways, which were all down-regulated. However, there is little research on aminoacyl-tRNA biosynthesis in plants, thus measures of comparison are limited. For example, after gravitropic stimulation, twenty pathways, including aminoacyl-tRNA biosynthesis, pentose phosphate pathway, carbon fixation, and starch and sucrose metabolism, were identified in peanuts (*Arachis hypogaea* L.) from transcriptome data (Li *et al.* 2013).
the thylakoid lumen, is a soluble copper-containing protein, which transfers electrons to photosystem I. A previous research demonstrated that, in Arabidopsis, only plastocyanin can donate electrons to photosystem I in vivo (Weigel et al. 2003).

It has also been reported that the oxygen-evolving enhancer protein 1 of Leymus chinensis (LeOEE1) responds to both salt and high pH stresses and is one of the mechanisms to maintain the capacity of photosystem II under environmental stresses (Yu et al. 2008). In addition, OEE 1 purified from Capsosiphon fulvescens is an excellent antioxidant (Kim et al. 2015), while OEE in green algae exhibits thiorredoxin activity (Heide et al. 2004).

Compared to darkness, the photosynthetic machinery is fully activated under light conditions. The highest up-regulation was observed in potato (Solanum tuberosum L.) tubers for the Rubisco activase (RCA), the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the photosystem II 22 kDa protein (CP22) genes (Grandellis et al. 2016).

Finally, photosynthesis is one of the most important physiological processes of plants, as it is the basis for the formation of crop yield and quality. Studies have shown that more than 90% of the dry matter in crops comes from photosynthetic products of the leaves, thus photosynthesis is crucial for the growth and development of crops. Photosynthesis is the process by which plants and other organisms convert light energy into chemical energy, subsequently releasing oxygen to promote organism activity. Sugar is also produced, which helps organic matter to support the growth and development of the plant. Abiotic stresses, such as osmotic stress, temperature, heavy metals, etc., have a direct effect on photosynthesis, mainly by interfering with all the major components of the process, including thylakoid electron transport, carbon reduction cycles, and stomatal control of carbon dioxide supply (Cheng et al. 2017). In this study, the DEPs in the photosynthesis pathway did not decline, which was contrary to the stress response. In addition, an increase rate of photosynthesis may lead to an increase in the final seed sugar content, which is consistent with the physiological test result that the sucrose content of the mature seed of the transgenic FGF21 gene is higher than that of the wild-type.

Starch and sucrose metabolism

Seven DEPs were found in the starch and sucrose metabolism pathway, which were all down-regulated except for the up-regulated expression of ADP-glucose pyrophosphorylase (AGPase).

AGPase is the key enzyme in the first step of starch synthesis, with sucrose as the substrate. The expression and activity of AGPase directly impact the content of starch, thus affecting starch accumulation and final yield of rice. Contrary to the up-regulation of AGPase in this study, the expression levels of AGPase, pyrophosphate-fructose-6-phosphate 1-phosphotransferase (PFP), and sucrose synthase in wheat cultivar Jing 411 were down-regulated under heat stress (Zhang et al. 2017).

Degradation of starch is a complex process requiring the catalysis and regulation of a series of enzymes, including α-amylase, glucon hydrolysis kinase, β-amylase, α-glucosidase, etc. As a restriction enzyme, α-amylase plays an important role in this process. It can hydrolyze the (1→4) glycosidic bonds of amylose and amylopectin to produce linear malt sugars and branched glucosides, which ultimately degrade starch into bioavailable monosaccharides (Nakata et al. 2017). Studies have shown that the enzyme activity is significantly positively correlated with many important agronomic traits (such as germination rate, seedling vigor, yield, cold tolerance, resistance to hypoxia stress, etc.). (Guglielminetti et al. 1995, Hwang et al. 1999, Karrer et al. 1992). In this study, α-amylase was down-regulated, contrary to the stress response.

α-glucosidase plays an important role in the hydrolysis of carbohydrates, such as sucrose, maltose, and starch, to produce glucose. It is widely present in all types of lower and higher organisms and participates in the synthesis of glycoproteins, glycolipids, and polysaccharides (Lovering et al. 2005).

β-glucosidases, mainly involved in sugar metabolism in vivo, hydrolyzes the non-reducing β-D-glucosidic bond at the end and releases the glucose and ligand, so as to maintain the normal physiological function of animals and plants. In plants, β-glucosidase participates in the formation and metabolism of cell walls, pigment metabolism, and fruit ripening. Specifically, the β-glucosidase/β-glycoside system is one of the major chemical weapons plants use to defend themselves against stress and pests (Wang et al. 2009).

As illustrated by the proteomic data, the expression of enzymes related to starch synthesis (AGPase) was up-regulated, while the expression of degradative enzymes (α-amylase, α-glucosidase and β-glucosidases) was down-regulated. It can be concluded that the starch content should be elevated. However, physiological data showed that the starch content and SBE activity of mature seeds of FGF21 transgenic rice was lower than that of non-transgenic wild-type rice, indicating that SBE and other related starch synthases may play important roles in the final starch content of mature rice seeds.

Comparison of proteome analysis results of different transgenic rice varieties

Profiling techniques, such as proteomics, are currently being used as complementary analytical tools to detect the unintended effects of genetically modified organisms. Vamvaka et al. reported the development of transgenic rice (Oryza sativa cv. Nipponbare) plants expressing the HIV-neutralizing antibody 2G12 in the endosperm. The comparison of wild-type and transgenic plants at the proteomic levels indicated that endogenous genes related to starch biosynthesis (AGPase and SBE) were downregulated (In our study, AGPase was up-regulated) in the endosperm of the transgenic plants, whereas genes encoding prolamin
and glutaredoxin-C8 were up-regulated (Vamvaka et al. 2016). Wang et al. analyzed the proteomic profiles of transgenic rice seeds containing the Cry1Ab/Ac protein to assess the safety of these transgenic seeds. By comparing proteomic profiles, they found that 20 to 22 protein (including AGPase) levels were differentially modulated in transgenic rice seeds in comparison to their non-transgenic counterparts (T01 vs. WT01; T02 vs. WT02) (Wang et al. 2012). Additionally, homozygous transgenic bacterial blight (BB) resistant rice line was developed by integration of Xa21 gene into the genome of elite indica rice cultivar IR72. Based on comparison of the wild-type and transgenic lines, 11 DEPs were found, with four proteins upregulated and seven down-regulated. Most of these DEPs involved in the carbohydrate metabolism pathway (Gayen et al. 2016). Another paper showed that the largest numbers of spots with changed expression between indica (varieties MH86, D68, and MH63) and japonica (ZH10) cultivars, and a lower number between the three indica varieties, a much lower number between MH86 and MH63, and the least between transgenic lines and their corresponding controls (Bar68-1 vs D68; 2036-1a vs MH86). Although they found several spots with changed expression between transgenic and non-transgenic lines, most of them (7/12 in Bar68-1, 11/17 in 2036-1a) showed nontransgenic varietal differences. After eliminating overlapped proteins spots, they identified only 5 and 6 specific spots with changed expression between Bar68-1 and D68, and 2036-1a and MH86, respectively. At last, they concluded that together with result from PCA results, rice seed proteomes were largely unchanged with transgenic modification as compared with conventional rice. In summary, the types and numbers of DEPs may be different due to differences in rice varieties, expression of foreign gene types, and proteomic analysis methods.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (NSFC) [grant number 31471423]. We are also grateful to the Guangzhou Gene Denovo Biotechnology Co., Ltd. for assisting in the proteome analysis.

Literature Cited

Boller, T. (1995) Chemoperception of microbial signals in plant cells. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46: 189–214.

Cheng, Z.W., Z.Y. Chen, X. Yan, Y.W. Bian, X. Deng and Y.M. Yan (2017) Integrated physiological and proteome analysis reveals underlying response and defense mechanisms of Brachypodium distachyon seedling leaves under osmotic stress, cadmium and their combined stresses. J. Proteomics 170: 1–13.

Cho, J.I., N. Ryoo, S. Ko, S.K. Lee, J. Lee, K.H. Jung, Y.H. Lee, S.H. Bhoo, J. Winderichx, G. An et al. (2006) Structure, expression, and functional analysis of the hexokinase gene family in rice (Oryza sativa L.). Planta 224: 598–611.

Córdoba-Cañero, D., T. Morales-Ruiz, T. Roldán-Arjona and R.R. Ariza (2009) Single-nucleotide and long-patch base excision repair of DNA damage in plants. Plant J. 60: 716–728.

Entesari, M., Y. Wakasa, B.M. Zanjani and F. Takaiwa (2018) Change in subcellular localization of over expressed vaccine peptide in rice endosperm cell that is caused by suppression of endogenous seed storage proteins. Plant Cell Tissue Organ Cult. 133: 275–287.

Fan, W., X. Li, M. Guan, L. Miao, J. Shi, S. Dou, L. Liu, L. Li and G. Liu (2014) Transcriptional and translational characterization of rice chitinase genes. Acta Agron. Sin. 40: 571–580.

Fang, J., A. Lin, W. Qiu, H. Cai, M. Umar, R. Chen and R. Ming (2016) Transcriptome profiling revealed stress-induced and disease resistance genes up-regulated in PRSV resistant transgenic papaya. Front. Plant Sci. 7: 855.

Gayen, D., S. Paul, S.N. Sarkar, S.K. Datta and K. Datta (2016) Comparative nutritional compositions and proteomics analysis of transgenic Xa21 rice seeds compared to conventional rice. Food Chem. 203: 301–307.

Grandellis, C., V. Giannimaria, E. Fantino, I. Cerrudo, S. Bachmann, F. Santin and R.M. Ulloa (2016) Transcript profiling reveals that cysteine protease inhibitors are up-regulated in tuber sprouts after extended darkness. Funct. Integr. Genomics 16: 399–418.

Guglielminnelli, L., J. Yamaguchi, P. Perata and A. Alpi (1995) Amylolytic activities in cereal seeds under aerobic and anaerobic conditions. Plant Physiol. 109: 1069–1076.

He, L.I., Y. Wang, L.I. Mei-Shan, X.U. Ming-Zi and X.H. Liu (2013) Cytological studies of lateral development in different varieties of rice glumes. J. Jilin Agr. Sci. 38: 6–10.

Heide, H., H.M. Kalis and H. Follmann (2004) The oxygen evolving enhancer protein 1 (OEE) of photosystem II in green algae exhibits thioredoxin activity. J. Plant Physiol. 161: 139–149.

Huang, W., C. Yu, J. Hu, L. Wang, Z. Dan, W. Zhou, C. He, Y. Zeng, G. Yao, J. Qi et al. (2015) Pentatricopeptide-repeat family protein RF6 functions with hexokinase 6 to rescue rice cytoplasmic male sterility. Proc. Natl. Acad. Sci. USA 112: 14984–14989.

Hwang, Y.S., B.R. Thomas and R.L. Rodriguez (1999) Differential expression of rice alpha-amylase genes during seedling development under anoxia. Plant Mol. Biol. 40: 911–920.

Karrer, E.E., J.M. Chandler, M.R. Foolad and R.L. Rodriguez (1992) Correlation between α-amylase gene expression and seedling vigor in rice. Euphytica 66: 163–169.

Kawakatsu, T., Y. Kawahara, T. Itoh and F. Takaiwa (2013) A whole-genome analysis of a transgenic rice seed-based edible vaccine against cedar pollen allergy. DNA Res. 20: 623–631.

Kim, E.Y., Y.H. Choi, J.I. Lee, I.H. Kim and T.J. Nam (2015) Antioxidant activity of oxygen evolving enhancer protein 1 purified from Capnosiphium fulvescens. J. Food Sci. 80: H1412–H1417.

Kohli, A., R.M. Twyman, R. Abranches, E. Wegel, E. Stoger and P. Christou (2003) Transgene integration, organization and interaction in plants. Plant Mol. Biol. 52: 247–258.

Latham, J.R., A.K. Wilson and R.A. Steinbrecher (2006) The mutational consequences of plant transformation. J. Biomed. Biotechnol. 2006: 25376.

Lb, D.A.G., G. Sachetto-Martins, M.G. Contarini, M. Sandroni, R. de P. Ferreira, V.M. de Lima, M.C. Cordeiro, D.E. de Oliveira and M. Margis-Pinheiro (1997) Arabidopsis italiana class IV chitinase is early induced during the interaction with Xanthomonas campestris. FEBS Lett. 419: 69–75.

Li, H.F., X.P. Chen, F.H. Zhu, H.Y. Liu, Y.B. Hong and X.Q. Liang (2013) Transcriptome profiling of peanut (Arachis hypogaea) gynophores in gravitropic response. Funct. Plant Biol. 40: 1249–1260.

Lin, H.Y., Y.H. Kao, S.T. Chen and M. Meng (2009) Effects of inherited

Analyses of transgenic FGF21 mature rice seeds
mutations on catalytic activity and structural stability of human glucose-6-phosphate isomerase expressed in Escherichia coli. Biochim. Biophys. Acta 1794: 315–323.

Lovering, A.L., S.S. Lee, Y.W. Kim, S.G. Withers and N.C.J. Strynadka (2005) Mechanistic and structural analysis of a family 31 α-glycosidase and its glycosyl-enzyme intermediate. J. Biol. Chem. 280: 2105–2115.

Luo, Y., S. Ye, X. Chen, F. Gong, W. Lu and X. Li (2017) Rush to the fire: FGF21 extinguishes metabolic stress, metflammation and tissue damage. Cytokine Growth Factor Rev. 38: 59–65.

Nakata, M., Y. Fukamatsu, T. Miyashita, M. Hakata, R. Kimura, Y. Nakata, M. Kuroda, T. Yamaguchi and H. Yamakawa (2017) High temperature-induced expression of rice α-amylases in developing endosperm produces chalky grains. Front. Plant Sci. 8: 2089.

Neale, A.D., J.A. Wahleithner, M. Lund, H.T. Bonnett, A. Kelly, D.R. Meeks-Wagner, W.J. Peacock and E.S. Dennis (1990) Chitinase, beta-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell 2: 673–684.

Nieelsen, K.K., K. Bojsen, P. Roepstorff and J.D. Mikkelsen (1994) A hydroxyproline-containing class IV chitinase of sugar beet is glycosylated with xylose. Plant Mol. Biol. 25: 241–257.

Qi, T., Z. Liu, M. Fan, Y. Chen, H. Tian, D. Wu, H. Gao, C. Ren, S. Song and D. Xie (2017) GDP-D-mannose epimerase regulates male gametophyte development, plant growth and leaf senescence in Arabidopsis. Sci. Rep. 7: 10309.

Rissel, D., J. Losch and E. Peiter (2014) The nuclear protein poly (ADP-ribose) polymerase 3 (ARTAPRP3) is required for seed storability in Arabidopsis thaliana. Plant Biol. (Stuttgart) 16: 1058–1064.

Robinson, S.P., A.K. Jacobs and I.B. Dry (1997) A class IV chitinase is highly expressed in grape berries during ripening. Plant Physiol. 114: 771–778.

Samac, D.A. and D.M. Shah (1991) Developmental and pathogen-induced activation of the Arabidopsis acidic chitinase promoter. Plant Cell 3: 1063–1072.

Schwanhäusser, B., D. Busse, N. Li, G. Dittmar, J. Schuchhardt, J. Wolf, W. Chen and M. Selbach (2011) Global quantification of mammalian gene expression control. Nature 473: 337–342.

Siahpoosh, M.R., D.H. Sanchez, A. Schlereth, G.N. Scofield, R.T. Furbank, J.V. van Dongen and J. Kopka (2012) Modification of OsSUT1 gene expression modulates the salt response of rice Oryza sativa cv. Taipei 309. Plant Sci. 182: 101–111.

Strzalka, W. and A. Ziemienowicz (2011) Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation. Ann. Bot. 107: 1127–1140.

Takahata, F., Y. Wakasa, S. Hayashi and T. Kawakatsu (2017) An overview on the strategies to exploit rice endosperm as production platform for biopharmaceuticals. Plant Sci. 263: 201–209.

Vamvaka, E., R.M. Twyman, A.M. Murad, S. Melnik, A.Y. Teh, E. Arcalis, F. Allmann, E. Stoger, E. Rech, J.K. Ma et al. (2016) Rice endosperm produces an underglycosylated and potent form of the HIV-neutralizing monoclonal antibody 2G12. Plant Biotechnol. J. 14: 97–108.

Wakasa, Y., H. Takagi, S. Hirose, L. Yang, M. Saeki, T. Nishimura, O. Kamimura, T. Hiroi and F. Takaiwa (2013) Oral immunotherapy with transgenic rice seed containing destructed Japanese cedar pollen allergens, Cry1 and Cry2, against Japanese cedar pollinosis. Plant Biotechnol. J. 11: 66–76.

Wang, W., C.Q. Li and X.L. Hu (2009) Developmental expression of β-glucosidase in olive leaves. Biol. Plantarum 53: 138–140.

Wang, Y.L., Y.L. Yao, T.Y. Li and J.Z. Cai (1995) An inquiring into grain characters and their relations with grain weight in rice (Oryza sativa L.). Acta Agronomica Sinica 21: 573–578.

Wang, Y.L., Y. Yamamoto, Y.L. Yao, J.K. Xu, Y. Bian, J.M. Jian, Y.J. Nitta, T.Y. Li and J.Z. Cai (1998) Effect of cultural conditions on grain weight and it’s causes in rice. Acta Agronomica Sinica 3: 280–290.

Wang, Y., W. Xu, W. Zhao, J. Hao, Y. Luo, X. Tang, Y. Zhang and K. Huang (2012) Comparative analysis of the proteomic and nutritional composition of transgenic rice seeds with Cry1ab/ac genes and their non-transgenic counterparts. J. Cereal Sci. 55: 226–233.

Weigel, M., C. Varotto, P. Pesaresi, G. Finazzi, F. Rappaport, F. Salamini and D. Leister (2003) Plastocyanin is indispensable for photosynthetic electron flow in Arabidopsis thaliana. J. Biol. Chem. 278: 31286–31289.

Wu, X.H. (2014) Cloning, structural evolution and expression analysis of the hexokinase gene family of cassava (D). Hainan University.

Yin, X., S. Hiraga, M. Hajika, M. Nishimura and S. Komatsu (2017) Transcriptomic analysis reveals the flooding tolerant mechanism in flooding tolerant line and abscisic acid treated soybean. Plant Mol. Biol. 93: 479–496.

Yu, X., H. Jin and S.K. Hong (2008) Isolation and characterization of oxygen evolving enhancer protein 1 gene in halophyte Leymus chinensis. J. Biotechnol. 136: S645.

Zhang, Y., J. Pan, X. Huang, D. Guo, H. Lou, Z. Hou, M. Su, R. Liang, C. Xie, M. You et al. (2017) Differential effects of a post-anthesis heat stress on wheat (Triticum aestivum L.) grain proteome determined by iTRAQ. Sci. Rep. 7: 3468.