Regulatory Toxicology and Pharmacology 76 (2016) 128–136

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Differential analyses of major allergen proteins in wild-type rice and rice producing a fragment of anti-rotavirus antibody

Yoshikazu Yuki [a, b, *], Shiho Kurokawa [a], Hiroko Kozuka-Hata [c], Daisuke Tokuhara [d], Mio Mejima [a], Masaharu Kuroda [e], Masaaki Oyama [c], Tomoko Nishimaki-Mogami [f], Reiko Teshima [g], Hiroshi Kiyono [a, b]

[a] Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, 108-8639, Tokyo, Japan
[b] International Research and Development Center for Mucosal Vaccine, The Institute of Medical Science, The University of Tokyo, 108-8639, Tokyo, Japan
[c] Medical Proteomics Laboratory, The Institute of Medical Science, The University of Tokyo, 108-8639, Tokyo, Japan
[d] Department of Pediatrics, Osaka City University Graduate School of Medicine, 545-8585, Osaka, Japan
[e] Crop Development Division, NARO Agriculture Research Center, 943-0193, Niigata, Japan
[f] Division of Biochemistry, National Institute of Health Sciences, 158-8501, Tokyo, Japan
[g] Division of Foods, National Institute of Health Sciences, 158-8501, Tokyo, Japan

ARTICLE INFO

Article history:
Received 4 September 2015
Received in revised form
28 January 2016
Accepted 29 January 2016
Available online 3 February 2016

Keywords:
Immunoelectron microscopy
Rice allergens
Rice-based antibody
Shotgun MS
2D-DIGE

ABSTRACT

To develop oral antibody therapy against rotavirus infection, we previously produced a recombinant fragment of llama heavy-chain antibody to rotavirus (ARP1) in rice seeds (MucoRice-ARP1). We intend to use a purification-free rice powder for clinical application but needed to check whether MucoRice-ARP1 had increased levels of known allergen proteins. For this purpose, we used two-dimensional fluorescence difference gel electrophoresis to compare the allergen protein levels in MucoRice-ARP1 and wild-type rice. We detected no notable differences, except in the levels of α-amylase/trypsin inhibitor–like family proteins. Because by this approach we could not completely separate ARP1 from the proteins of this family, we confirmed the absence of changes in the levels of these allergens by using shotgun mass spectrometry as well as immunoblot. By using immunoelectron microscopy, we also showed that RAG2, a member of the α-amylase/trypsin inhibitor–like protein family, was relocated from protein bodies II to the plasma membrane or cell wall in MucoRice-ARP1 seed. The relocation did not affect the level of RAG2. We demonstrated that most of the known rice allergens were not considerably upregulated by the genetic modification in MucoRice-ARP1. Our data suggest that MucoRice-ARP1 is a potentially safe oral antibody for clinical application.

1. Introduction

Rotavirus infection is a global disease that causes severe diarrhea in children (Parashar et al., 2003). Two oral rotavirus vaccines, the pentavalent vaccine RotaTeq and the monovalent vaccine Rotarix, are currently available (Glass and Parashar, 2006). These vaccines have been licensed in more than 100 countries; however, the first dose is recommended to be administered in a narrow time window (between the ages of 6 and 14 weeks) to avoid the risk of intussusception (Glass et al., 2014), and these vaccines are not recommended for infants with severe immunodeficiency (Lee et al., 2013). In addition, clinical trials have indicated that the efficacies of both vaccines are substantially lower in developing than in developed countries (Armah et al., 2010; Zaman et al., 2010). It may therefore be necessary to create alternative treatments.

We have previously developed oral passive immunotherapy

Abbreviations: CTB, cholera toxin B-subunit; DTT, dithiothreitol; GM, genetically modified; IEF, isoelectric focusing; MALDI-TOF, matrix-assisted laser desorption/ionization–time of flight; PB-I, protein body I; PB-II, protein body II; PBS, phosphate-buffered saline; pI, isoelectric point; PSM, peptide spectrum match; RNAi, RNA interference; RA, rice seed allergenic protein; RAG2, rice seed allergenic protein RAG2; T-DNA, transfer DNA; MS/MS, tandem mass spectrometry; 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; WT, wild type.

* Corresponding author. Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, 108-8639, Tokyo, Japan.
E-mail address: yukiy@ims.u-tokyo.ac.jp (Y. Yuki).

http://dx.doi.org/10.1016/j.yrtph.2016.01.023
0273-2300/

against rotavirus by using a rice-based fragment of llama heavy-chain antibody to rotavirus (ARP1) (Tokuhara et al., 2013). The variable domain of llama heavy-chain antibodies consists of the smallest single antigen-binding domain and has unique characteristics, including resistance to pepsin, acid environments, and heat (Hamers-Casterman et al., 1993; van der Linden et al., 1999; Dolk et al., 2005). In a phase II clinical trial conducted in Bangladesh, oral ARP1 produced in yeast was demonstrated to be safe and effectively reduced the severity of rotavirus-induced diarrhea in children (Sarker et al., 2013). By using an overexpression system together with RNAi technology to suppress the production of the major endogenous storage proteins prolamin and glutelin (Yuki et al., 2013), we have developed a water-soluble ARP1 named MucoRice-ARP1 that is produced at a very high level in rice seed. We have demonstrated that oral administration of a phosphate-buffered saline (PBS) extract of MucoRice-ARP1 seed protects cells against different serotypes of rotavirus in vitro and reduces virus shedding and disease in both immunocompetent and immunodeficient mice (Tokuhara et al., 2013).

Five allergen families have been identified in the salt-soluble protein fraction extracted from rice seed, namely the α-amylase/trypsin inhibitor–like protein family (14–16 kDa), glyoxalase I (33 kDa), and 19-kDa, 52-kDa, and 63-kDa globulins (Adachi et al., 1993; Nakamura and Matsuda, 1996; Usui et al., 2001; Satoh et al., 2011). Because in our clinical study we intend to use a PBS suspension of a polished rice-powder preparation of MucoRice-ARP1 without purification, we needed to check whether MucoRice-ARP1 had unexpectedly increased levels of known allergen proteins (Goodman et al., 2008). Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) is one approach to estimating protein levels in transgenic and non-transgenic plants (Nakamura et al., 2010, 2014; Teshima et al., 2010, 2012). In an earlier study, we used 2D-DIGE to compare allergen protein levels in the salt-soluble fractions of wild-type (WT) rice and purification-free oral rice-based cholera vaccine (MucoRice-CTB), which expressed cholera toxin B subunit (CTB) in a CTB overexpression system together with RNAi to suppress the expression of major endogenous storage proteins. In MucoRice-CTB, not only was the level of RAG2 (a member of the α-amylase/trypsin inhibitor–like protein family) decreased, but also its location was changed (Kurokawa et al., 2013, 2014). We have also analyzed the same salt-soluble fraction samples from MucoRice-CTB and WT rice by using label-free shotgun mass spectrometry (MS); the results were consistent with those of 2D-DIGE reported by us (Kurokawa et al., 2013) and other groups (Liu et al., 2004; Mueller et al., 2008).

Because the molecular weights of proteins of the α-amylase/trypsin inhibitor–like family (14–16 kDa) are almost the same as that of ARP1 (Adachi et al., 1993; Tokuhara et al., 2013), here we performed differential analyses of major allergen proteins in WT rice and MucoRice-ARP1 by using label-free shotgun MS and 2D-DIGE. We showed that the levels of rice allergens, including those of the α-amylase/trypsin inhibitor–like protein family, did not differ considerably between MucoRice-ARP1 and WT seed. By using immunoelectron microscopy, we also showed that RAG2 was relocated from protein bodies II (PB-II) to the plasma membrane or cell wall in MucoRice-ARP1 seed but not in WT seed; therefore, relocation of RAG2 in MucoRice-ARP1 did not affect its production level.

2. Materials and methods

2.1. Production of an antibody fragment against rotavirus in rice (MucoRice-ARP1)

A sequence encoding ARP1 with optimized codon usage for rice (Tokuhara et al., 2013) was synthesized and inserted into the binary T-DNA vector pZ2028 with an overexpression cassette for ARP1 and a combination cassette for RNAi-mediated suppression of the major rice endogenous storage proteins 13-kDa prolamin and glutelin A, as described previously (Tokuhara et al., 2013). The RNAi trigger sequences were a 45-bp fragment of the rice 13-kDa prolamin gene (coding sequence positions 1–45) and a 129-bp fragment of the rice glutelin A gene (coding sequence positions 142–270) (Kuroda et al., 2010). The expression vectors were used to transform the rice (*Oryza sativa* ssp. *japonica*) cultivar Nipponbare by using an *Agrobacterium*-mediated method described previously (Nochi et al., 2007). The MucoRice-ARP1 line with the highest level of ARP1 in the seed was selected and advanced to the T6 generation by self-crossing to obtain homozygous lines. Plants were grown in soil in a growth chamber (27 °C, 12 h day/22 °C, 12 h night). The average ARP1 yield (170 μg per seed, 11.9% of the total seed protein, 0.85% of seed weight) was determined by densitometry (Tokuhara et al., 2013).

2.2. Extraction of rice salt-soluble proteins

Mature brown seeds of non-transgenic rice (Nipponbare, WT) and transgenic rice lines (MucoRice-ARP1) of the sixth generation onward were harvested. Each grain was pulverized separately with a Multi Beads Shocker (Yasui Kikai Corp., Osaka, Japan). Because many rice allergens have been found in the salt-soluble fraction (1 M NaCl extract) of rice seed (Nakamura and Matsuda, 1996), we extracted salt-soluble proteins from 0.2 g of the rice fine powder with 3 mL of 1 M NaCl for 3 h at 4 °C on a rotator (TAITEC Corp., Osaka, Japan). The extracts were centrifuged at 20,400 × *g* for 10 min at 4 °C, and the supernatants were then filtered through a 0.45-μm syringe filter and stored in aliquots at −80 °C until use. Concentrations of the extracted proteins were measured with a 2-D Quant Kit (GE Healthcare Biosciences, WI, USA). Extracted proteins were concentrated by using a 2-D Clean-Up Kit (GE Healthcare Biosciences) according to the manufacturer's protocol and then used as salt-soluble proteins for allergen analyses by 2D-DIGE, 2D-immunoblotting, and shotgun MS.

2.3. 2D-DIGE analysis of WT rice and MucoRice-ARP1

2D-DIGE analysis was carried out as described previously (Nakamura et al., 2010, 2014; Teshima et al., 2010, 2012). A mixture of equal amounts of salt-soluble proteins (25 μg) from WT rice and MucoRice-ARP1 (an internal standard) was labeled with Cy2 fluorescent dye, whereas the protein samples were labeled with Cy3 and Cy5 fluorescent dyes according to the manufacturer's protocol (GE Healthcare Biosciences). The Cy-labeled proteins (25 μg per sample) were mixed together and loaded onto an Immobiline DryStrip (pH 3–10 non-linear, 13 cm; GE Healthcare Biosciences), where they were rehydrated overnight at 20 °C. First-dimension isoelectric focusing (IEF) was conducted at 20 °C with an Ettan IPGphor (GE Healthcare Biosciences) at 500 V for 4 h, 1000 V for 1 h, and 8000 V for 4 h. The strips were incubated for 15 min in equilibration buffer (100 mM Tris–HCl [pH 8.0], 6 M urea, 30% [v/v] glycerol, 2% [w/v] SDS, and a small amount of bromophenol blue) containing 0.5% (w/v) dithiothreitol (DTT) and then for 15 min in the same buffer containing 4.5% (w/v) iodoacetamide. Second-dimension SDS polyacrylamide gel electrophoresis (PAGE) was performed at 220 V for 3 h through 10%–20% gradient acrylamide gels (14 × 14 cm; DRC Co., Ltd., Tokyo, Japan). Each gel was scanned at three different wavelengths with a Typhoon 9400 variable image analyzer (GE Healthcare Biosciences) to generate Cy2, Cy3, and Cy5 images. The spots were matched by using DeCyder software version 7 (GE Healthcare Biosciences). Because we had previously