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REVIEW

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Abstract Plants provide a promising expression platform for producing recombinant proteins with several advantages in terms of high expression level, lower production cost, scalability, and safety and environment-friendly. Molecular pharming has been recognized as an emerging industry with strategic importance that could play an important role in economic development and healthcare in China. Here, this review represents the significant advances using transgenic rice endosperm as bioreactor to produce various therapeutic recombinant proteins in transgenic rice endosperm and large-scale production of OsrHSA, and discusses the challenges to develop molecular pharming as an emerging industry with strategic importance in China.

Keywords Molecular pharming · Transgenic rice endosperm · Plant-made pharmaceutical (PMP) · Manufacturing of PMP

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Introduction

Plants provide a promising expression platform for producing recombinant proteins since last two decades, which was called molecular pharming or molecular farming. The plants have high capacity to generate antibodies, vaccines, enzymes, and peptides, which have been tested in various plant species, including rice, tobacco, maize, soybean, potato, barley, carrot and safflower (Obembe et al. 2011). Up to now, there are at least 108 pharmaceutical proteins or peptides which have been successfully produced in plant cells (Table 1 and Supplementary Table 1); more than 30 plant-made pharmaceutical (PMP) products, including protein and peptide, have entered into clinical trial and nine went into markets (Supplementary Table 2). Although plants used as bioreactors for molecular pharming have advantages in terms of scalability, safety, and cost effectiveness (Paul and Ma 2011), they may be an alternative to conventional fermentation system using bacteria, yeast, and mammalian cells (Daniell et al. 2009; Sharma and Sharma 2009; Greenham and Altosaar 2013). However, the cost effectiveness is being a critical issue to encounter the development of molecular pharming (He et al. 2011). Three major issues, expression level, processing (including the isolation and the purification) and manufacturing, directly affected the marketing of PMP products. To solve the issues above, the extensive studies have been made (Greenham and Altosaar 2013).Those efforts included increasing expressing level via using stronger promoter (Yang et al. 2001, 2003, 2006, 2007; Greenham and Altosaar 2013; Horvath et al. 2000; Hwang et al. 2002; Qu and Takaiwa 2004; Takagi et al. 2005; Wu et al. 1998, 2007; Furtado et al. 2008), organelle targeting (Avesani et al. 2003; Benchabane et al. 2008; Boothe et al. 2010), improve protein trafficking environment (Tada et al. 2003)

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Table 1 The recombinant proteins expressed in plants bioreactors

Product categories	No. of PMP products
Pharmaceutical proteins	15
Vaccines	44
Antibodies	21
Cytokines	28

and improving translational efficiency (Greenham and Altosaar 2013), etc. Although the achievements have been made, marketing of PMP products, particularly for clinical application, was still limited. Recently, a PMP product, *Elelyso*, developed by Pfizer was approved by FDA of USA for clinical application in 2012 (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm302549.htm). It is surprising that the data from clinical trial showed some co-effects were less than that of the same product produced in Chinese Hamster Ovary (CHO) cell (Zimran et al. 2011). It was a milestone event for molecular pharming and strongly demonstrated that PMP products would be safer than the current expression systems. Recently, the molecular pharming in China has been recognized as an emerging industry with strategic importance that could play an important role in economic development and healthcare. Some scientists involved in the investigations of molecular pharming made very aggressive progress in molecular pharming using rice as bioreactor for large-scale production of recombinant proteins (Ning et al. 2008; Xie et al. 2008; He et al. 2011; Zhang et al. 2012; An et al. 2013).

Advantages of transgenic rice as bioreactor for molecular pharming

As discussed above, three major issues have encountered the marketing of PMP products from molecular pharming. These include lower expression level, downstream processing and manufacturing and scaling. To solve those problems, rice endosperm is the best choice in terms of some advantages over other plant species.

Rice seed has more biosafety than other species

When transgenic plant is used as bioreactor to produce large amount of pharmaceutical proteins, the public concerns of biosafety in field trial are merged. Therefore, choosing an appropriate host is a critical strategy to prevent from the escape from environment. Considering this issue, rice as a self-pollination crop is the best choice for molecular pharming. Rice is a highly self-pollinated crop. The lifetime of rice pollen grain is about 5 minutes when it is out of anther (Song et al. 2012). The biosafety assessment for transgenic rice has indicated that the maximum escape distance of rice pollen grains is less than 30 meters (Gealy et al. 2003; Song et al. 2003). Cross-pollination frequency of transgenic rice is less than 1 % (Messeguer et al. 2001; Chen et al. 2004; Rong et al. 2004). It is obvious that its biosafety is much more than that of cross-pollination crops. One strategy to express and accumulate the pharmaceutical proteins in the endosperm is driven by the endosperm-specific promoters (Ning et al. 2008), which reduced the risk of pharmaceutical proteins to the environment comparing with the bioreactors using plant leaf or root. In addition, the regulations of biosafety management of transgenic plants for industry and pharmaceutical uses in China are more strict. The regulation required the transgenic plant should be fencing with more than 1.8 m high with video monitor at the entire growth stage, which could prevent from the potential accident escape via humans and animal activities.

Rice genome sequence database benefits to downstream processing design

The bioinformation of rice genome sequencing database provides detailed information for downstream processing and molecular characterization of the transgenic rice. For instance, it provides whole genomic sequencing information that encodes all genes, including the storage protein genes, which are very useful to design cloning the promoters, downstream processing protocol and manufacturing based on those bioinformation. Moreover, the biochemical and physical characters of four main storage proteins have been extensively investigated. Those information could be used to easily design the protocol to remove the host cell proteins and characterize the residual host cell proteins from final PMP products. It is the essential information that is required by FDA for evaluating the safety of PMP product for therapeutic applications. Up to now, the processing protocols to isolate and purify the recombinant proteins from rice seed have successfully been developed using those information (Table 2). Furthermore, several other advantages of transgenic rice used as bioreactor, which includes that rice has been planted in Asia as a main crop for long history (Yang et al. 2008). Rice, unlike tobacco, is the main food of human beings without any harmful components, which is generally considered as generally recognized as safe (GRAS) by the Food and Drug Administration; the cultivation technology and processing machinery of rice is available, which make it easier to reach a large-scale production; transgenic rice seeds containing pharmaceutical proteins are able to store for years without significant protein activity losts

Table 2 Summary of pilot purification data of different pharmaceutical proteins in transgenic rice seeds

PMP Products	Molecular mass (kDa)	Electronic point (pI)	Expression level (mg^{-1}/g brown rice)	No. of chromatography	Recovery (%)	Purity (%)
OsrHSA	65	5.3	8.0	3	55.8	>99.45[a]
OsrAAT	52	5.2	2.2	3	18.89	>97[a]
OsrbFGF	17	9.6	0.18	1	4.49	>95[b]
OsrLF	80	8.7	7.7	1	11.8	>95[b]
HSA:hIGF-1	74	5.6	6.6	2	1.8	>95[b]
OsraFGF	13	6.2	0.01	NA	NA	NA
OsrGM-CSF	16	4.9	7.5	NA	NA	NA
BipC-hIGF-1	36	4.6	0.7	NA	NA	NA
OsrlZ	14	10.2	5.0	NA	NA	NA

[a] Purity of the product was determined by size-exclusion HPLC

[b] SDS-PAGE gel electrophoresis with Coomassie blue G250 staining

(Tackaberry et al. 2008; Takaiwa et al. 2007). These advantages make rice as one of the best bioreactors for industrial manufacturing of pharmaceutical proteins.

Rice endosperm has high capacity to highly express recombinant proteins

Rice belongs to monocot plant as a main food crop in the world. There are entire protein synthesis and modification machinery in rice cell. Targeting recombinant proteins to subcellular compartments in plants can increase accumulation levels by several-fold (Yang et al. 2008; Sharma and Sharma 2009; Boothe et al. 2010; Greenham and Altosaar 2013). In rice endosperm, four storage proteins are targeted into two protein storage vacuoles, called protein body I (PBI) and protein body II (PBII) (Yamagata et al. 1982; Krishnan et al. 1986; Ogawa et al. 1987). Glutelin and globulin are destined in PB II through ER via Golgi apparatus and prolamin stores in PBI via budding from the ER (Muntz 1998) (Fig. 1). The proteins are synthesized during endosperm development, then the proteins enter into the ER lumen via signal peptide, where the signal peptide is cleaved by the peptidase and then into the endomembrane system for further modifications and folding. Finally, the proteins are sorted into PBI or PBII following different mechanisms (Li et al. 2013). The protein synthesis and modification machinery in rice endosperm cell provide an important mechanism for a strategy to improve the expression level of recombinant protein. First, rice endosperm cell has the similar protein synthesis and modification machinery as animal or humans, where the recombinant protein synthesized could properly be folded; second, as soon as recombinant protein synthesized and folded, it is sorted into endomembrane system to further modifications and could be prevented from the proteinase attacking in cytosol; third, getting rice seed maturation, in general, will be taken 30–45 days after pollination. Recombinant proteins could continually be synthesized and accumulated in matured seeds with a very high level (Yang et al. 2001). Several human proteins were expressed in transgenic rice endosperm. The expression level was arranged from 0.18 to 8.0 % brown rice (Ning et al. 2008; Xie et al. 2008; He et al. 2011; Zhang et al. 2012; An et al. 2013) (Table 2). These data indicated transgenic rice endosperm, as a bioreactor, has a capacity to highly express various foreign proteins.

In addition, the strategies to improve the expression level are always to increase the transcription level via using high transcription activity promoter and various positive regulation elements (Torres et al. 1999; Yang et al. 2001, 2003, 2006, 2007; Stoger et al. 2002; Greenham and Altosaar 2013; Horvath et al. 2000; Hwang et al. 2002; Qu and Takaiwa 2004; Takagi et al. 2005; Wu et al. 1998, 2007; Furtado et al. 2008). However, the protein trafficking into protein storage vacuoles and the ER stress become the limited factors when recombinant proteins are highly expressed in plant cell, which could affect recombinant proteins accumulation with a high level (Luo et al. 2009; Zhang et al. 2013). Several attempts to improve the expression level via the approaches of alleviating the ER stress and help folding have been made and achieved success (Oono et al. 2010; Yasuda et al. 2009; Shigemitsu et al. 2012; Yang et al. 2012; Kim et al. 2012). These results suggested that the expression level of recombinant proteins in grain is regulated not only on transcriptional, but also on post-translational level.

Rice endosperm has capacity expressing complicated and functional proteins

For molecular pharming, recombinant proteins should be bio-functional and have proper tertiary structure. The current data indicated that rice endosperm is capable of expressing the recombinant proteins with the molecular mass range from 13 to 65 kDa. As shown in Table 2, those recombinant proteins are enzymes, plasma protein, small peptide, growth factors and milk proteins. These proteins

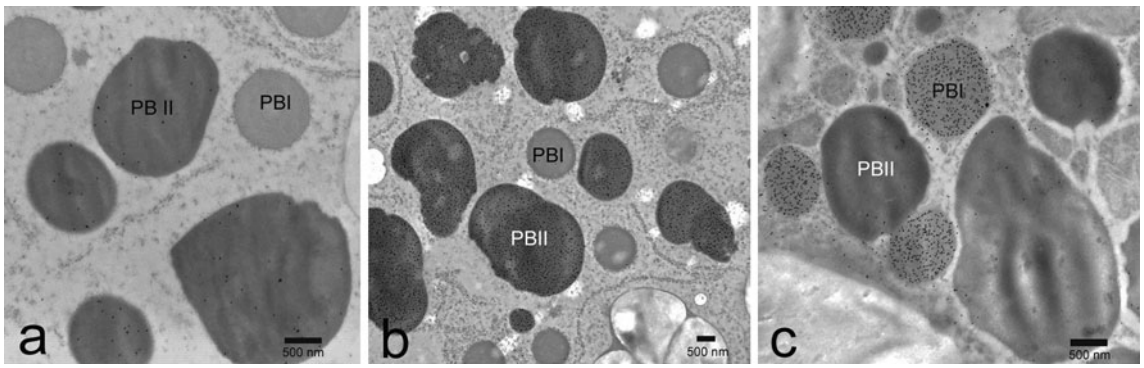

Fig. 1 Subcellular localization of storage proteins in rice endosperm cell. Salt-soluble globulin located in PBII (**a**); basic/acidic soluble glutelin in PBII (**b**) and ethanol-soluble prolamin in PBI (**c**)

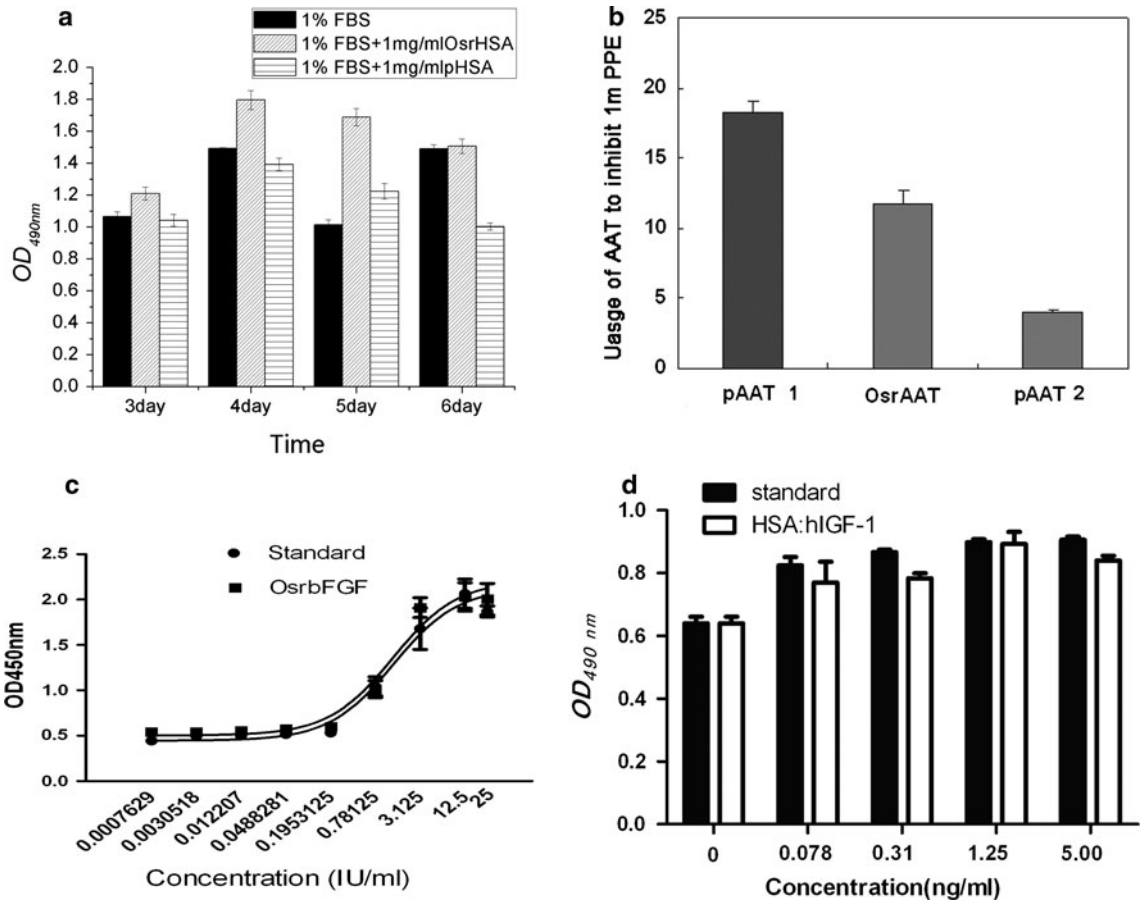

Fig. 2 Bioactivities of the pharmaceutical proteins expressed in the transgenic rice seeds. Comparison of OsrHSA and pHSA on CHO cell culture with 1 % FBS (**a**), comparison between OsrAAT and two commercial products from human plasma (**b**), the OsrbFGF stimulating 3T3 cell proliferation (**c**), effects of the HSA:hIGF-1 on stimulating 3T3 cell proliferation (**d**)

expressed in rice endosperm cell are not only having high expression level, but also having bioactivities (Fig. 2). Furthermore, rice endosperm can express very complicated structure proteins. For instance, human serum albumin (HSA) is structurally complicated protein in human plasma. Its molecular mass is about 65 kDa with 17 disulfide bonds. The expression level reached more than 10 % total soluble protein or 0.6 % dry weight of brown rice. The data indicated that OsrHSA has not only proper tertiary structure and 17 disulfide bonds, but also the same functions as plasma HSA in vitro and in vivo (He et al. 2011). For another example, human alpha antitrypsin

(AAT) is a protein binding onto the trypsin to inhibit its proteinase activity. It is very difficult to express in other bioreactors (Bollen et al. 1983; Karnaukhova et al. 2006). Human AAT has successfully been expressed in rice endosperm cell. The results indicated that OsrAAT has proper structure and high bioactivity compared to plasma AAT (Zhang et al. 2012). Currently, other recombinant proteins expressed in rice endosperm all showed the functional and proper structures, suggesting that rice endosperm cell has capacity to folding and processing foreign proteins with proper structure and the functions.

Manufacturing of recombinant protein from rice endosperm

The cost-effective and high recovery of the recombinant protein production depending on the expressed protein could be used in commercial application. For example, the expression level for the industrial production of rHSA needed to exceed the estimated cost-efficacy threshold (0.1 g/kg) (Fernandez-San Millan et al. 2007; He et al. 2011). There are many challenges to establish a simple procedure for isolation and purification of recombinant proteins from the expression host. These include host selection, optimizing the isolation and purification protocols. Therefore, in general, selecting an expression host or organ becomes very important. As mentioned above, most of the host proteins in rice endosperm are the storage proteins of which biochemical and physical features have been characterized and documented. Expression of recombinant proteins in rice endosperm cell is a good choice for downstream processing and manufacturing.

Rice storage proteins are easily removed during processing

More than 90 % of the protein contents in rice seed come from storage proteins. Four types of main storage proteins are in rice endosperm cells, including basic/acidic soluble glutelin, salt-soluble globulin, water-soluble albumin and ethanol-soluble prolamin. Of them, except for albumin, other three storage proteins have distinguished biochemical features. It provides convenience to separate via changes of buffer system, pH value and salt concentration and different resins for chromatography. For example, basic subunit of glutelin is soluble in basic solution, and acidic subunit of glutelin is soluble in acidic solution. The other major storage protein is ethanol-soluble prolamin. Those proteins could not be well soluble in PB buffer or Tris buffer systems. Optimizing pH, salt types or/and concentration, different buffer systems or the combinations can be used to selectively isolate the target proteins based on the difference among those storage proteins from rice grain extract. As shown in Fig. 3, several recombinant proteins are successfully isolated and purified from rice endosperm based on the principle mentioned above. Those data demonstrated that rice endosperm is an ideal bioreactor to produce the recombinant proteins for molecular pharming.

Purification of recombinant protein from rice endosperm

The downstream processing of recombinant protein is time-and capital-consuming due to the difficulty to separate the recombinant proteins from host cell proteins. It largely depends on the expression host or organs. Purification cost was estimated to be approximately 80 % of total cost (Kusnadi et al. 1997). As much, less purification steps would reduce the manufacturing cost and simultaneously increase the recovery of the protein. Therefore, developing a cost-effective processing protocol becomes very important for molecular pharming.

In general, the downstream processing of recombinant proteins from rice seeds could be a very good system to meet those requirements. The processing can be divided into two parts, pretreatment and chromatography. Pretreatment includes grinding, extraction and clarification. For extraction of recombinant proteins from rice powder, the strategy is to maximally increase extraction of the target protein and to minimally decrease the host cell protein extraction. So, the extraction buffer, extraction temperature, pH, salt concentration/types, ion strength, chelating agent, and reductant are generally considered based on the host cell proteins and interest of proteins when extracting and stabilizing the host cell protein or target proteins. Due to the well-documented storage proteins in rice grain, for example, the combination of high temperature, lower pH and NaCl concentration in extraction solution was optimized based on the HSA biochemical features and contractly, storage proteins could be denatured at high temperature and precipitated at lower pH, which maximally extracted recombinant HSA and maximally removed the host storage proteins from the rice grain extracts (Fig. 4). For the other example, these documented information were used to optimize the protocol to increase OsrbFGF (recombinant fibroplast growth factor from *Oryza sativa)* solubility and develop one step purification to obtain high purity and bioactivity OsrbFGF and OsrLF (recombinant lactoferrin from *Oryza sativa*) from rice seeds (An et al. 2013) (Fig. 3). The results from various recombinant proteins expressed demonstrated that rice seeds as bioreactor for molecular pharming have great advantages on downstream processing of the recombinant protein over other plant and yeast systems (Ning et al. 2008; Xie et al. 2008; He et al. 2011; Zhang et al. 2012; An et al. 2013).

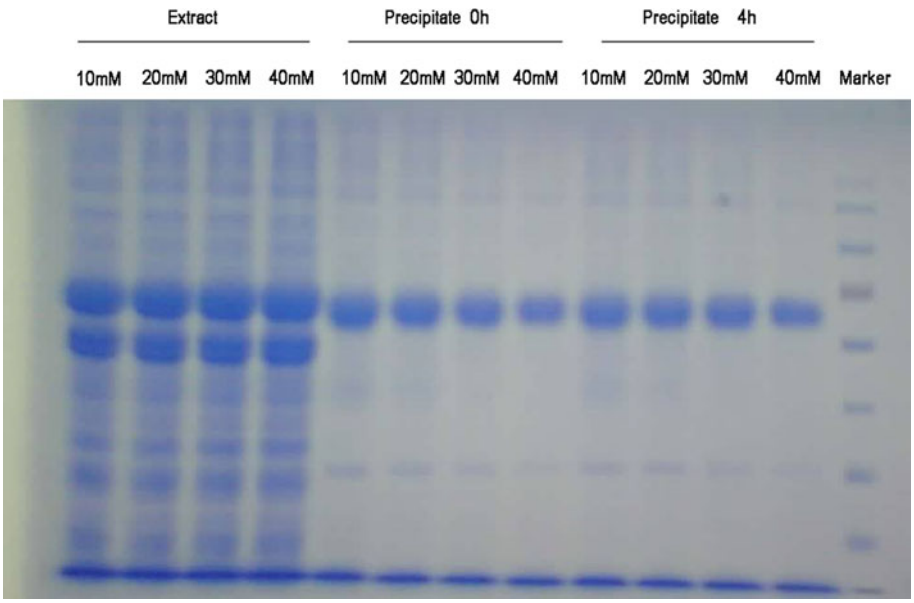

Fig. 3 Optimized extraction conditions of OsrHSA from the transgenic rice seeds. OsrHSA was extracted in 65 °C with 10–40 mM sodium-caprylate for 1 h and precipitated at pH 4.5 for 0–4 h

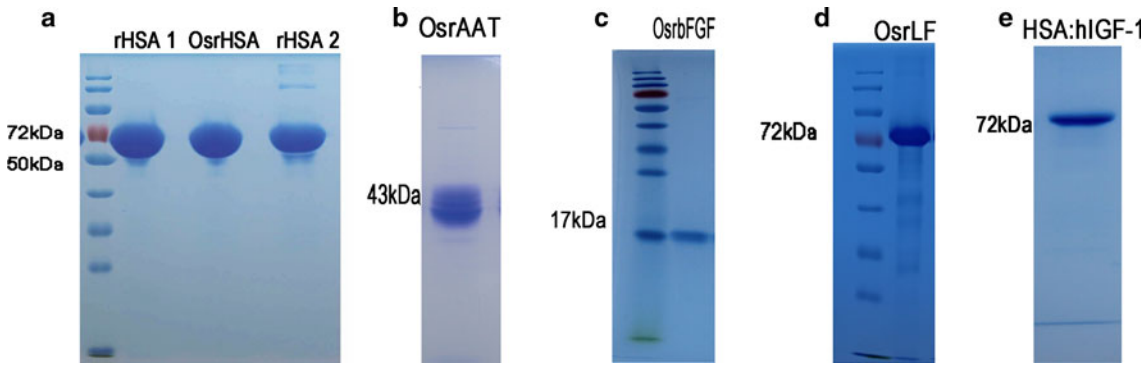

Fig. 4 Purified PMP products produced from the transgenic rice seeds. The SDS-PAGE images of OsrHSA and two commercial rHSA from yeast (**a**), OsrAAT (**b**), OsrbFGF (**c**), OsrLF (**d**) and HSA:IGF-1 (**e**)

Challenges of rice endosperm as bioreactor for molecular pharming

Though the expression platform and standard procedures for large-scale production of recombinant proteins were well established from rice endosperm cell, several challenges were met for developing PMP products, including releasing the transgenic rice into field trial, the manufacturing of recombinant protein from rice endosperm cell and absence of regulation for assessment of PMPs for clinical applications. Although molecular pharming has been recognized by Chinese government as an emerging industry with strategic importance that could play an important role in economic development and healthcare, those issues should be solved shortly.

Regulations require releasing transgenic rice and assessment of pharmaceutical use in China

With regard to the concerns of biosafety for releasing transgenic rice for pharmaceutical and industrial use, Chinese government have developed extensive experience and expertise in the risk assessment and management of the transgenic plant for pharmaceutical and industrial uses. Although transgenic rice showed a very low frequency (0.04–0.80 %) of pollen-mediated gene flow between genetically modified (GM) rice and adjacent non-GM plants (Messeguer et al. 2001; Rong et al. 2005), the producers should follow the regulation and should satisfy the requirements for releasing the transgenic rice into the field trial. To prevent from this kind of transgenic rice escape and potential possibility entering food and feeding chains, Chinese government requests the producer to take the very strict measures to prevent any possible escape of the transgenic rice for pharmaceutical and industrial uses into food and feeding chains. Those measures are: an isolation zone should be more than 100 m apart from non-transgenic rice, a buffer zone around the field (>1.5 m), fencing with a video monitoring in the field trial at whole rice growth stages and established standard operation protocols for sowing, planting, harvesting, drying, transporting,

Fig. 5 Restrict measures to prevent from the potential accident escape via humans and animal activities. **a**, **b** The fencing of transgenic rice in field trial; **c** video monitoring whole growth stages of transgenic rice and **d** transgenic rice in field trial

processing and storage etc. (Fig. 5). These approaches will largely diminish the environmental impacts. Furthermore, there are no specific regulations to guide the evaluation of molecular pharming, manufacturing or products for clinical application. It helps to develop the regulations and establish the assessment protocol of PMP products in clinical applications as the strategic importance of an emerging industry in China.

Manufacturing of recombinant proteins from rice endosperm

As a novel platform as the strategic importance of an emerging industry in China, it becomes critical to establish the large-scale manufacturing of PMPs. Although the processing protocol of the recombinant proteins was developed at laboratory and pilot scales, manufacturing of the recombinant proteins at kilograms level/lot is still challenging. Those include automatical facilities for extraction, filtration, processing, and establishing good manufacturing practice (GMP) management for downstream processing of PMPs, which were able to meet the requirement by SFDA. To practice and establish GMP of PMP products, such as OsrHSA, the guidelines of drug assessment should be followed and a GMP-compliant bioprocess in production of OsrHSA from rice seed and a good agricultural practices (GAP) for seed production have been established. Quality assurance (QA) and quality control (QC) of OsrHSA have been established. The standard operation protocols for the transgenic rice growth, harvesting, protein extraction, purifications and formulation have been established. Large-scale manufacturing of OsrHSA (>1.2 kg/lot) has been established. Recently, a manufacturing facilities of OsrHSA meet GMP requirment with yields of 8 kgs/lot has being constructed (Fig. 6). The large-scale manufacturing of OsrHSA from the transgenic rice seed will be a milestone event for molecular pharming and becomes a strategic importance of an emerging industry in China, which could play an important role in economic development and healthcare in future.

Challenges of plant type of glycosylation in transgenic rice grain

Up to now, about 30 % pharmaceutical protein drugs are glycoproteins. The potential immunogenicity and allergenicity from plant-specific glycans becomes a concern although no evidence has showed harmful to humans.

Fig. 6 Pilot facility and large scale manufacturing of OsrHSA from transgenic rice grain. **a**, **b** The processing of transgenic rice and purification of OsrHSA as pilot scale; **c** purification facility for manufacturing of OsrHSA

Higher plant cell has the capacity for glycosylation modification. A kind of plant-specific N-glycosylation patterns is different from humans, but the main sugar backbone is highly conserved from plant to human beings (Rayon et al. 1998). In general, the plant-specific glycosylation are β-1,2-xylose, core α-1,3-fucose and the Lewis a epitope (van Ree et al. 2000), while mammalian are α-1,6-fucose, β-1,4-galactose, sialic acids, and lack of β-1,2-xylose (Andersen et al. 2011). Therefore, the potential antigenicity of plant-specific glycosylation residues is a major concern for plant-derived therapeutic recombinant glycoproteins (Bardor et al. 2003; Ma et al. 2003). 32.8 % of OsrAAT expressed in the transgenic rice seed were glycosylated and 64.8 % of the N-glycan structures were vacuole-specific paucimannosidic molecules (Zhang et al. 2012). To solve the problems, many attempts have been taken to reduce the plant-specific glycans using various approaches, including keeping the glycoproteins in the endoplasmic reticulum (ER) using endoplasmic reticulum targeting signal KDEL (Napier et al. 1992), overexpression or inhibition of Golgi glycosyltransferase by knockout mutants and RNA interference (RNAi) technologies (Schähs et al. 2007). Recent results indicated that α-1,3-fucose gylcan type is about 7.9 % in rice grains, which is much lower than that of 47.1 % in rice leaf. Although the immunogenicity and allergenicity concerns of plant-specific glycans would be concerns for therapeutic recombinant glycoproteins, no direct evidence of plant-specific glycan on glycoprotein showed immunogenicity or allergenicity. The glycosylation of plant-derived antibodies seems to not interfere with successful passive antibody therapy (Ma et al. 1998; Zeitlin et al. 1998). Therefore, more scientific evidence is needed to explore the immunogenicity of plant-specific glycan to humans.

Perspectives

Transgenic rice seeds as bioreactor for molecular pharming show great promise system for the production and processing of recombinant pharmaceutical protein with large scale. Many advantages over other plant host or animal bioreactors are obvious. Those include (a) capacity to obtain high expression level from 0.18 to 8.0 % brown rice, which economically reach marketing requirement; (b) production cost is as 10- to 1,000-folds lower than that of conventional fermentation or animal cell culture that was replaced by field trial for transgenic seed production; (c) transgenic rice has high capacity to reproduction of seed with >1,000-folds. As soon as a homozygous transgenic plant is obtained, it could scale up to 40–45 tons transgenic rice seed within two generations, which could at least produce 10–50 kg recombinant proteins; (d) it is environment-friendly due to replacement of high energy consuming fermentation or animal cell culture. The recombinant protein synthesis in the transgenic rice used solar energy. For example, the energy consuming of 1 g of OsrHSA is about 1/28 over yeast fermentation; (e) it is no contamination of any prions or pathogens for humans. Therefore, based on the advantages and the practices using transgenic rice as bioreactor for molecular pharming, it will develop a strategic importance of an emerging industry in future when the assessment regulation of PMP product is established in China.

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