

Avidin Expressed in Transgenic Rice Confers Resistance to the Stored-Product Insect Pests *Tribolium confusum* and *Sitotroga cerealella*

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Rice (*Oryza sativa* var. Nipponbare) was transformed with an artificial avidin gene. The features of this construct are as follows: (1) a signal peptide sequence derived from barley alpha amylase was added at the N-terminal region, (2) codon usage of the gene was optimized for rice, and (3) the gene was driven by rice glutelin *GluB-1*, an endosperm-specific promoter. Avidin was produced in the grain of the transgenic rice but not in the leaves. The concentration of avidin in the kernels was about 1,800 ppm. All larvae of the confused flour beetle (*Tribolium confusum*) and Angoumois grain moth (*Sitotroga cerealella*) died when fed transgenic avidin rice powder or kernels, respectively, whereas most of the test insects developed into adults when they were fed a nontransgenic rice control diet. Avidin extracted from the transgenic rice kernel lost most biotin-binding activity after 5 min heating at 95 °C.

Key words: avidin; transgenic plant; rice kernel; stored-product insect pests; plant resistance

Stored-product insect pests cause substantial damage to many crops, but the use of fumigants for protection from these pests remains a concern because of unwanted toxic residues. Furthermore, it is known that methyl bromide, which is a highly effective fumigant, contributes to depletion of Earth's ozone layer and has negative effects on the environment.

Transgenic biotechnology can be utilized as an alternative choice for preservation of crops. We are interested in protecting crops during storage from attack by insect pests using insect growth-inhibiting proteins, e.g., proteinase inhibitors.^{1–3)} If genes coding for these proteins are introduced into crops, the transformant might produce enough of the protein to provide resistance against pests. There are many biocidal proteins that occur in nature that are potential biopes-

ticides for stored grain protection.

The insecticidal activity of chicken avidin has been known since 1959, when it was first reported that the protein is toxic to the housefly, *Musca domestica* L. when administered in the diet to larvae.⁴⁾ Avidin is a glycoprotein found in chicken egg white that has a molecular weight of approximately 17 kDa and is composed of four subunits, yielding a tetramer of approximately 67 kDa. It binds biotin strongly ($K_d = 10^{-15}$ M) and prevents acquisition of biotin by many organisms. Biotin is a cofactor needed for four kinds of carboxylase reactions⁵⁾ and essential for all organisms. Insects do not have a biosynthetic pathway for biotin and must obtain it from outside sources. Hence, diets containing avidin are toxic to a wide range of insects.⁶⁾ Avidin is one of the most promising candidate insect control proteins because of its efficacy and broad spectrum of activity. Studies of transgenic avidin plants, including maize (*Zea mays*)⁷⁾ and tobacco (*Nicotiana tabacum*),⁸⁾ have indicated that they are toxic to insect pests or that they suppress the growth of insects when they feed on the transgenic plants.

Rice is an important staple crop in Japan and other Asian countries. We introduced the avidin gene into rice to protect it from stored-product insects and investigated the insecticidal activity of the avidin rice grain on larvae of the confused flour beetle, *Tribolium confusum*, and the Angoumois grain moth, *Sitotroga cerealella*, which are economically important stored-product insect pests.

Materials and Methods

Materials. Seeds of rice, *Oryza sativa* L. cv. Nipponbare, were used for the transformation. The transformed plants were grown in a greenhouse. Avidin from chicken egg white was purchased from Sigma-Aldrich, St. Louis, MO. DNA oligomers were synthesized by

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; ELISA, Enzyme-linked immunosorbent assay; PBS, phosphate buffered saline

Espec Oligo Service, Tsukuba, Japan.

The confused flour beetle, *Tribolium confusum* Jacquelin du Val, and the Angoumois grain moth, *Sitotroga cerealella* (Olivier), were maintained at the National Food Research Institute and used for the bioassays.

Binary constructs and transformation. As shown in Fig. 1, an artificially fused gene comprised of the alpha amylase signal sequence (N-terminal 25 amino acid residues) from barley, *Hordeum vulgare* (AAA32925), and mature avidin from chicken, *Gallus gallus* (X05343),⁹ was designed based on the codon usage

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      10      20      30      40      50      60
ATGGCTAACAAAGCACCTCAGCCTGTCTTCTCCTGCTTCTCGGCTGTGACGCTCG
M A N K H L S L S L F L V L L G L S A S
      70      80      90      100     110     120
CTAGCGTCTGGACAGCCAGGAAGTCTCCCTACCGGCAAGTGGACGAATGACCTGGGT
L A S G Q A R K C S L T G K W T N D L G
      130     140     150     160     170     180
AGCAACATGACCATTGGCGCTGTGAAGTCTCGCGGAGAGTTCAGTGGGACATACATTAC
S N M T I G A V N S R G E F T G T Y I T
      190     200     210     220     230     240
GCAGTCAAGCGACTTCAAACGAAATCAAAGAGTCGCGATTGCACGGCACCCAGAATACA
A V T A T S N E I K E S P L H G T Q N T
      250     260     270     280     290     300
ATCAACAAGCGTACCGAGCCGACCTCGGGTTTACTGTGAATTGGAAGTTCTCCGAGAGT
I N K R T Q P T F G F T V N W K F S E S
      310     320     330     340     350     360
ACAACCGTCTTACCGGGCAATGCTTTATCGATCGAAATGGTAAAGAAGTTCTCAAGACG
T T V F T G Q C F I D R N G K E V L K T
      370     380     390     400     410     420
ATGTGGCTTCTACCGAGCTCCGTGAACGACATAGCGGATGACTGGAAGCCACTCGGGTA
M W L L R S S V N D I G D D W K A T R V
      430     440     450     460
GGAATCAACATCTTACAAGACTGAGGACCCAGAAGGAGTGA
G I N I F T R L R T Q K E *

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Fig. 1. Avidin Artificial Gene Sequence.

Barley alpha amylase signal peptide and mature avidin gene sequences were fused. The codon usage was changed to optimize for expression in rice plants. The underline indicates the amylase signal sequence. For experimental details, refer to "Materials and Methods".

table (NCBI-GenBank) of rice, *Oryza sativa*. That is, codon usage was changed according to the average frequency of rice genes. The sequence was divided into six parts, each consisting of about 90 bases of nucleotides, including some overlapping sequences, and each part was synthesized by the Espec Oligo Service. Finally, the artificial gene was constructed using PCR from these oligomers according to the method of Fujimoto *et al.*¹⁰

The artificial gene was transcriptionally fused to the 1.3 kb *GluB-1* promoter from rice.¹¹ Thereafter, the chimeric gene was introduced into a binary vector modified from pIG121Hm¹² (Fig. 2). The gene-introduced vector was transferred into *Agrobacterium* (EHA101) by electroporation. The transformation was carried out according to a modification of the standard *Agrobacterium* method¹² designed by one of the authors (K.A.). Transformants, which were infected by *Agrobacterium* harboring the vector without the *GluB-1* promoter and the avidin gene, were used as the control in western blotting, Enzyme-linked immunosorbent assay (ELISA), and insect bioassay experiments.

Confirmation of introduced gene in rice. Polymerase chain reaction (PCR) was carried out to check for the introduction of the avidin gene into the rice plant. The reaction was done for 30 cycles under conditions of annealing at 55 °C for 30 sec, extension at 72 °C for 3 min, and denaturation at 94 °C for 30 sec. The following primers were used: 5'-GTAACACTATTATGCTCCCTTCGTTAC-3' and 5'-GCCGCTCTAGAAGTAGTGG-3'. These primer sequences were derived from the binary vector sequences, which are just outside of the introduced *GluB-1* promoter (1.3 kbp) and the avidin gene (0.5 kbp).

SDS-PAGE and western blot analysis. Each rice grain was cut into two pieces, soaked in 300 microliters of extraction buffer, 100 mM sodium phosphate (pH 7) at 4 °C overnight. The grain was ground with a pestle and the homogenate was centrifuged at 15,000 rpm in a microcentrifuge for 15 min. The leaf sample was ground with a pestle, in liquid nitrogen, mixed with 9 volumes (v/w) of the extraction buffer, and centrifuged. The

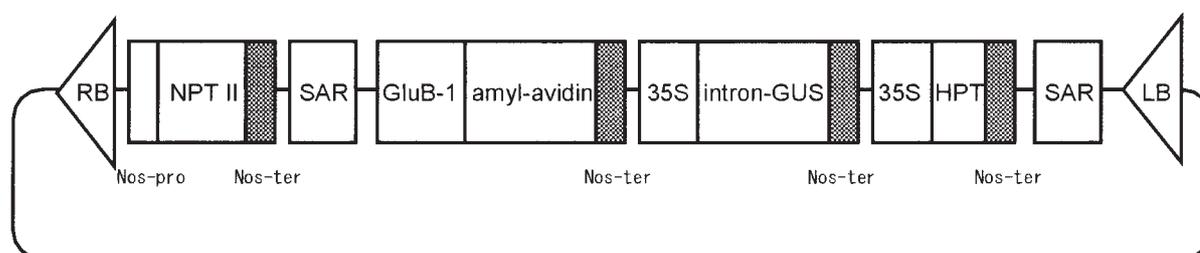


Fig. 2. Binary Vector Construction to Introduce the Avidin Gene.

Vector construction: RB, right boundary; NPT II, neomycin phospho transferase gene; SAR, scaffold-associated region; GluB-1, GluB-1 promoter; amyI-avidin, fusion gene of amylase signal sequence and avidin gene; 35S, 35S promoter; intron-GUS, beta-glucuronidase gene containing intron; HP, hygromycin phospho transferase gene; LB, left boundary. For experimental details, refer to "Materials and Methods".

supernatants were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) using 15% slab gels, according to the method of Laemmli.¹³⁾ Proteins were stained with Coomassie Brilliant Blue R-250. For western blot analysis, proteins separated by SDS–PAGE were blotted onto a PVDF membrane (Immobilon-Psq, Millipore, Billerica, MA) for 1 h at 0.77 mA/cm² in 20 mM glycine, 25 mM Tris, and 20% methanol using a semidry electroblotting apparatus (NA1512, Nihon Eido, Tokyo).

Anti-avidin rabbit serum (Biogenesis, Poole, England) was used as the primary antibody, and anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad Laboratories, Hercules, CA) was used as the secondary antibody. Immunoreacting proteins were detected using the alkaline phosphatase conjugate substrate kit (Bio-Rad).

ELISA. Samples were prepared as described above under “SDS–PAGE and western blot analysis” and subjected to ELISA.¹⁴⁾

Dilution buffer for avidin standard was prepared as follows: Rice grains (Nipponbare) were ground using a food mill (Millser IFM-100, Iwatani International, Tokyo). The rice powder was mixed with 15 times volume of extraction buffer, 100 mM sodium phosphate (pH 7), and the mixture was centrifuged at 15,000 rpm for 15 min. The supernatant was used as the dilution buffer for standard avidin solution. Chicken avidin was diluted in the buffer and used as the standard avidin solution.

Anti-avidin rabbit serum was used as the primary antibody and anti-rabbit IgG alkaline phosphatase conjugate as the secondary antibody. Immunoreactive proteins were detected using the alkaline phosphatase substrate kit (Bio-Rad). Absorbance at 405 nm was determined after 15–30 min using a microplate reader (MPR A4, Tosoh, Tokyo).

Protein assay. Protein content was determined using the DC protein assay kit (Bio-Rad). Bovine gamma globulin was used as the standard protein.

Larval-feeding bioassays. An insect-feeding bioassay of *T. confusum* was conducted based on the methods described by Kramer *et al.*⁷⁾ Genetically transformed rice grains from the no. 17 line were ground using a food mill (IFM-100, Iwatani International). The rice powder was used for the insect diet after equilibration in a rearing chamber for 10 d. Each newly hatched larva was placed on 50 mg of the diet in a 1.5 ml microcentrifuge tube with the lid pierced with a needle for air exchange. Twenty larvae each were used with avidin rice and control rice, and reared at 30 °C and 70% relative humidity. After 2 weeks, the mortalities and weights of the larvae were recorded, and the numbers of adults were counted after four weeks.

The insect-feeding bioassay of *S. cerealella* was conducted as follows: Each newly hatched larva was placed with a rice kernel in a 1.5 ml microcentrifuge

tube, as described above. Thirty larvae were tested using avidin rice kernels from the no. 17-10 line or control rice, and reared at 30 °C and 70% relative humidity. The numbers of adults to emerge from the kernels were counted.

Residual avidin activity after heating. The percentage of residual active avidin in the avidin rice kernels was estimated using an ELISA-like method. Samples were prepared as described above under “SDS–PAGE and western blot analysis” and heated at 95 °C for 0 to 60 min.

The blocking solution contained 1% bovine serum albumin in phosphate buffered saline (PBS), which consists of 137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, and 1.47 mM KH₂PO₄ (pH 7.4). Anti-avidin rabbit serum was added to the wells of an ELISA plate, incubated for 2 h, and removed. Then blocking solution was added, left overnight at 4 °C, and removed. Samples were loaded, incubated for 2 h, and removed. The wells were washed 5 times with PBS. Biotinyl-alkaline phosphatase was placed each well, incubated at 37 °C for 1 h, and removed. Then the wells were washed 5 times with PBS. Active avidin was detected using the alkaline phosphatase substrate kit, as described above.

Results and Discussion

Transformation of artificial avidin gene

It has been reported that the expression level of an introduced gene is often remarkably low when a foreign gene derived from insects, bacteria, or animals is introduced into plants.¹⁵⁾ Optimization of the codon usage of the synthetic gene for the host plant is sometimes used to increase the expression level. We designed a synthetic gene to enhance the expression level, as shown in Fig. 1. The signal sequence of alpha amylase, which secretes protein outside of the cell, was added at the N-terminal site of the mature avidin gene. The codon usage of the fused gene was optimized for rice. Another feature was the use of the glutelin *GluB-1* promoter, which directs high expression in endosperm tissue. Endosperm-specific expression has an advantage because the use of a constitutive promoter like CaMV35S or ubiquitin can cause a detrimental effect on growth or pollination of the transformant.¹⁶⁾

After transformation, DNA was extracted from shoots regenerated from calluses and the introduction of gene was checked by PCR (Fig. 3). Finally, 18 independent transformants were obtained. The morphological and developmental characters of the transformant plants looked normal compared to the control. The appearance of the brown rice of the transgenic plants was also normal. The ripening percentage of transgenic avidin rice (T₁) was 42% on the average (in a range of 10 to 77%), and that of the control rice was 63% (in a range of 44 to 79%). The production of avidin protein can slightly affect the fertility. Expression of avidin in pollen

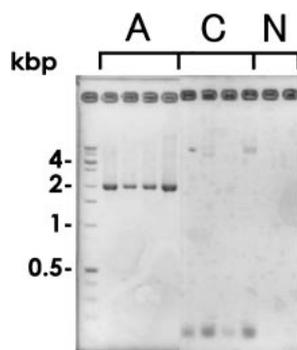


Fig. 3. Detection of Introduced Gene in Rice Plants Using PCR.

The binary vector sequences, which are just outside of the introduced *GluB-1* promoter (1.3 kb. p.) and avidin gene (0.5 kb. p.), were used as primer sequences. The length of the amplified DNA band (1.8 kb. p.) was that of the promoter and avidin gene with avidin rice DNA. The length of the amplified band with the control was short because its DNA does not contain the promoter and the avidin gene. No band was detected in non-transformant rice DNA. A, avidin rice; C, control rice; N, non-transformant rice. For experimental details, refer to "Materials and Methods".

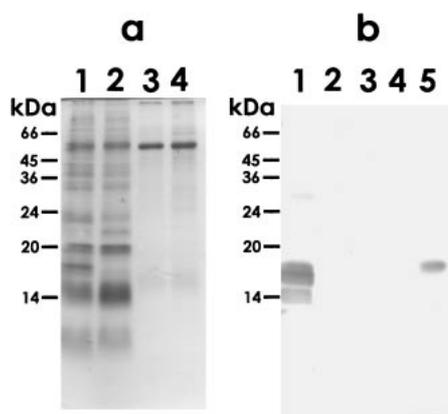


Fig. 4. SDS-PAGE and Western Blot Analysis of Transgenic Avidin Rice.

Ten micrograms of soluble protein were loaded. (a), SDS-PAGE; (b), western blotting; 1, avidin rice grain; 2, control grain; 3, avidin rice leaves; 4, control leaves; 5, authentic avidin. For experimental details, refer to "Materials and Methods".

caused a male sterility phenomenon with avidin corn when the ubiquitin promoter was used.¹⁶⁾ Since the *GluB-1* promoter is endosperm-specific and does not express in pollen, the transgenic avidin rice was fertile. Rice is self-compatible and male sterility would cause a fatal problem.

Detection of avidin and its concentration in the grain

Western blot analysis indicated that the avidin gene was expressed in the grain of the transformant but not in the leaves (Fig. 4). This result is consistent with the evidence that the *GluB-1* promoter directs endosperm-specific expression.¹¹⁾ The estimated molecular weight of avidin expressed in the transgenic rice grain was 16 kDa, which was the almost same as for standard

avidin.

The avidin content in the grain was estimated using ELISA. Seeds (T_1) from the no. 17 line (T_0) showed the highest concentration of all of the 18 lines. The avidin concentration was variable in the kernels, with levels ranging from 761 to 2,604 ppm. The mean level and SD of avidin in the seeds from the no. 17 line were $1,834 \pm 782$ ppm ($n = 10$). The variation in the avidin content among the T_1 seeds from no. 17 was large. The reason for the variation with T_1 is probably segregation, because T_0 plants are usually heterogeneous with T-DNA.

A line from the next generation, no. 17-10, was selected for further analysis. The average avidin content and SD were $1,741 \pm 262$ ppm ($n = 10$) with the seeds (T_2) from no. 17-10. The variation in avidin content in the T_2 seeds was small compared to that in the T_1 seeds. The homozygous individuals with T-DNA would have increased in the later generation though the copy number of the introduced T-DNA was unknown. Some T_1 seeds from no. 17, in which avidin was not detected by western blotting analysis, were found. In contrast, all T_2 seeds from no. 17-10 contained avidin as far as we assayed (data not shown).

The avidin concentrations in rice (761 to 2,604 ppm) were much higher than those in tobacco (46 to 63 ppm) and apple (30 to 175 ppm).¹⁷⁾ The *GluB-1* promoter and alpha amylase signal sequence were used with rice. The *GluB-1* promoter caused high-level accumulation of avidin in the transgenic rice kernels. On the other hand, the CaMV 35S promoter and vacuolar targeting sequence were used with the other plants. Transgenic avidin corn (T_1), in which the ubiquitin promoter was used, contained approximately 80 ppm of avidin, but subsequent generations were selected that exhibited substantially higher levels of avidin.¹⁶⁾ After a selection process with seven generations in the field, the corn contained as much as 3,000 ppm avidin.¹⁸⁾ The level is comparable to that of avidin rice. Storage organs like seeds might be suitable for accumulation of introduced gene products compared to leaves. In theory, half of the kernels of the avidin corn should not contain avidin because of male sterility. Since such a phenomenon was not found in avidin rice (T_2) with the *GluB-1* promoter, it is suitable for protecting seeds from stored-product insect pests.

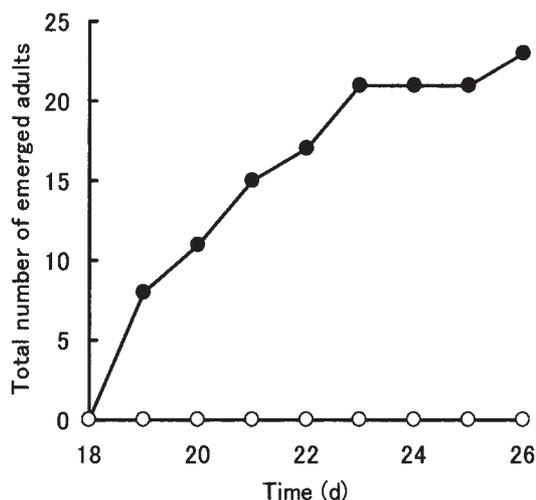
Insect bioassay

T. confusum was reared on transgenic avidin rice or control rice powders. After 2 weeks, only 2 of 20 larvae survived on the avidin rice (Table 1). On the contrary, 18 larvae were alive after consuming the control rice powder. Additionally, the growth of larvae fed avidin rice was suppressed as compared to that of the control larvae. The average larval weight was only 30% of the control larval weight. After one month, all 20 of the larvae had died when the transgenic rice powder was fed as their diet. On the other hand, 16 of 20 insects survived

Table 1. Effect of Avidin on Survival and Larval Weight of *Tribolium confusum* Reared on Transgenic Avidin Rice Powder^a

Diet	Number of surviving insects after		Weight (mg) of larvae after
	2 weeks	4 weeks	2 weeks
Avidin rice powder	2 (10%)	0 (0%)	0.13
Control rice powder	18 (90%)	16 (80%)	0.43

^aTwenty larvae each were inoculated. Percent survival given in parentheses.

**Fig. 5.** Total Number of Emerged *Sitotroga cerealella* Reared on Transgenic Avidin Rice and Control Rice.

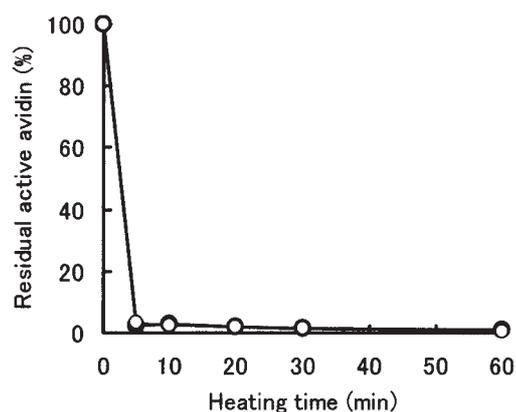
Accumulated numbers of adults were counted after newly hatched larvae ($n = 30$) were added to the rice. ○, reared on avidin rice; ●, reared on control rice. For experimental details, refer to "Materials and Methods".

and developed into adults in the control rice powder.

S. cerealella was reared on avidin rice or control rice grains. Total emerged numbers of *S. cerealella* during the experiment are shown in Fig. 5. Finally, adults fed on avidin rice did not emerge. On the other hand, 23 of 30 insects emerged as adults in the control.

The avidin rice exhibited resistance to both *T. confusum* and *S. cerealella*. This result was expected, since avidin corn is resistant to both of these stored-product pests.⁷⁾ The insecticidal activity of avidin against both coleopterans and lepidopterans is an advantage when compared with the spectrum of activity of Bt endotoxins, which are generally effective only against lepidopterous pests.

The avidin concentration in the rice was about 1,800 ppm, which was sufficient to inhibit the growth of the insects and eventually kill them. This level was much higher than that required for insect toxicity in semi-artificial diets⁶⁾ and transgenic avidin tobacco.⁸⁾ Although we used the most abundant avidin rice lines, no. 17 and no. 17-10, for the insect tests, other lines that contain lower levels avidin might be sufficient to prevent the growth of pest insects.

**Fig. 6.** Residual Active Avidin after Heating.

The percentage of residual active avidin after heat treatment is shown. ○, avidin extracted from transgenic rice; ●, authentic avidin. For experimental details, refer to "Materials and Methods".

Heat denaturation of avidin

It is known that avidin is denatured by heat and loses biotin-binding activity. Avidin in cooked rice would lose activity and be safe for humans and other organisms to eat. To confirm heat denaturation of avidin produced in the kernel, we estimated residual active avidin after heating, as shown in Fig. 6. Avidin extracted from the transgenic rice kernel lost most biotin-binding activity after 5 min of heating at 95 °C. Active avidin was only 3% of non-heated avidin rice. A similar result was obtained with authentic avidin.

Since avidin produced in rice kernels lost most activity after heating, avidin rice might be usable as a food. Of course further research is necessary to confirm the safety of avidin rice after cooking.

Rice is an important crop in Asia, including Japan. Controlling insect pests of stored rice and rice products can be very difficult because of the variety of species that can infest the grain. The manufacture and importation of the insecticidal fumigant methyl bromide will be completely phased out in developed countries in 2005. In developing countries, phase-out will be complete in 2015. As an alternative approach, insect pest management using avidin-biotin transgenic technology might be useful in countries where rice is not stored at low temperatures in warehouses.

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