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# Expression of α-1,6-fucosyltransferase (FUT8) in rice grain and immunogenicity evaluation of plant-specific glycans

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ABSTRACT

Rice seed is a cost-effective bioreactor for the large-scale production of pharmaceuticals. However, convincing evidence of the immunogenicity of plant-specific glycans is still limited although plant-specific glycans are considered potential allergic antigens. In the present study, we found that the α-1,3-fucose content of the glycoprotein produced from rice seed was much lower than that in leaf, and conversely, a higher β-1,2-xylose content was detected in seed than that in leaf. We detected the α-1,6-fucose content in the glutelin and recombinant human α1-antitrypsin (OsrAAT). The further results in a line containing AAT and FUT8 genes indicated that the α-1,6-fucose content of modified glycosylated recombinant α1-antitrypsin (mgOsrAAT) was 38.4%, while glutelin was only 6.8%. Interestingly, the α-1,3-fucose content of mgOsrAAT was significantly reduced by 59.8% compared with that of OsrAAT. Furthermore, we assessed the immunogenicity of OsrAAT, mgOsrAAT and human α1-antitrypsin (hAAT) using an animal system. The PCA results indicated no significant differences in the IgG, IgM and IgE titers among OsrAAT, mgOsrAAT and hAAT. Further studies revealed that those antibodies were mainly from α-1,3-fucose, but not from β-1,2-xylose, indicating that α-1,3-fucose was the major immunogenic resource. Our results demonstrated that α-1,3-fucose contents in seed proteins was much less than that of leaf, and could not be a plant-specific glycan because it also exists in human proteins.

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## 1. Introduction

Over the past two decades, interest in the production of biopharmaceutical proteins in plant cells has grown (Bakker et al., 2006; Da Cunha et al., 2014; Loos et al., 2011a,b; Ma et al., 2015; Nagels et al., 2011; Stoger et al., 2014; Strasser et al., 2009). This technique is effective for the production of biopharmaceutical proteins in terms of cost-effectiveness and versatility, thus allowing rapid and economical scaling up, as well as safety, owing to a lack of harbored microbial or mammalian pathogens compared with conventional approaches (Daniell et al., 2001; Sack et al., 2015). Since the first report of the expression of functional monoclonal antibodies in transgenic tobacco (Hiatt et al., 1989) and the subsequent approval of such methodologies in clinical applications (Hollak, 2012), a range of proof-of-principle studies conducted on pharmaceutically relevant proteins have successfully expressed in a diverse range of plant-based production platforms, such as tobacco, *Arabidopsis*, rice, *maize*, soybean, *lemna, alfalfa* and others (Bouquin et al., 2002; Cox et al., 2006; Daniell et al., 2001; Drakakaki et al., 2006; Egelkrout et al., 2012; He et al., 2011; Karg and Kallio, 2009; Loos et al., 2011a,b; Ma et al., 2003; O'Keefe et al., 2015; Ou et al., 2014; Sharma and Sharma, 2009; Shin et al., 2011; Song et al., 2011; Strasser et al., 2009; Strasser et al., 2008). In 2012, *Elelyso*, a plant-made pharmaceutical (PMP) approved through the United States Food and Drug Administration (FDA), which was developed by Protalix and Pfizer (Sack et al., 2015). In 2015, the human immunodeficiency virus (HIV)-neutralizing human monoclonal antibody, 2G12, expressed in transgenic tobacco obtained regulatory approval, entering a phase I safety study of single vaginal administration in healthy female subjects. The successful completion of the clinical trial marked a significant milestone in the commercial development of PMP proteins (Ma et al., 2015).

Several plant expression platforms using different tissues or organs have been investigated, including suspension cell culture, leaves, seeds, fruits and roots, to determine differences in sorting, modification and protein accumulation capacities through different expression strategies. Each platform has unique properties with specific benefits and drawbacks in terms of production time, environmental containment, scalability and costs (Twyman et al., 2003; Ou et al., 2014). Among these platforms, leaves and seeds are two

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promising organs that have become alternative choices for molecular farming because of their large amounts of biomass, greater stability and capacity for scaling up (Fischer et al., 2012). Indeed, the high grain yield, rich protein storage compartment and well-established production and processing of cereal crop seeds, such as rice, maize, wheat and barley, suggest that these may be preferential potential production hosts (Da Cunha et al., 2013; Hood, 2002; Stoger et al., 2002). The increased advantages of highly accumulating recombinant proteins and the increased stability of these molecules at ambient temperatures facilitate the separation of cultivation from purification processes in terms of augmenting flexibility and reducing costs (Stoger et al., 2005; Vianna et al., 2011). Moreover, seed is directly edible and can be orally administered without further purification and formulation (Peters and Stoger, 2011).

Approximately 30% of pharmaceutical proteins are glycoproteins. Plant-specific glycosylation influences the properties of the proteins (Daniell et al., 2001; Mann and Jensen, 2003). For example, the absence of the mammalian terminal β-1,4-galactose influences the serum clearance rate of plant-derived monoclonal antibodies (Bakker et al., 2001). Plant-specific α-1,3-fucose and β-1,2-xylose have been implicated as potential allergenic sources (Jin et al., 2008a,b). Therefore, modifications of plant-specific glycosylation are considered to be obstacles for PMP product applications. To overcome these obstacles, recombinant DNA technologies have been used to modify the *N*-glycosylation pathway in plants, thus demonstrating the feasibility of overcoming these limitations and paved the way for the humanization of the plant glycosylation pathway (Loos and Steinkellner, 2014; Steinkellner and Castilho, 2015; Castilho and Steinkellner, 2016). These approaches can be summarized as removal and insertion of glycosylation reactions by knock-out/knock-down approaches and introduction of glycosylation enzymes (Loos and Steinkellner, 2014; Dicker et al., 2016). For example, several strategies have been attempted to introduce foreign enzymatic activities into plant cells through the expression of genes that catalyze humanized glycans, including β-1,4-galactosyltransferase (*GalT*) to produce β-1,4-galactose residues (Bakker et al., 2001; Bakker et al., 2006), and β-1,4-*N*-acetylglucosaminyltransferase III (*GnTIII*) to compete plant-specific β-1,2-xylose and α-1,3-fucose modifications (Frey et al., 2009). KDEL endoplasmic reticulum (ER)-retention signals have also been added to prevent processing of the *N*-glycans in the Golgi apparatus (Triguero et al., 2011). The activities of α-1,3-fucosyltransferase (α-1,3-*FucT*) and β-1,2-xylosyltransferase (β-1,2-*XylT*) have been suppressed through RNA interference (RNAi) technology (Strasser et al., 2008), or been knocked out by gene-editing (Ozawa et al., 2012). Furthermore, entire sialylation-associated biosynthetic pathway could be introduced to transiently express proteins decorated with terminal sialic acid (Castilho et al., 2010, 2014). α-1,6-fucose is also important for human glycoproteins since this specific N-glycan residue has a great impact in the activity of monoclonal antibodies (Mori et al., 2007; Yamane-Ohnuki and Satoh, 2009). However, the studies on α-1,6-fucosyltransferase (FUT8) is limited regarding to the studies on GalT and other glycosyltransferases. Recently, transient overexpression of FUT8 in *N. benthamiana* lacking β-1,2-xylose and α-1,3-fucose allowed the synthesis of plant-derived proteins with humanized fucosylation (Forthal et al., 2010; Castilho et al., 2011). Until now, molecular modeling of immunoglobulin G1 suggests that the changes in the interactions of the Fc carbohydrate chain depending on the presence of core fucose, possibly changing the accessibility (Castilho et al., 2015; Castilho and Steinkellner, 2016). Therefore, fucosylation is a very important issue in *N*-glycosylation and need to be further studied.

On the other hand, the evidence of plant-specific glycans as allergenic sources is still limited. In a previous study, BALB/c mice have not been found to develop any humoral immune response to these plant-specific N-glycan residues (Chargelegue et al., 2000; Jin et al., 2006). The previous studies have indicated that the IgGs with plant-specific *N*-glycosylation should be acceptable for oral application because human beings are constantly exposed to plant glycoproteins through their diets (Ma et al., 1998). However, many biopharmaceutical biologics, such as monoclonal antibodies, are administered at high dosages through intravenous injection; thus, the presence of non-human glycan epitopes might provoke unwanted side effects in humans. Several studies have shown that plant *N*-glycoproteins bound to IgE from sera of allergic patients and core α-1,3-fucose might be responsible for this binding (Bencúrováet al., 2004; Jin et al., 2008a), demonstrating that both plant-specific α-1,3-fucose and β-1,2-xylose present on plant glycoproteins elicit specific antibodies in rabbits after parenteral application. IgE immunoblotting with sera derived from allergic patients revealed the binding to plant-produced antibodies carrying core α-1,3-fucosylated *N*-glycan structures (Jin et al., 2008a). In clinical practice, when the subjects were monitored with plant-made influenza virus-like particle (VLP) vaccines for 6 months, no one developed allergic/hypersensitivity symptoms (Ward et al., 2014). A first evaluation of plant-made conjugate vaccines in phase I safety and immunogenicity clinical trial revealed no serious adverse event related to vaccination in inoculated patients (Tusé et al., 2015). Moreover, clinical data have shown that the plant-made drug *Elelyso* has far fewer side effects than those of ERT produced from CHO cells (Sack et al., 2015).

In the present study, we observed that the content of proteins with α-1,3-fucose in rice seed was much lower than that in leaf, and conversely, the content of proteins with β-1,2 xylose in rice seed was higher than that in leaf. To evaluate the fucosylation effects of α-1,6-fucosyltransferase (FUT8) in endosperm cell, we stably expressed *FUT8* gene controlled by an endosperm-specific promoter in the rice endosperm cell, and crossed it with transgenic plant expressing recombinant human α1-antitrypsin (OsrAAT) (Wang et al., 2015). We detected 6.8% α-1,6-fucose in the native storage protein glutelin and 38.4% α-1,6-fucose in mgOsrAAT. Interestingly, the α-1,3-fucose content was significantly reduced from 82.5% in OsrAAT to 22.7% in mgOsrAAT. Furthermore, we assessed the immunogenicities of OsrAAT, mgOsrAAT and hAAT using animal model. No significant difference in immunogenicity was observed among these three types of glycosylated AAT. Further analysis of the antibodies for α-1,3-fucose- and β-1,2-xylose-specific glyco-epitopes indicated that α-1,3-fucose was the major immunogenic resource from plant-specific *N*-glycans. These results provided evidence of the immunogenicity of recombinant proteins with plant-specific glycosylation in rice seed.

## 2. Materials and methods

### 2.1. Plant materials

Two *indica* rice varieties, 9311 and Yuetai and two *japonica* rice varieties, TP309 and Nipponbare provided by Professor Zhu's laboratory, were used for glycosylation pattern analysis. A transgenic line (132-17) expressing human alpha-antitrypsin (AAT) was used from our laboratory (Zhang et al., 2012).

### 2.2. Generation of transgenic rice plants and crossing

α-1,6-Fucosyltransferase gene (*FUT8*; GenBank accession No. D89289.1) were synthesized by GenScript Corporation (Piscataway, NJ, USA) using rice-preferred genetic codons. A complete sequence of *FUT8* driven by a globulin (*Glb*) promoter was cloned into a binary vector. It was co-transformed with a selective marker gene into

rice calli derived from TP309 via agrobacterium-mediated transformation. The positive plants were identified by PCR and Western blotting. Three single copy lines (515-8, 515-25 and 515-32) with different expression levels were determined by Western blotting. A line 515-32 with the highest expression level was used for further study and for crossing with 132-17. After self-crossing, a maker-assistance selection with FUT8- and AAT-specific primers were used to identify the homozygous line by described in Wang et al. (Wang et al., 2015). Finally, a homozygous line 515-32/132-17 containing both FUT8 and AAT genes was used for glycosylation analysis.

### 2.3. Protein extraction and purification

For total leaf protein preparation, five grams of rice fresh leaves was ground with liquid nitrogen, suspended in phosphate buffer (0.075 M $Na_2HPO_4$-$NaH_2PO_4$, pH 7.2) and mixed at 4 °C for 30 min. The crude extracts were centrifuged at 12,000*g* for 5 min, and the supernatant was transferred to a new tube for use.

For total seed protein extraction, 200 mg of seed powder was extracted with total protein extraction buffer (66 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS], and 2% β-ME). The sample was centrifuged at 12,000*g* for 5 min, and the supernatant was transferred to a new tube for use.

For AAT purification, 100 g of seeds was extracted with 20 mM phosphate-buffered saline (PBS) and was then purified through three steps of diethylaminoethanol (DEAE; GE Healthcare, Fairfield, CT, USA), ceramic hydroxyapatite type I (CHT-I; Bio-Rad, Hercules, CA, USA) and Capto Adhere (GE Healthcare, Fairfield, CT, USA) chromatography as previously described (Zhang et al., 2012).

### 2.4. Western blotting

The protein samples were separated via 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) as previously described (Zhang et al., 2012). For the detection of FUT8 and AAT, rabbit anti-FUT8 (1:5000), mouse anti-AAT (1:5000), goat anti-rabbit-IgG (1:10,000), and goat anti-mouse-IgG (1:10,000) antibodies coupled to alkaline phosphatase were purchased from Abcam (Cambridge, MA, USA).

### 2.5. N-*Glycan preparation*

One microgram of the protein was dissolved in 100 μl of $H_2O$. Subsequently 900 μl of pre-cooled acetone (10% trichloroacetic acid [TCA]) was added, and the mixture was left standing at 4 °C for 1 h. The protein was centrifuged at 12,000*g* for 15 min. The supernatant was discarded, and the pellet was washed 3 times with 1 ml of pre-cooled acetone. The pellet was dried and re-dissolved in 75 μl of 0.75 M urea, and 25 μl of 200 mM $NH_4HCO_3$ (pH 8.0) and 1 μl of 1 M dithiothreitol (DTT) were then added. The sample was denatured at 100 °C for 1 min and then at 0 °C for 1 min; these steps above were repeated three times. The protein was digested with 20 μl of trypsin (Sigma, St. Louis, MO, USA) solution (1 μg/μl) at 37 °C for 16 h, and then the digestion was terminated after boiling for 5 min. The peptides were separated with a pre-packed C18 column (Thermo Fischer, Waltham, MA, USA) and were eluted in 50% acetonitrile. The elution was used for MALDI-TOF MS analysis. After lyophilization, the sample was dissolved in 100 μl of 50 mM $CH_3COOH/CH_3COONH_4$ (pH 5.0). The *N*-glycans were released after digesting with 3 μl (0.1 mU) of peptide-N-glycosidase A (PNGase A; Roche, Basel, Basel-Stadt, Switzerland) or PNGase F (New England Biolabs, Ipswich, MA, USA) at 37 °C for 24 h and then purified with a C18 column. The flow-through faction was loaded onto a porous graphitic carbon (PGC) column (Thermo Fischer, Waltham, MA, USA), and the *N*-glycans were eluted in 25% acetonitrile. The oligosaccharides were lyophilized for subsequent use.

### 2.6. MALDI-TOF MS analysis

The purified *N*-glycans were dissolved in 10 μl of 5 mM NaAc and mixed with an equal volume of 1% 2,5-dihydroxybenzoic acid (DHB) in 50% acetonitrile. Two-microliter aliquots were loaded onto a stainless-steel sample plate and dried at room temperature. The positive-ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectra of $[M+Na]^+$ adducts were recorded on an Ultraflex mass spectrometer (ABI, New York City, NY, USA) fitted with delayed extraction and a nitrogen laser (337 nm). A maltodextrin series was used as an external molecular weight standard. The spectra were generated from the sum of 200–300 laser pulses. The data was collected from at least three individual measurements.

### 2.7. Calculation of the relative content of α-1,6-fucose

To selectively discriminate α-1,6-fucose from α-1,3-fucose for quantitative analysis, the protein sample was digested by PNGase A that release all kinds of N-linked glycans, including α-1,3-fucose and α-1,6-fucose, and PNGase F that exclusively release fucose with α-1,6-linkage, respectively (van Ree et al., 2000). To qualitatively analyze the content of α-1,6-fucose from total glycans, an *N*-glycan structure MMX was chosen as an internal reference because this glyco-form did not contain any fucose, it could be digested by both PNGase A and PNGase F. If the percentage of MMX released through PNGase A and PNGase F digestion is A and B, respectively, the total fucose released through PNGase A digestion is C, and the α-1,6-fucose released through PNGase F digestion is D, then the following formula can be used to calculate the relative content of α-1,6-fucose:

$$P_{\alpha\text{-}1,6} = D/B \times A$$

and the relative content of α-1,3-fucose should be:

$$P_{\alpha\text{-}1,3} = C - P_{\alpha\text{-}1,6}$$

### 2.8. Immunogenicity assay

New Zealand rabbits aged 14 weeks with 2.69–3.01 kg body weight were randomly divided into three groups containing five rabbits each. The rabbits were inoculated with OsrAAT (AAT from 132-17), mgOsrAAT (AAT from 515-32/132-17) and hAAT (AAT from human plasma, Sigma, USA) with incomplete Freund's adjuvant, respectively. One milligram was used for the first subcutaneous inoculation, and this was followed by boosts with 0.3 mg with incomplete Freund's adjuvant every two weeks. Animal death, health status and allergic reactions were observed every morning and afternoon. The body weights were measured, and blood samples were collected from the central auricular arteries at days 0 (before the first inoculation and after inoculation), 8, 22, and 36, respectively.

The IgG, IgM and IgE titers were measured using enzyme-linked immunosorbent assay (ELISA) as previously described (He et al., 2011). Briefly, the microtiter plate was blocked with a blocking solution (PBS, 3% bovine serum albumin [BSA], and 0.1% Tween 20) at 37 °C for 2 h, and 100 μl of serum was successively added to the microtiter plate, followed by incubation at 37 °C for 2 h and washing with the blocking solution. Subsequently, the microtiter plate was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, IgM or IgE (Abcam, Cambridge, MA, USA) at 37 °C for 1 h. The total titers of IgG, IgM and IgE were detected according to

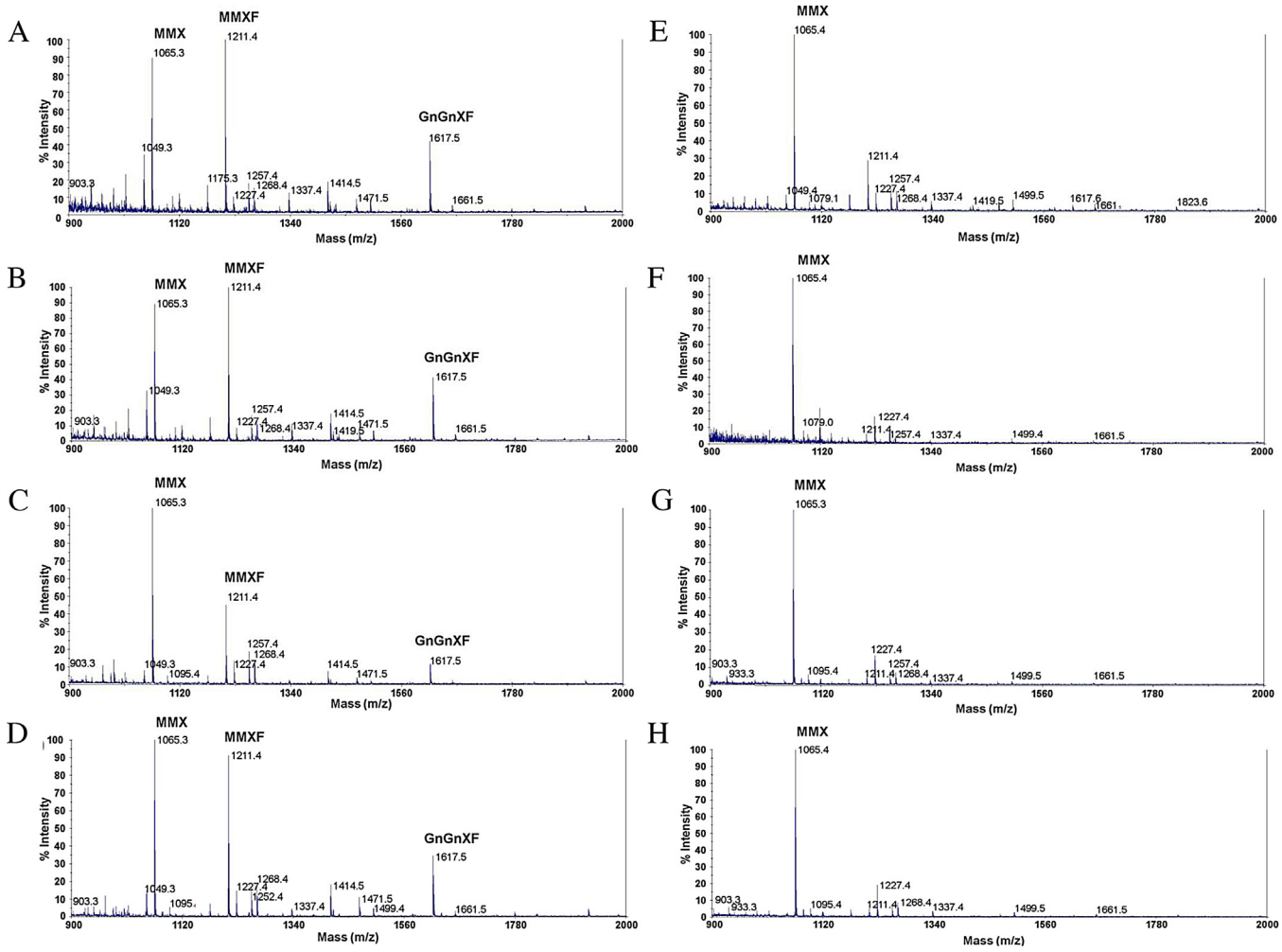



**Fig. 1.** MALDI-TOF spectra of $[M+Na]^+$ ions of the N-glycans in the leaf and seed cells from four *Japonica* and *indica* cultivars. 9311 leaf (A) and seed (E); YB leaf (B) and seed (F); TP leaf (C) and seed (G); Nipponbare leaf (D) and seed (H).

the manufacturer's instructions (Shanghai Yuanye Biotechnology Co, Shanghai, China).

Male 6- to 8-week-old guinea pigs were used for the passive cutaneous anaphylaxis (PCA) assay. Antisera against OsrAAT, mgOsrAAT and hAAT were collected from D36 and diluted with PBS at half of the titer of the corresponding serum and were subsequently mixed with an equal volume of PBS (negative control). OsrAAT, mgOsrAAT or hAAT were used as sensitinogens. Approximately 100 μl of the antigens was intradermally inoculated at four independent spots. After 4 h of sensitization, 0.3 mg of each antigen, i.e., OsrAAT, mgOsrAAT or hAAT, was challenged through intravenous injection, and 0.5 ml of 20 mg/ml of Evans blue (Sigma, St. Louis, MO, USA) was intravenously injected into each animal. After 30 min, the animals were slaughtered, and the dorsal skin was removed for observation of the blue spot size.

### 2.9. Measurement of concentrations of plant-specific glycan antibodies

To find out the concentrations of antibodies against plant-specific glycans in serum, we separated the antibodies from experimental animal serum using affinity chromatography. Two antigens, HRP (Sigma, St. Louis, MO, USA; Lot no. 77332) or $PLA_2$ (Sigma, St. Louis, MO, USA; Lot No. P9279) was coupled to Agarose 4B. Coupling reaction was carried out according to the manufacturer's instructions (GE Healthcare, Fairfield, CT, USA). The affinity chromatographic columns (7 mm × 25 mm) were packed with 1 ml coupled resin and equilibrated with 10-fold column size of a equilibrate buffer (20 μM $Na_2HPO_4$, 0.5 M NaCL, pH 7.6). Then 1 ml of animal serum was loaded and washed by 20 μM $Na_2HPO_4$, 0.5 M NaCl, (pH 2.7). The captured antibody was eluted by 0.1 M glycine (pH 2.7). The concentration antibodies in the eluent was measured by ELISA Kit For Immunoglobulin G (Cloud-Clone Corp, Houston, Texas, USA). A serial concentration of the standards (1.23 μl/ml to 100 μl/ml) and 50 μl of sera were loaded to the microtiter plate, and then incubated with 50 μl detection solution A for 1 h at 37 °C. Then, added 90 μl TMB substrates and incubated at 37 °C for 10–20 min. 50 μl stop solution were added to stop reaction and read the UV absorption at 450 nm (BioTek, ELX808). Each sample was repeated for three times.

## 3. Results

### 3.1. The glycosylation profiles of rice leaf and seed

A previous study has indicated that the glycosylation pattern of the protein in tobacco leaf shows complex glycans and has demonstrated immunogenicity in rabbits (Jin et al., 2008a). Our previous results have indicated that rice seed is a cost-effective expression system (Abe et al., 2014; He et al., 2011; Ou et al., 2014). To determine whether the glycosylation patterns in rice show tissue- and species-specificity, total proteins of leaf and

**Table 1**
N-glycoforms of leaf and seed from 9311, Yuetai, TP 309 and Nipponbare.

| $m/z$ (M+Na)$^+$ | Glycan structures | Leaf (%) | | | | Seed (%) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 9311 | Yuetai | TP | NP | 9311 | Yuetai | TP | NP |
| 1048.9 | $Man_2XylFucGlcNAc_2$ (MXF) | 8.1 ± 0.2 | 8.7 ± 0.3 | 4.3 ± 0.3 | 4.6 ± 0.4 | 4.3 ± 0.4 | 3.6 ± 0.5 | 1.7 ± 0.3 | 2.0 ± 0.3 |
| 1211.1 | $Man_3XylFucGlcNAc_2$ (MMXF) | 20.0 ± 1.0 | 20.9 ± 1.3 | 14.0 ± 0.1 | 21.9 ± 0.7 | 5.6 ± 0.4 | 4.2 ± 0.3 | 3.2 ± 0.2 | 2.8 ± 0.3 |
| 1414.3 | $GlcNAcMan_3XylFucGlcNAc_2$ (GnXF) | 5.0 ± 0.2 | 4.9 ± 0.7 | 4.5 ± 0.5 | 5.2 ± 0.3 | 2.8 ± 0.3 | ND | ND | ND |
| 1617.5 | $GlcNAc_2Man_3XylFucGlcNAc_2$ (GnGnXF) | 8.2 ± 0.2 | 9.7 ± 0.6 | 5.1 ± 0.2 | 8.8 ± 0.3 | 1.8 ± 0.2 | ND | ND | ND |
| Fucose and Xylose | | 41.3 ± 0.4 | 44.2 ± 1.6 | 27.9 ± 0.9 | 40.5 ± 1.0 | 14.5 ± 0.3 | 7.8 ± 0.2 | 4.9 ± 0.2 | 4.8 ± 0.1 |
| 902.8 | $Man_2XylGlcNAc_2$ | 2.0 ± 0.2 | 3.4 ± 0.4 | 2.0 ± 0.2 | 1.7 ± 0.4 | 2.9 ± 0.4 | 5.0 ± 0.2 | 4.2 ± 0.3 | 4.0 ± 0.1 |
| 1064.9 | $Man_3XylGlcNAc_2$ (MMX) | 22.7 ± 0.3 | 20.4 ± 0.9 | 29.5 ± 0.7 | 25.4 ± 0.6 | 43.5 ± 1.1 | 51.8 ± 1.1 | 56.8 ± 1.0 | 51.6 ± 0.7 |
| 1106.0 | $GlcNAcMan_2XylGlcNAc_2$ | ND | 2.3 ± 0.3 | ND | 1.2 ± 0.3 | 2.1 ± 0.2 | 2.6 ± 0.2 | 4.2 ± 0.3 | 1.8 ± 0.2 |
| 1227.1 | $Man_4XylGlcNAc_2$ | 3.9 ± 0.3 | 2.5 ± 0.5 | 7.4 ± 0.3 | 4.9 ± 0.1 | 5.1 ± 0.4 | 9.7 ± 0.2 | 10.7 ± 0.4 | 11.2 ± 0.8 |
| 1268.1 | $GlcNAcMan_3XylGlcNAc_2$ | 5.9 ± 0.1 | 4.1 ± 0.3 | 8.9 ± 0.3 | 5.5 ± 0.5 | 5.3 ± 0.3 | 2.0 ± 0.2 | 4.3 ± 0.2 | 4.9 ± 0.3 |
| 1471.3 | $GlcNAc_2Man_3XylGlcNAc_2$ | 2.2 ± 0.3 | 2.4 ± 0.3 | 3.1 ± 0.5 | 3.0 ± 0.2 | 2.2 ± 0.2 | ND | ND | 1.0 ± 0.1 |
| Xylose only | | 36.7 ± 0.9 | 35.1 ± 1.1 | 50.9 ± 1.2 | 41.7 ± 0.7 | 61.1 ± 0.7 | 71.1 ± 0.9 | 80.2 ± 0.2 | 74.5 ± 1.6 |
| 1079.0 | $Man_3FucGlcNAc_2$ | 1.6 ± 0.2 | 1.5 ± 0.2 | ND | 1.8 ± 0.3 | 2.1 ± 0.2 | 5.4 ± 0.3 | ND | 3.5 ± 0.3 |
| Fucose only | | 1.6 ± 0.2 | 1.5 ± 0.2 | 0.0 | 1.8 ± 0.3 | 2.1 ± 0.2 | 5.40±0.3 | 0.0 | 3.5 ± 0.3 |
| 932.8 | $Man_3GlcNAc_2$ | 2.0 ± 0.1 | 1.8 ± 0.3 | 2.2 ± 0.2 | 2.3 ± 0.2 | 3.1 ± 0.1 | 4.2 ± 0.2 | 4.2 ± 0.3 | 4.6 ± 0.4 |
| 1095.0 | $Man_4GlcNAc_2$ | 2.8 ± 0.3 | 2.0 ± 0.3 | 3.6 ± 0.3 | 1.8 ± 0.2 | 3.3 ± 0.3 | 3.6 ± 0.4 | ND | 3.2 ± 0.5 |
| 1257.1 | $Man_5GlcNAc_2$ | 6.7 ± 0.4 | 4.3 ± 0.3 | 9.5 ± 0.5 | 5.0 ± 0.3 | 5.7 ± 0.3 | 4.7 ± 0.3 | 4.7 ± 0.4 | 3.6 ± 0.4 |
| 1419.2 | $Man_6GlcNAc_2$ | 2.2 ± 0.3 | 3.0 ± 0.1 | 2.4 ± 0.3 | 1.5 ± 0.3 | 2.2 ± 0.3 | ND | 1.3 ± 0.2 | 1.2 ± 0.3 |
| Oligomannose | | 13.7 ± 0.4 | 11.1 ± 0.4 | 17.7 ± 0.9 | 10.6 ± 0.2 | 14.3 ± 0.2 | 12.5 ± 0.1 | 10.2 ± 0.2 | 12.6 ± 2.3 |
| 1339.2 | $GlcNAc_2Man_3GlcNAc_2$ | 4.0 ± 0.4 | 3.7 ± 0.3 | 1.1 ± 0.2 | 2.9 ± 0.4 | 3.0 ± 0.1 | 1.8 ± 0.3 | 1.9 ± 0.2 | 2.0 ± 0.2 |
| 1501.4 | $GlcNAc_2Man_4GlcNAc_2$ | 2.7 ± 0.3 | 2.2 ± 0.3 | 1.3 ± 0.2 | 1.4 ± 0.3 | 2.8 ± 0.2 | 1.4 ± 0.2 | 1.8 ± 0.2 | 1.5 ± 0.2 |
| 1663.5 | $GlcNAc_2Man_5GlcNAc_2$ | ND | 2.2 ± 0.3 | 1.1 ± 0.2 | 1.1 ± 0.1 | 2.2 ± 0.2 | ND | 1.0 ± 0.1 | 1.1 ± 0.2 |
| Others | | 6.7 ± 0.3 | 8.1 ± 0.3 | 3.5 ± 0.3 | 5.4 ± 0.7 | 8.0 ± 0.4 | 3.2 ± 0.4 | 4.7 ± 0.2 | 4.6 ± 0.4 |

ND: no detected.

seed from two *japonica* and *indica* varieties were analyzed. As shown in Supplementary Fig. S1, no obvious differences in the protein patterns were observed between *japonica* and *indica* varieties in either the leaf or the seed by SDS-PAGE. Furthermore, the similar MS profiles were observed between *japonica* variety TP309 and *indica* variety 9311, but a little difference was found in several MS peak height (Supplementary Fig. S2). So, we analyzed the glycan structures of the proteins between leaf and seed. As expected, the glycan compositions exhibited an obvious difference. As shown in Fig. 1 and Table 1, the glycan structures of $Man_3XylFucGlcNAc_2$ (MMXF), $GlcNAc_2Man_3XylFucGlcNAc_2$ (GnGnXF) and $GlcNAcMan_3XylFucGlcNAc_2$ (GnXF) in the leaf were significantly higher than those in the seed (Fig. 1 and Table 1). The glycan structure of $Man_3XylGlcNAc_2$ (MMX) in the seed was higher than that in the leaf. Two glycan structures, GnXF and GnGnXF, were detected in the leaf, but fewer or none of these structures were detected in the seed (Table 1). Furthermore, the glycan structure of $Man_2XylFucGlcNAc_2$ (MXF) in the *indica* variety was higher than that in the *japonica* variety in either the leaf or the seed. The three predominant glyco-structures, MMX, MMXF and GnGnXF, accounted for more than 50% of the total peak area in the leaf. The results indicated that MMX and MMXF were major glycan types in rice leaf, whereas MMX was the only major glycan type in the seed.

Notably, α-1,3-fucose and β-1,2-xylose are plant-specific glycans catalyzed in the Golgi apparatus. To determine whether differences in glycosylation patterns exist between the leaf and seed, we analyzed the glyco-forms with α-1,3-fucose and/or β-1,2-xylose. All types of xylose and fucose structures in the leaf were much higher than that in the seed, ranging from 27.9% to 44.2% in the leaf and 4.8% to 14.5% in the seed. In contrast, the xylose without fucose structure in the seed was much higher than that in the leaf, ranging from 61.1% to 80.2% in the seed and from 35.1% to 50.9% in the leaf (Table 1). Other glycan structures, including α-1,3-fucose only, oligomannose and other complex glycans showed no obvious differences between the leaf and the seed. Our results showed that fewer fucose-containing glycans in the rice seed than in the leaf. We therefore speculated that the protein produced in rice seed may have fewer plant immune response than in the leaf if α-1,3-fucose could be the main resource for the immunogenicity.

### 3.2. Engineered human glyco-modification in transgenic rice endosperm

To introduce α-1,6-fucose structure of glycoprotein in rice seed, *FUT8,* encoding α-1,6-fucosyltransferase gene from human controlled by an endosperm-specific promoter, was introduced into the rice genome. Total of 53 independent transgenic lines were obtained. Out of 32 lines showed to express FUT8 proteins, three independent lines (515-8, 515-25 and 515-32) showed different expression levels. As shown in Fig. 2, the protein encoded FUT8 was obviously detected in the rice endosperm of the transgenic lines. A line 515-32 showed the highest expression level of FUT8 and was chosen for further study. To monitor whether FUT8 could catalyze α-1,6-fucose linkage in endosperm cell, a native storage protein, glutelin, from 515-32 was extracted and digested by PNGase A and PNGase F, which hydrolyzes all types of N-linked oligosaccharides and N-linked oligosaccharides without α-1,3-linkage oligosaccharides from glyco-peptides, respectively. The profile of *N*-glycans released through PNGase A was shown in Fig. S3A. MALDI-TOF analysis revealed that the major type of *N*-glycan in the line 515-32 was MMX, occupied 50.1% of the total peak area (Table 2). All fucose glycan structures, including α-1,3-fucose and α-1,6-fucose, accounted for 16.9% of the total peak area. MMX accounted for 55.6% in PNGase F digestion (Fig. S3 and Table 2). The qualitative analysis of

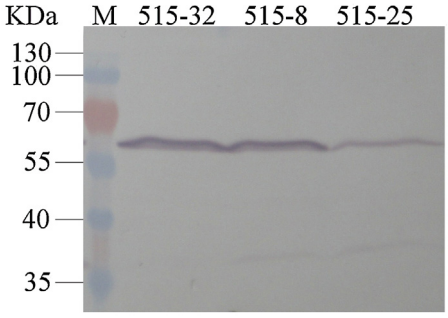



**Fig. 2.** Western blot analysis of FUT8 expression in the transgenic seeds. M, molecular mass marker.

**Table 2**
N-glycoforms of glutelin from 515-32.

| $m/z$ $(M+Na)^+$ | Glycan structures | PNGase A digestion (%) | PNGase F digestion (%) |
|---|---|---|---|
| 1048.9 | $Man_2XylFucGlcNAc_2$ (MXF) | 3.2 ± 0.2 | 1.8 ± 0.3 |
| 1211.1 | $Man_3XylFucGlcNAc_2$ (MMXF) | 9.8 ± 0.2 | 4.6 ± 0.3 |
| 1414.3 | $GlcNAcMan_3XylFucGlcNAc_2$ (GnXF) | 2.0 ± 0.3 | ND |
| 1617.5 | $GlcNAc_2Man_3XylFucGlcNAc_2$ (GnGnXF) | 1.9 ± 0.2 | 1.1 ± 0.1 |
| Fucose and Xylose | | 16.9 ± 0.4 | 7.5 ± 0.1 |
| 902.8 | $Man_2XylGlcNAc_2$ | ND | 2.0 ± 0.1 |
| 1064.9 | $Man_3XylGlcNAc_2$ (MMX) | 50.1 ± 1.2 | 55.6 ± 1.4 |
| 1227.1 | $Man_4XylGlcNAc_2$ | 5.3 ± 0.4 | 5.8 ± 0.2 |
| 1268.1 | $GlcNAcMan_3XylGlcNAc_2$ | 5.4 ± 0.5 | 3.9 ± 0.2 |
| 1471.3 | $GlcNAc_2Man_3XylGlcNAc_2$ | 3.9 ± 0.2 | 3.9 ± 0.1 |
| Xylose only | | 64.7 ± 0.8 | 71.2 ± 1.2 |
| 932.8 | $Man_3GlcNAc_2$ | 1.8 ± 0.2 | 2.3 ± 0.3 |
| 1053.9 | $Man_5GlcNAc$ | 2.0 ± 0.3 | 1.2 ± 0.1 |
| 1095.0 | $Man_4GlcNAc_2$ | 1.2 ± 0.3 | 2.2 ± 0.3 |
| 1175.0 | $GlcNAc_2Man_2GlcNAc_2$ | 2.1 ± 0.2 | 2.1 ± 0.2 |
| 1257.1 | $Man_5GlcNAc_2$ | 5.2 ± 0.3 | 6.3 ± 0.2 |
| 1339.2 | $GlcNAc_2Man_3GlcNAc_2$ | 2.8 ± 0.2 | 2.3 ± 0.3 |
| 1419.2 | $Man_6GlcNAc_2$ | 3.3 ± 0.3 | 2.0 ± 0.1 |
| 1501.4 | $GlcNAc_2Man_4GlcNAc_2$ | ND | 1.5 ± 0.2 |
| 1663.5 | $GlcNAc_2Man_5GlcNAc_2$ | ND | 1.4 ± 0.1 |
| Others | | 18.4 ± 0.4 | 21.3 ± 1.2 |

ND: no detected.

α-1,6-fucose using MMX as an internal reference indicated that the oligosaccharides with α-1,6-fucose in 515-32 occupied 6.8% of total *N*-glycans, whereas oligosaccharides with α-1,3-fucose accounted for 10.1% (Table 4). Other two lines (515-8 and 515-25) with lower expression level of FUT8 showed a slightly fewer contents of α-1,6-fucose (Table S1). The results demonstrated that FUT8 was highly expressed and can effectively catalyze α-1,6-fucose linkage in rice endosperm cell.

### 3.3. Glyco-forms of OsrAAT produced in different transgenic backgrounds

To distinguish the difference between native storage protein and foreign recombinant protein, we characterized the glycan profile of recombinant *alpha* antitrypsin (AAT) in rice seed. Two transgenic lines, 132-17 and 515-32, carrying the genes for *AAT* and *FUT8* were stacked by crossing (Wang et al., 2015; Zhang et al., 2012). After marker-assistance selection (MAS), a homozygous line 515-32/132-17 was obtained. Western blot analysis showed that the AAT expression levels in 515-32/132-17 (mgOsrAAT) and 132-17 (OsrAAT) were nearly equal (Fig. S4A). Two types of recombinant AAT from the transgenic lines 515-32/132-17 and 132-17, was obtained as described in Material and Methods (Wang et al., 2015; Zhang et al., 2012). The results indicated that the molecular masses of OsrAAT and mgOsrAAT were less than that of hAAT (Fig. S4B), suggesting the heterogeneity of glycosylation patterns between plant and human cells. To characterize the glycan structures of mgOsrAAT from line 515-32/132-17, mgOsrAAT was digested by PNGase A and PNGase F, respectively, and was subsequently analyzed through MALDI-TOF MS. We found that the glycan profiles were largely distinct between OsrAAT and mgOsrAAT, showing high heterogeneity (Fig. 3). The main proportion of oligosaccharides in OsrAAT was MXF, accounting for 62.8% of the total oligosaccharides (Fig. 3A and Table 3). The α-1,3-fucose and β-1,2-xylose contents accounted for 82.5% and 94.1% of the total oligosaccharides, respectively (Tables 3 and 4). In mgOsrAAT, the total fucose oligosaccharides accounted for 61.1%. Difucosylated *N*-glycans were detected at $m/z$ 1196.1 and 1358.2, which corresponded to $Man_2XylFuc_2GlcNAc_2$ ($MXF_2$) and $Man_3XylFuc_2GlcNAc_2$ ($MMXF_2$), accounted for 13.8% and 7.8%, respectively. The results indicated that FUT8 was able to catalyze the monofucosylated oligosaccharides. The MMX structure

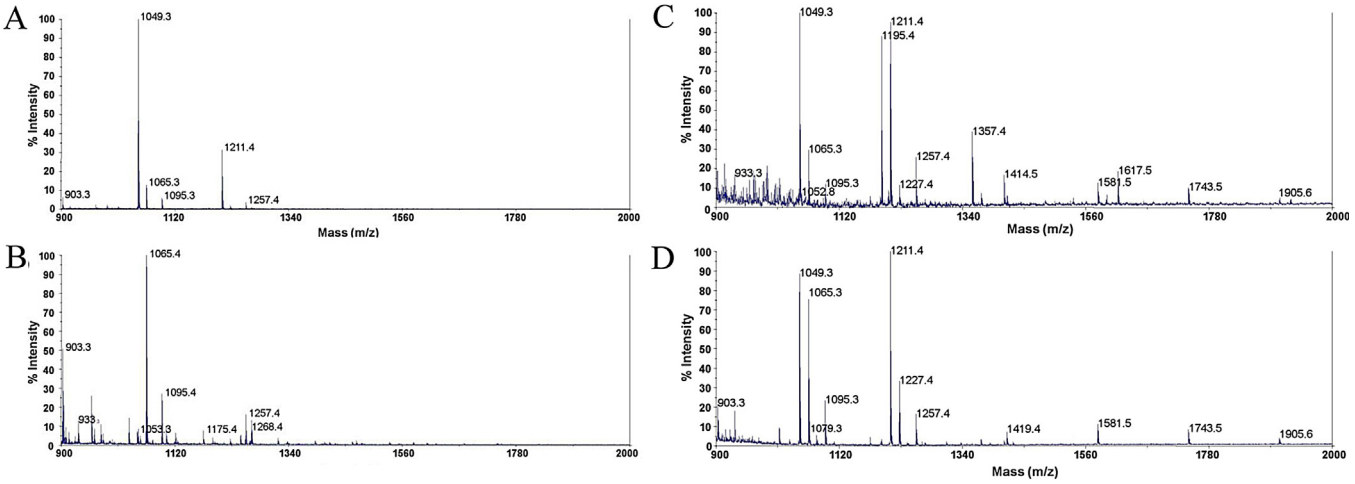



**Fig. 3.** MALDI-TOF spectra of the N-glycans from OsrAAT (132-17) and mgOsrAAT (515-32/132-17). OsrAAT was digested by PNGase A (A) and PNGase F (B); mgOsrAAT was digested by PNGase A (C) and PNGase F (D).

**Table 3**
N-glycoforms of OsrAAT and mgOsrAAT.

| $m/z$ (M+Na)$^+$ | Glycan structures | PNGase A digestion (%) | | PNGase F digestion (%) | |
|---|---|---|---|---|---|
| | | OsrAAT | mgOsrAAT | OsrAAT | mgOsrAAT |
| 1048.9 | $Man_2XylFucGlcNAc_2$ (MXF) | 62.8 ± 1.6 | 17.9 ± 1.2 | ND | 21.9 ± 0.7 |
| 1196.1 | $Man_2XylFuc_2GlcNAc_2$ | ND | 13.8 ± 0.7 | ND | ND |
| 1211.1 | $Man_3XylFucGlcNAc_2$ (MMXF) | 19.7 ± 1.3 | 16.2 ± 0.8 | ND | 22.1 ± 1.2 |
| 1358.2 | $Man_3XylFuc_2GlcNAc_2$ | ND | 7.8 ± 0.4 | ND | ND |
| 1414.3 | $GlcNAcMan_3XylFucGlcNAc_2$ (GnXF) | ND | 2.5 ± 0.4 | ND | ND |
| 1617.5 | $GlcNAc_2Man_3XylFucGlcNAc_2$ (GnGnXF) | ND | 2.9 ± 0.3 | ND | ND |
| Fucose and xylose | | 82.5 ± 0.3 | 61.1 ± 1.1 | 0.0 | 44.0 ± 0.5 |
| 902.8 | $Man_2XylGlcNAc_2$ | 3.6 ± 0.4 | 6.5 ± 0.5 | 23.0 ± 1.2 | 6.4 ± 0.5 |
| 1064.9 | $Man_3XylGlcNAc_2$ (MMX) | 6.7 ± 0.5 | 7.0 ± 0.2 | 44.0 ± 1.5 | 18.3 ± 1.1 |
| 1227.1 | $Man_4XylGlcNAc_2$ | 1.3 ± 0.2 | 3.3 ± 0.4 | 1.7 ± 0.3 | 8.0 ± 0.3 |
| 1268.1 | $GlcNAcMan_3XylGlcNAc_2$ | ND | ND | 6.0 ± 0.5 | ND |
| Xylose only | | 11.6 ± 0.5 | 16.8 ± 0.3 | 74.7 ± 0.3 | 32.7 ± 0.6 |
| 932.8 | $Man_3GlcNAc_2$ | ND | 4.5 ± 0.6 | 4.3 ± 0.3 | 5.3 ± 0.8 |
| 1095 | $Man_4GlcNAc_2$ | 3.5 ± 0.2 | 3.0 ± 0.1 | 13.0 ± 0.5 | 5.4 ± 0.8 |
| 1175 | $GlcNAc_2Man_2GlcNAc_2$ | ND | ND | 2.7 ± 0.3 | ND |
| 1257.1 | $Man_5GlcNAc_2$ | 2.4 ± 0.6 | 5.5 ± 0.2 | 5.3 ± 0.4 | 4.0 ± 0.2 |
| 1419.2 | $Man_6GlcNAc_2$ | ND | 2.0 ± 0.2 | ND | 1.8 ± 0.2 |
| 1581.4 | $Man_7GlcNAc_2$ | ND | 2.8 ± 0.3 | ND | 2.9 ± 0.2 |
| 1743.5 | $Man_8GlcNAc_2$ | ND | 2.6 ± 0.4 | ND | 2.6 ± 0.4 |
| 1905.7 | $Man_9GlcNAc_2$ | ND | 1.7 ± 0.3 | ND | 1.3 ± 0.2 |
| Others | | 5.9 ± 0.8 | 22.1 ± 1.0 | 25.3 ± 0.3 | 23.3 ± 0.7 |

ND: no detected.

accounted for 7.0% of total oligosaccharides when digested by PNGase A and accounted for 18.3% when digested by PNGase F (Fig. 3 and Table 3). No difucosylated glycans was detected in PNGase F digestion because this modification conferred protection to the glycans against various glycosidases. It could be protected oligosaccharides from extensive degradation to GlcNAc by hexosamidase and lysozyme, and also even from defucosylation by fucosidase (Ihara et al., 2013). The results revealed that α-1,6-fucose content accounted for 16.8% and the total of α-1,6-fucose contents plus 21.6% difucosylated glycans reached 38.4% in mgOsrAAT. The α-1,3-fucose content was 22.7% of total fucose contents in mgOsrAAT, which decreased 59.8% compared with OsrAAT, while the β-1,2-xylose structure accounted for 77.9%, which decreased 16.2% compared with OsrAAT (Table 4). No α-1,6-fucose was detected in OsrAAT as expected (Fig. 3B). In addition, the mannose-containing glycans, including high-mannose glyco-forms were increased in mgOsrAAT compared with OsrAAT (Table 3). These results indicated that expression of *FUT8* in rice endosperm cell effectively increased α-1,6-fucose contents and difucosylated structures, as well as the mannosidic glycans. Meanwhile, an increase of the α-1,6-fucose content simultaneously inhibited both α-1,3-fucose and β-1,2-xylose contents.

### 3.4. Evaluation of the immunogenicity of OsrAAT and mgOsrAAT

To understand whether the immunogenicity differences exist between OsrAAT and mgOsrAAT, we performed animal tests. No obvious anaphylaxis reactions were observed after the administration of OsrAAT, mgOsrAAT and hAAT. However, these results do not indicate that no immunoreactions occurred *in vivo*. To monitor the plant-glycan-specific IgG, IgM and IgE titers, we analyzed the titers of these antibodies in rabbit serum following immunization.

**Table 4**
Summary of N-glycoforms of glutelin and AATs.

| Saccharides | Glutelin (515-32) (%) | OsrAAT (%) | mgOsrAAT (%) |
|---|---|---|---|
| α-1,6-fucose | 6.8 | ND | 38.4 |
| α-1,3-fucose | 10.1 | 82.5 | 22.7 |
| Total fucose | 16.9 | 82.5 | 61.1 |
| β-1,2-xylose | 81.6 | 94.1 | 77.9 |

ND: no detected.

Total IgG and IgM titers against OsrAAT, mgOsrAAT or hAAT rapidly increased at D8 after the first immunization, while no IgE antibody was detected. IgG and IgM titers against OsrAAT, mgOsrAAT or hAAT peaked at D22 and then stabilized at D36. The IgE titers against OsrAAT, mgOsrAAT or hAAT increased at D22 and then stabilized until D36 (Fig. 4). The highest titer of IgE was 1:1600 during the whole process. The level of IgE was a main characteristic of hypersensitivity and played an important role in inflammatory response. These results indicated that there were no significant differences in IgG, IgM and IgE titers between OsrAAT, mgOsrAAT and hAAT. The lower IgE titer may ensure rice endosperm a safe platform for biopharmaceutical.

To further assess plant glycan-specific immunogenicity of OsrAAT and mgOsrAAT, a PCA assay was performed (He et al., 2011). The sera from the sensitization of OsrAAT, mgOsrAAT and hAAT were neutralized by using the antigens OsrAAT, mgOsrAAT and hAAT, respectively. The results indicated that mgOsrAAT and OsrAAT were completely neutralized anti-OsrAAT serum (Fig. 4D-2 and 3, Fig. 4E-2-3), but hAAT only partially neutralized anti-OsrAAT or anti-mgOsrAAT sera, suggesting that the plant-specific antibodies could present in anti-OsrAAT and anti-mgOsrAAT sera (Fig. 4D and E). Similarly, hAAT completely neutralized anti-hAAT serum (Fig. 4F 4), while OsrAAT and mgOsrAAT only partially neutralized anti-hAAT sera (Fig. 4F 2 and 3). In addition, we observed that mgOsrAAT neutralized anti-hAAT serum to a higher degree than OsrAAT (Fig. 4F 3 versus 2), suggesting that mgOsrAAT containing humanized α-1,6-fucose partially neutralized anti-hAAT serum. The hAAT serum that was not completely neutralized with mgOsrAAT may have contained plant-specific glycans, such as α-1,3-fucose and β-1,2-xylose or human plasma impurities. According to the results of 22.7% (mgOsrAAT) and 82.5% (OsrAAT) of α-1,3-fucose and 94.1% (OsrAAT) and 77.9% (mgOsrAAT) of β-1,2-xylose (Table 4), we speculated that the immunogenicity could be mainly responding for α-1,3-fucose rather than β-1,2-xylose.

To confirm this hypothesis, two known glycoproteins, horseradish peroxidase (HRP) that carries *N*-glycans with both α-1,3-fucose and β-1,2-xylose and honeybee venom phospholipase $A_2$ ($PLA_2$) that carries *N*-glycan with α-1,3-fucose only were used to couple with Agarose 4 B for affinity chromatography, respectively (Bardor et al., 2003; Jin et al., 2008a; van Ree et al., 2000). The antibody concentrations from both HRP- and $PLA_2$-coupled

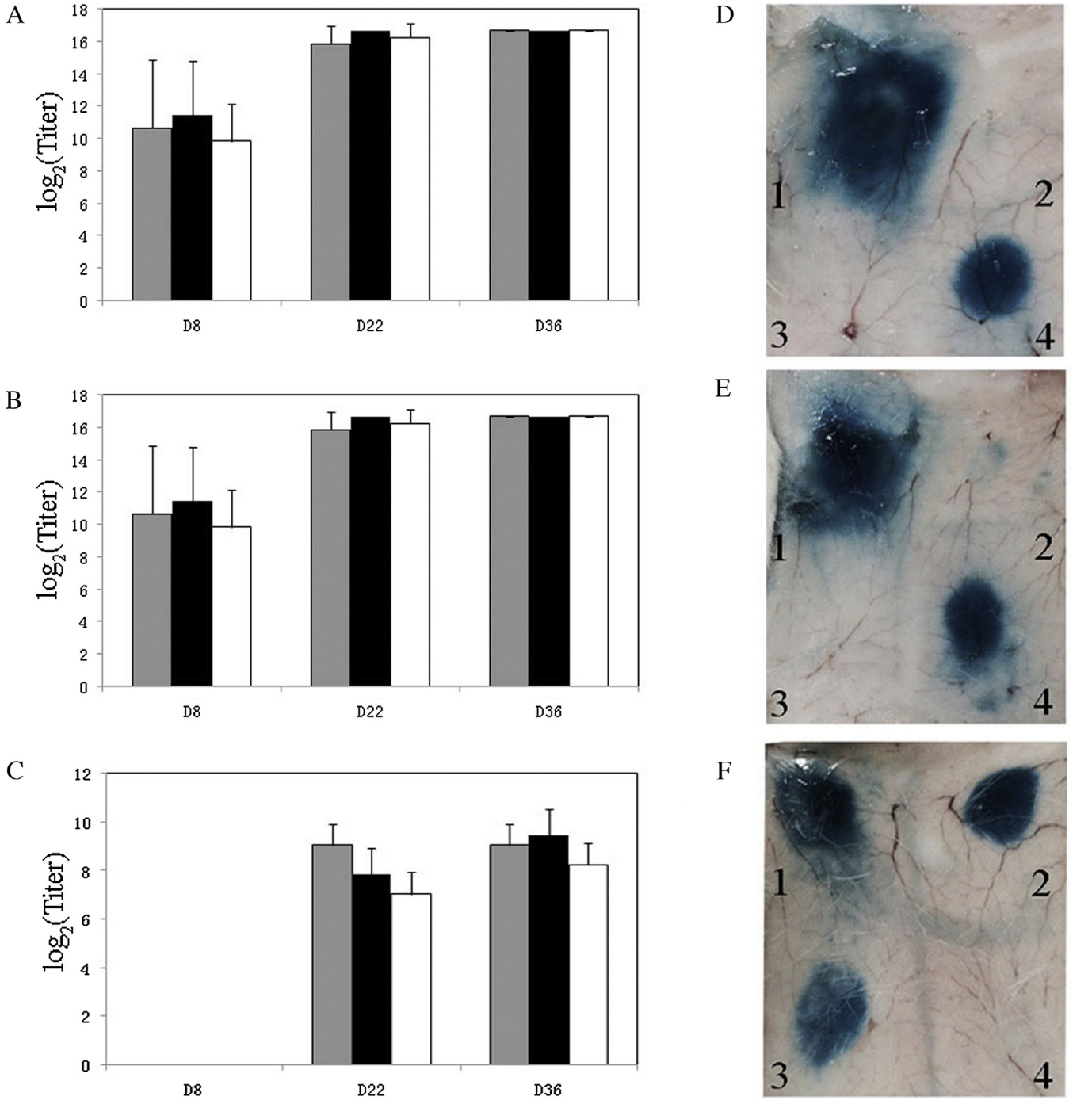



**Fig. 4.** IgG, IgM and IgE titers and PCA assays of different sources of AAT. (A–C) IgG (A), IgM (B) and IgE (C) titers against OsrAAT (gray), mgOsrAAT (dark) and hAAT (white) on days 8, 22 and 36. (D–F) PCA assay. (D) The sera against OsrAAT (1, CK) were neutralized with OsrAAT (2), mgOsrAAT (3) and hAAT (4). (E) The sera against mgOsrAAT (1, CK) were neutralized with OsrAAT (2), mgOsrAAT (3) and hAAT (4). (F) The sera against hAAT (1, CK) were neutralized with OsrAAT (2), mgOsrAAT (3) and hAAT (4).

affinity chromatography were measured. The results indicated that no significant difference of the antibody concentrations was found between HRP and $PLA_2$ affinity chromatography in three treatments (Table 5). It was noticed that the antibody concentration of hAAT was no significant difference between HRP and $PLA_2$ compared with OsrAAT and mgOsrAAT because hAAT simultaneously contains both α-1,3-fucose ($4^+$%) and α-1,6-fucose ($6^+$%) (Kolarich et al., 2006a,b). It is well explained why hAAT has similar antibody concentration to OsrAAT and mgOsrAAT. As shown in Table 5, β-1,2-xylose contents in OsrAAT and mgOsrAAT are 94.1% and 77.9%, respectively, and no β-1,2-xylose in hAAT. However, the antibody contents have no significant difference, indicating that the antibodies in the serum is elicited by α-1,3-fucose rather than β-1,2-xylose. It confirms our hypothesis that the antibody in animal was mainly elicited by α-1,3-fucose.

## 4. Discussion and conclusion

Initially, synthesized proteins are translocated into the ER lumen. However, the final destinations of the proteins are different. The diversity of N-glycan populations suggested that the distinct subcellular localizations or alternative trafficking routes of proteins

**Table 5**
Concentrations of antibodies purified by HRP and $PLA_2$.

| Treatments | Tested animal No. | Conc. of Ab specifically bound to HRP (μg/ml) | | Conc. of Ab specifically bound to $PLA_2$ (μg/ml) | | Significance test (HRP versus $PLA_2$) | Total fucose contents | β-1,2-xylose |
|---|---|---|---|---|---|---|---|---|
| OsrAAT | 1 | 146.9 | | 136.8 | | | | |
| | 2 | 117.4 | | 107.2 | | | | |
| | 3 | 156.8 | 147.7 ± 21.3 | 152 | 137.6 ± 24.2 | $P = 0.25$ | 82.5% (α-1,3-fucose) | 94.1% |
| | 4 | 141.9 | | 122.8 | | | | |
| | 5 | 175.5 | | 169.2 | | | | |
| mgOsrAAT | 6 | 155.7 | | 143.9 | | | | |
| | 7 | 126.6 | | 128.7 | | | | |
| | 8 | 132.2 | 133.3 ± 16.8 | 107.2 | 126.3 ± 18.0 | $P = 0.27$ | 38.4% (α-1,6-fucose) 22.7% (α-1,3-fucose) | 77.9% |
| | 9 | 141.3 | | 143.2 | | | | |
| | 10 | 110.6 | | 108.3 | | | | |
| hAAT | 11 | 142.6 | | 122.8 | | | | |
| | 12 | 111.9 | | 99.9 | | | | |
| | 13 | 116.0 | 125.1 ± 17.8 | 109.5 | 114.6 ± 15.7 | $P = 0.45$ | $4^+$% (α-1,6-fucose) $6^+$% (α-1,3-fucose) (Kolarich et al., 2006a,b) | ND |
| | 14 | 109.0 | | 101.0 | | | | |
| | 15 | 146.1 | | 181.0 | | | | |
| Significance test (OsrAAT versus mgOsrAAT versus hAAT) | | $P = 0.41$ | | | | | | |

differ between leaf and seed cells. In leaf, there is only one type of vacuole, i.e., an acidic vacuole, whereas there are two major vacuoles in seed cells: protein-storage vacuoles (PSVs; protein body II [PBII]) and ER-derived vacuoles (protein body I [PBI]) (Vitale and Hinz, 2005). The two destinations in the leaf cell are either apoplast, cytosol or secretion. If the secretory pathway is chosen, then the protein might be secreted to apoplast or retained in the ER. In addition, the endomembrane system in seed cells is highly specialized. Two protein storage vacuoles derived from either the ER or the vacuole, traffic through the endomembrane system to the protein bodies (Müntz, 1998). However, proteins can also accumulate in ER bodies (Chavan and Lennarz, 2006) or small vesicles when the proteins remain in the early Golgi apparatus (Vitale and Hinz, 2005). The different protein sorting routes could sort to different vacuoles, as the results, it could produce different glycan structures. Furthermore, the organ functional diversity between seed and leaf can also lead to modification differences. For example, the acidic vacuole in the leaf primarily functions in the cleavage and secretion of proteins out of the cytosol, whereas the vacuoles in seed function as storage organ to provide energy for the germination of the next generation. In the present study, we presented the results of the glycan profiles of the glycoproteins in the leaf and seed of different cultivars, and found that the glycan content was similar between varieties, but there were dramatic differences in the glycosylation patterns between leaf and seed.

In our study, we identified large amounts of *N*-glycans MMX and MMXF in both the leaf and the seed consistence to previous study (Léonard et al., 2004). These structures are typically observed for vacuolar glycoproteins (Lerouge et al., 1998), suggesting that these proteins traffic to the PSV via the Golgi complex, in where terminal GlcNAc residues were removed. Another major glycan, GnGnXF, in the leaf was typically observed on secreted glycoproteins. Minor peaks corresponded to the trimmed glycans, some of which contained fucose on the core GlcNAc, representing degradation products of Golgi-modified complex glycans. Secreted glycoproteins containing terminal GlcNAc residues were added to the core fucose and xylose, and these terminal residues were subsequently trimmed off by enzymes either outside or within the vacuole (Lerouge et al., 1998). Our results showed that the glycoproteins were targeted and modified differently between the leaf and seed and potential immunogenic glycan structures, α-1,3-fucose or β-1,2-xylose in seed were much less than that in leaf.

Recently, rice seeds have been demonstrated to be an effective bioreactor for molecular pharming (Abe et al., 2014; He et al., 2011; Ou et al., 2014). We observed that the *N*-glycosylation of α-1,3-fucose in seed cells was as low as 10%, and *N*-glycosylation patterns of the proteins in endosperm cells were much simpler than that in leaf cells. Less α-1,3-fucose contents might make rice seed a much more favorable platform for the production of recombinant proteins. Recently, the scientists in Protalix found that the glycoproteins produced from the transgenic plant did not provoke more adverse reactions in human than those from mammalian system. Although all recombinant therapeutic proteins were expected to have some degree of allergenicity or immunogenicity, it was clear that it was not a property that could be attributed only to plant-derived proteins (Shaaltiel and Tekoah, 2016). In our case, we also found no significant difference on immunogenicity among three types of AATs. In fact, hAAT contains $6^+$% α-1,3-fucose (Kolarich et al., 2006a,b). Therefore, we think α-1,3-fucose could not be a plant-specific glycan. Due to hAAT has been applied for therapy of AAT deficiency disease, α-1,3-fucose is safe for humans.

In plants, the *N*-glycosylation of proteins is catalyzed through several steps in the ER and Golgi apparatus, including the sequential addition of different glycan residues, such as α-1,2-mannose, β-1,2-xylose and α-1,3-fucose, by different enzymes involved in *N*-glycan biosynthesis. Glucosidase I is found in the ER, whereas α-1,2-mannosidase I and N-acetylglucosaminyltransferase I (*GnTI*) are localized in both the ER and Golgi apparatus. The α-1,3-FucT and β-1,2-XylT enzymes are found exclusively in the Golgi stacks (Saint-Jore-Dupas et al., 2006). The processing of *N*-glycans in the Golgi apparatus displays major differences depending on cell type. Protein trafficking in the endomembrane system is largely affected through the subcellular locations of the proteins, leading to changes in protein glycosylation in different compartments. When OsrAAT is expressed in rice seeds, this protein is primarily detected in precursor accumulating (PAC) vesicle-like structures in the ER and vacuoles (Zhang et al., 2012), whereas glutelin is primarily detected in PBII (Tanaka et al., 1980). Trafficking of both OsrAAT and glutelin through the Golgi apparatus simultaneously shared the same glycosylation patterns of uniform glyco-structures. The α-1,3-fucose in OsrAAT were present in higher quantities than that in glutelin, suggesting that OsrAAT exhibited a high diversity of glycan structures. Our results indicated that OsrAAT glycosylation might exhibit more heterogeneity, it was consistence to the results of multiple subcellular localizations in seed cells (Zhang et al., 2012; Vamvaka et al., 2016). Owing to *N*-glycosylation patterns of the recombinant proteins are dependent on the traffic route and target destinations, therefore, *N*-glycosylation patterns of recombinant protein

are protein-dependent. It is necessary to characterize the heterogeneity of recombinant proteins when it is expressed either in leaf or in endosperm.

Fucosylation is one of the most common post-translational modifications. Fucosylated glycans are generated through fucosyltransferases and are involved in various biological processes (Becker and Lowe, 2003; Zhao et al., 2008). FUT8 catalyzes the transfer of fucose from GDP-fucose to the innermost N-linked N-acetylglucosamine (GlcNAc) of glycoproteins via an α-1,6-linkage in mammalian cells. After stable transformation of the transgenic line with FUT8, we developed a line 515-32/132-17 by crossing 132-17 with 515-32. The two main glycan types, MXF and MMXF, constituted most of the glycan of OsrAAT, whereas the glycan pattern of mgOsrAAT showed high heterogeneity. We found two difucosylated glyco-forms in mgOsrAAT, which did not detect in either glutelin or total proteins in 515-32 (Table 3). It might be because of the different pathways and modifications between native protein and recombinant AAT. For recombinant protein AAT, the total fucose was reduced from 82.5% in OsrAAT to 61.1% in mgOsrAAT, of which 38.4% harbored α-1,6-fucose, possibly because FUT8 competes with native α-1,3-FuT activity. Both acceptor glycan and donor substrate, as well as the interference with the endogenous glycosylation machinery may influence the final glycosylation profile (Loos and Steinkellner, 2014).

In the present study, we found that the α-1,3-fucose content of the glycoprotein produced from rice seed was much lower than that in leaf and higher β-1,2-xylose content in seed than that in leaf. We found that different glycosylation profiles between the native storage protein and mgOsrAAT in plants expressing *FUT8*. The α-1,3-fucose content of mgOsrAAT was significantly reduced compared with that of OsrAAT. The immunogenicity of OsrAAT, mgOsrAAT and hAAT is no obvious differences in the IgG, IgM and IgE titers in animal system. We further demonstrated that α-1,3-fucose was the major immunogenic resource from plant-specific *N*-glycans.

## Conflict of interest statement

The authors declare no conflict.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2016.12.017.

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