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Preparation of Monoclonal Antibody Against HPT and Its Application to Detecting Marker Protein in Genetically Modified Rice[1]

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Objective To produce the monoclonal antibodies (mAbs) against hygromycin B phosphotransferase (HPT) and to develop immunoassay based on mAbs for biosafety assessment of HPT in genetically modified rice (GM rice). **Methods** BALB/c mice were immunized with purified recombinant 6His. HPT protein, and the conventional hybridoma technology was used to generate the monoclonal hybridoma cells. ELISA and Western blot were used to analyze the specificity of mAbs recognizing HPT and the cross reaction with other proteins. A double-Ab sandwich ELISA method was established to detect HPT expression level in the *sck* gene-modified rice plants. **Results** Four hybridomas, named F1, D4-2, D4-4, and D4-5, producing the mAbs against HPT were successfully obtained with the titer of ascetic mAbs ranging from 1×10^{-4} to 1×10^{-5}. Identification of subclass showed that all the produced mAbs belonged to IgG1. Western blot showed specific binding reaction between the mAbs to the HPT proteins expressed in the GM rice. A double sandwich ELISA coated with anti-HPT polyclonal antibody was established with mAbs as sandwich antibody, which showed a sensitivity of 30ng/mL and did not crossreact with other proteins. The expression level of HPT in the leaves of *sck*-transformed lines was detected (80-150ng/mL). But HPT protein in the grain and seed of GM rice could not be detected using this ELISA assay. **Conclusion** Anti-HPT mAbs prepared herein have a high specificity and can be used for rapid assay of HPT antigen. The expression level of HPT in the GM rice grain and seed is lower than our ELISA detection limit.

Key words: HPT; Monoclonal antibody; Preparation; Application

INTRODUCTION

The enzyme hygromycin B phosphotransferase (HPT) is a selectable marker used widely in various animal and plant transformation systems. It has proven that *hpt* is a highly effective marker gene for rice transformation in comparison with the traditional kanamycin antibiotic gene (*npt*II)[1]. On the other hand, cowpea trypsin inhibitor from cowpea seeds[2], a member of the serine protease inhibitor family, is resistant to a wide range of insects. Constitutive expression of the *cpti* gene in genetically modified rice (GM rice) plants confers resistance to two major rice insect pests[3].

As we know, rice is one of the most important crops in the world. In China, the rice planting area is vast (30 million hectare) and accounts for nearly one fourth of the agricultural fields[4]. The loss of rice yield due to direct insect pest is estimated to be at least several billion US dollars[5]. It is also known that several devastating diseases in rice, such as rice tungro disease and yellow dwarf disease, are caused by viruses and transmitted by insects. Fortunately, modern biotechnology makes it possible to provide resistance to crop pests to improve production and reduce chemical pesticide usage, thereby making major improvements in both food quality and nutrition. Scientists in the Institute of Genetics, Chinese Academy of Sciences, have developed a highly insect-resistant GM rice plant, which contains both *hpt* marker gene and modified *cpti* gene (named *sck*)[6]. Bioassay showed that *sck*-modified plants have enhanced insect resistance ability to Lepidoptera pest due to the increased accumulation level of foreign CpTI protein[7]. Up to now, several types of *sck*-modified rice line, including the excellent

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restorers Minghui81 and Minghui86 have been obtained, and the hybridized combination has been made and released for 5 years with the approval of the Ministry of Agriculture. Performance trial has shown that this GM rice has excellent insecticidal attribute and retains their eminent agricultural traits[7]. In this study, *sck*-modified rice plants were used as the experimental sample.

Because the genetic modification ultimately results in the introduction of new proteins, including the marker gene protein, into the food plant, in order to ensure the wholesomeness and safety of the food supply, the safety of the introduced proteins should be subjected to a careful and complete assessment before commercialization. However, there are no reports regarding the biosafety of the HPT protein and no appropriate and well-established method is available for detecting this protein in foods. Furthermore, because of the extremely low level of the foreign protein (only comprised of 0.0001%-0.01% of total soluble plant protein), the HPT content in GM rice can be detected only by immunoassay, especially in case of a large number of samples and different tissues. To detect the HPT content in genetically modified crops, we prepared the HPT monoclonal antibody in this study and developed some specific and sensitive methods.

MATERIALS AND METHODS

Chemicals

Freund's complete and incomplete adjuvants were purchased from Sigma. HRP (RZ⩾3.0) was obtained from Biozyme. Iso Strip Mouse Monoclonal Antibody Isotyping Kit was from Roche. HRP labelled goat-anti-mouse IgG was bought from Zhongshan Biotechnology Ltd.

Animals

Female BALB/c mice, five to six weeks old, were purchased from Institute of Laboratory Animals, Academy of Military Medical Sciences.

Antigen Development and Purification

The purified recombinant 6His-HPT with a relative Mr of 37000 kD with a purity of 95% was prepared in this laboratory and stored at -20℃. It was used to immunize rabbits to generate high titer polyclonal antibody against HPT[8].

Procedure of BALB/c Mouse Immunization

After being diluted to the concentration of 0.1 mg/mL using phosphate-buffered saline, the antigen was mixed with Freund's complete adjuvants (v/v=1:1). This mixture, at dose of 30 μg per mouse, was injected to subcutaneous lymph nodes in legs of BALB/c mice (first immunization). Two booster immunizations were performed using the mixture of antigen protein and Freund's incomplete adjuvants at 30-day intervals. Two weeks later, a test of blood was carried out[9]. Blood was collected from the mouse eye sockets and centrifuged at 1000 r/min for 15 min. Sera were tested for anti-HPT antibody titer using indirect ELISA. The mice with the highest antibody titer received a final booster (via caudal vein and intraperitoneal inoculation) of protein (50 μg antigen/mouse) without Freund's adjuvants three days before cell fusion.

Cell Fusion and Screening of Hybridomas

The immunized mice were killed and splenocytes were collected to fuse with myeloma cells. Polyethylene glycol (PEG) was used as fusing agent, and hybridoma cells were screened on HAT medium according to standard method[10]. Positive myeloma-lymphocytes were screened using indirect ELISA and cloned by limited dilution four times continuously to obtain the steady hybridomas-producing antibodies against HPT.

Production and Purification of Ascitic mAbs Against HPT[11]

Old BLAB/c mice were inoculated with 5×10^5 hybrid cells (i.p.) to induce ascites. After 7-10 days, ascites were removed and centrifuged for 10 min at 3000 r/min until the mice were on the brink of death. The supernatant was stored at -70℃.

Ascitic mAbs were precipitated by 50% saturated ammonium sulphate (SAS). The precipitate was washed, redissoloved in PBS (pH 7.4), and purified by 33% SAS twice more. MAbs were desalted by filtration on DEAE-52 ion exchange column.

The concentration of protein in ascites was determined by Bradford method[12] and the purity of purified mAbs was analyzed by 12% SDS-PAGE.

Purified mAbs were labelled with horseradish peroxidase (HRP) by $NaIO_4$ method. Conjugated ELISA (one mAb as the coated antibody, and the other HRP-mAb as the secondary antibody) was used to detect the titer of HRP-mAb and its proper dilution.

Characterization and Properties of mAbs

Conventional ELISA and Western blot were used to analyze the specificity of mAbs recognizing HPT, and the cross reaction of mAbs with other proteins.

HRP-labelled goat anti-mouse IgG was used as the secondary antibody.

The class and subclass of mAbs were determined using Iso Strip Mouse Monoclonal Antibody Isotyping Kit according to manufacturer's protocol.

Rice Protein Extraction and Quantification

Rice proteins were prepared from the leaves, grains and seeds of *sck*-modified rice plants as well as non-transformed control (provided by the Institute of Genetics and Developmental Biology, CAS). In brief, each material (2 g) was ground into powder with liquid nitrogen. Extraction buffer (0.1 g/0.1 mL, 0.3 mol/L $NaHCO_3$, 0.5 mol/L NaCl, 1% β-mercaptoethanol, 10% glycerin, 1% PMSF, 0.1% Tween-20) was added into the powder and incubated overnight at 4℃. The extract was centrifuged for 15 min at 12 000×g and the protein in the supernatant was quantified using Bradford method[12].

Detection of HPT *Protein in SCK-Modified Rice*

Protocol of double sandwich ELISA technique

(1) Standard curve: The anti-HPT pAb coated at 1:100 dilution was added (100 μL) to each well and incubated overnight at 4℃. Coating solution was aspirated and unbound sites on the plastic were blocked by incubating 100 μL blocking solution (1% BSA) for 1 h at 37℃. The unbound protein was washed three times with washing buffer (0.05% PBS-Tween), serial dilutions of standard HPT protein (double wells) were added and incubated for 1 hour at 37℃. The unbound HPT protein was washed, HRP labeled mAb was added at its proper dilution in blocking solution (100 μL per well) and incubated for 1 hour at 37℃. The unbound IgG-HRP was washed four times with washing buffer, TMB enzyme substrate was added (100 μL per well) and incubated at 37℃ until color developed sufficiently (approx.10 min). The reaction was stopped with 2 mol/L H_2SO_4, and serial dilutions of BSA were added with the same concentration of HPT standard protein as the negative control. The positive well was defined as the dilution when the positive OD_{450nm} was 2.1 times that of negative control (P/N≥2.1).

(2) Detection of HPT proteins in *sck*-modified rice: The extracted proteins were concentrated and added into the wells coated with anti-HPT pAb on the plastic. Quantification protocol was as the same as described in (1).

HPT concentration in the well was calculated according to the OD_{450nm} of extracted sample protein well and the standard curve. Then the expression level of HPT in the *sck*-modified rice plants was calculated by comparing the HPT content in the positive well and the total protein in the extracted sample.

Western blot analysis The extracted proteins from GM rice plants as well as non-transformed control (including grains, seeds and leaves), and the purified 6His.HPT were separated by 12% SDS-PAGE. The proteins in the gel were transferred to a PVDF membrane by Bio-Rad's transblot. The anti-HPT antibody is a monoclonal antibody cell line, and the secondary antibody is HRP labelled goat-anti-mouse IgG.

RESULTS

Characteristics of Anti-HPT mAbs

After selection of hybrid cells and cloning, four hybridoma cell lines, named F1, D4-2, D4-4, and D4-5, were successfully established by conventional hybridoma technology. The characteristics of these hybrid cell lines are shown in Table 1.

TABLE 1

Characteristics of Different Cell Lines of Anti-HPT mAbs

Hybridoma Cell Lines	Culture Supernatant ELISA Titer	Mouse Ascites ELISA Titer	Class and Subclass of mAbs	Chromosome Number
F1	1:3200	1:100 000	IgG-1	123
D4－2	1:3200	1:100 000	IgG-1	116
D4－4	1:3200	1:10 000	IgG-1	135
D4－5	1:3200	1:10 000	IgG-1	108

The four mAbs cell lines could recognize HPT proteins in indirect ELISA (P/N≥2.1). There was a specific Ag-Ab binding band at the relative molecular weight 37 kD position between ascites mAbs to the purified 6His-HPT protein, the recombinant plasmid expressed product (bacterial protein), and the extracted proteins from GM rice plants (Fig. 1). ELISA results also showed that the mAbs had no

cross reaction with other foreign proteins in GM rice and the lysate of recombinant plasmid before induction ($OD_{450} \leqslant 0.1$). These results revealed that the four mAbs were specific to HPT.

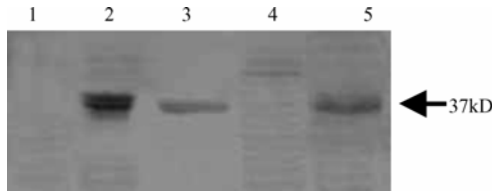

FIG. 1. Western bolt analysis of HPT. 1. Extracted proteins of non-transformed rice leaves. 2. Extracted proteins of GM rice leaves. 3. Purified 6His-HPT protein. 4. Cell lysate of *E. coli* DH5α (PBV222-HPT) before induction. 5. Cell lysate of *E.coli* DH5α (PBV222-HPT) after induction.

Purification and Labelling of mAbs

After ammonium sulfate precipitation and ion exchange chromatography, the purity of mAbs was higher than 90% according to densitometric analysis (Fig. 2).

Ascitic mAbs were labelled with HRP, the mean molar ratio of HRP/IgG was 1.61, and the mean labelling efficiency was 35.2%. Table 2 shows the titer and the proper dilution of labelled HRP-mAbs.

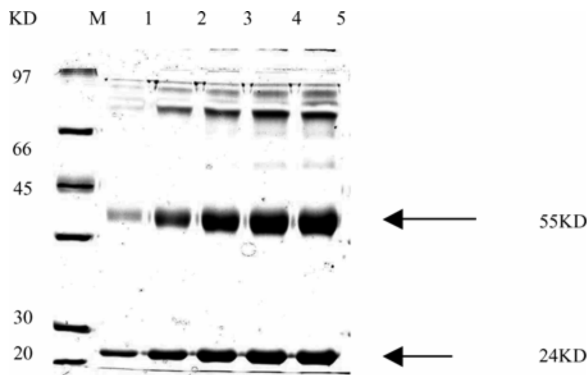

FIG. 2. Purified mAbs by DEAE-52 ion exchange column. M: standard middle weight proteins marker. 1-5: mAbs (9 μg/μL) 0.5 μL, 2 μL, 3 μL, 4 μL, 5 μL, respectively.

TABLE 2

Titer and Proper Dilution of Labelled HRP-mAbs

	D4-2	D4-4	D4-5	F1
Titer	1:6000	1:4000	1:5000	1:4000
Proper Dilution	1:4000	1:2000	1:3000	1:2000

Development of Sandwich Antibody ELISA

The standard curve was constructed with a sensitivity of 30 ng/mL, and showed a linear gradient reaction in the range of 30 to 800 ng/mL.

HPT expression level in the leaves of *sck*-modified rice was in the detection range of our standard curve, the content of which was about 80-150 ng/mL. Compared with the total protein content of the extracted sample, the expression level of HPT in leaves was about 0.0007%-0.005%.

HPT protein from the seeds and rice of the *sck*-modified plants could not be detected, and there were no differences in the OD_{450} between GM rice plants and non-transformed control. The whole experiment was repeated for several times and gave similar results.

Results of Western Blot

Fig. 3 shows the Western blot results based on F1-mAb cell line. There was a specific Ag-Ab binding band at the relative molecular weight 37 kD position between ascites mAbs to the purified 6His-HPT protein, and the extracted proteins from *sck*-modified rice.

DISCUSSION

Antibodies, especially mAbs, are critical reagents used in immunoassay. The development of mAbs against HPT provides a useful tool to quantitate and monitor HPT protein in the genetically modified organisms (GMOs).

In this study, we prepared the anti-HPT mAbs with the recombinant protein 6His-HPT antigen and acquired four hybrid cell lines with a high titer and specificity. In the purification procedure, the simple ammonium sulfate precipitation and ion exchange chromatography, instead of the affinity purification (e.g. protein A or G), were used to gain the high purity IgG (>90%). The results indicate that ion exchange materials have high capacity and are inexpensive enough to be disposable, and can easily be cleaned with 0.5 mol/L NaOH.

In the present study, monoclonal antibody was used to prepare horseradish peroxidase (HRP) conjugate. Since the mAbs do not react with otherforeign proteins introduced in the genetically modified rice (such as CpTI) and HPT originally comes from *E. coli*[13], the use of this antibody in conjunction with HRP as secondary antibodies can minimize the background interference and experimental time. Based on this fact, we established a new system to detect the expression level of HPT in GM rice. Conjugated mAbs-HRP was demonstrated

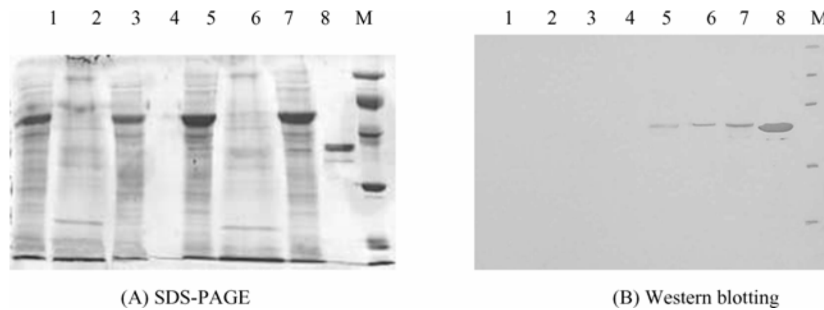

FIG. 3. Western blot analysis of HPT in GM rice. Lane 1: Extracted proteins of non-transformed rice. Lane 2: Extracted proteins of non-transformed rice seeds. Lane 3: Extracted proteins of non-transformed rice leaves. Lane 4: Blank contrast. Lane 5: Extracted proteins of *sck*-modified rice. Lane 6: Extracted proteins of sck-modified rice seeds. Lane 7: Extracted proteins of *sck*-modified rice leaves. Lane 8: 6His-HPT standard protein. M: Standard middle weight proteins marker.

to be excellent secondary antibodies in the double Ab sandwich ELISA system. This system has good specificity and high detection limit (30 ng/mL). The way of sample extraction is easy. The assay can be used in the management, labelling and other safety assessment items of HPT in GM crops.

In addition, we used herein this system to detect HPT protein in the *sck*-modified rice plants. The expression level of HPT in the leaves was 0.0007%-0.005%, which is consistent with the level of another marker protein NPTII in GM plants (0.00005%-0.001%) reported by other researchers[14]. Therefore, it could roughly evaluate the daily intake of HPT protein derived from the consumption of *sck*-modified rice and the potential implications to human health. However, HPT in the grains and seed of *sck*-modified rice could not be detected, indicating that the expression level of HPT in these tissues is lower than the detection limit. But it is important to note that many factors may affect the results of ELISA, such as choice of the parameters and controls, cut-off value enactment, experimental circumstances, *etc*. Besides, the extraction methods, the specificity and sensitivity of the detection methods are also important factors for protein measurement[15]. Further research is needed to check our results.

REFERENCES

1. Jia, Shirong. Safety evaluation of marker genes in transgenic food plants (1997). *Chinese Agriculture Science* **30**(2), 1-15.
2. Hilder, V. R, Gatehouse, A. M. R, Sheerman, S. E. (1987). A novel mechanism of insect resistance engineered into tobacco. *Natur*. **330**, 160-163.
3. Xu, D., Xue, Q., McElory, D., Mawal, Y., Hilder, V. A., and Wu, R. (1996). Constitutive expression of a cowpea trypsin inhibitor gene Cpti, in transgenic rice plants confers resistance to two major rice insect pests. *Mol. Breed* **2**, 167-173.
4. Jia, H. (2004) China ramps up efforts to commercialize GM rice. *Nature Biotechnology* **22**(6), 642
5. Heinrichs, E. A., Medrano, F. G., Rapusas, H. R. (1985). Genetic evaluation for insect resistance in rice. Manila (Philippines): International Rice Research Institute.
6. Zhu, Z., Deng, Z. Y., Wu, Q., Xu Honglin and Xiao, Guifang (1999). Production the high efficient insect-resistant transgenic rice plants. *Journal of Yunnan University (Natural Science Edition)* **21**, 146-147.
7. Zhu, Z. (2001). Research and development of highly insect-resistant transgenic rice. *Bulletin of the Chinese Academy Sciences* **17**(5), 353-357.
8. Yang, lichen, Zhu, zhen, Yang, Xiaoguang. (2003). Purification and Immunity Analysis of Recombinant $(His)_6$-HPT protein expressed in *E.coli*. *Biomedical and Environmental Sciences* **16**(2), 149-152.
9. Van Regenmortel, M. H. (1988). Which value of antigenic valency should be used in antibody avidity calculations with multivalent antigens? *Mol. Immunol.* **25**(6), 565-567.
10. J. V. Gooding (1986). Monoclonal Antibodies: Principles and Practice, 2nd ed., Academic Press, London.
11. Ba Denian (1998). Contemporary immunological technology and application, 1st ed. Beijing Medical University Press.
12. Wang, J. Z and Fan, M. (2000) Manual of Protein techniques, 1st ed. Science Press. Protein quantification by Bradford –method.
13. Rao, R. N., Allen, N. E., Hobbs, J. N., Alborm, W. E., Kirst, H. A., Paschal, J. W. (1983). Genetic and enzymatic basis of hygromycin B resistance in *Escherichia coli*. *Anitimicrob Agents Xhemoth*. **24**, 689-695.
14. Fuchs, R. L., Heeren, R. A., Gustafson, M. E., Rogan, G. J., Bartnicki, D. E., Leimgruber, R. M., Finn, R. F., Hershman, A., Berberich, S. A. (1993). Purification and characterization of microbially expressed Neomycin Phosphotransferase II (NPTII) protein and its equivalence to the plant expressed protein. *Bio/Technology* **11**, 1537-1542.
15. Lu, T. C. and Wang, X. Z. (2003). Evaluation of the detection methods of genetically modified plants. *Molecular plant breeding* **3**(1), 361-366.

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