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Strategies in Development of High Insect-resistant GM Rice and its Biosafety Assessment

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Rice – The Most Important Crop in China

(http://www.cfiin.com, China Food Industry Network)

- ***Rice planting area:*** ***about 29-33 (1996-2005) million hectares, accounting for about 20% of the total rice growing area of the world.***
- ***The total brown rice production: 195 (1996-2005) million tons, accounting for about 34-36% (1996-2005) of the total rice production in the world, about 40% of the total grain production in China.***
- ***Rice variety: more than 55% of the rice planting area is Hybrid Rice, and increase 15-20% production than conventional rice variety.***
- ***Expenditure for rice insect pest control: Expenditure of chemical insecticide and application will cost about 3 billion US $ per year.***
- ***Rice production losses caused by insect pests: More than 5% of the total brown rice production, i.e. about 10 million tons or 1.5 billion US $ even under control.***

The Rice Production Lost Caused by Pests & Disease in Rice

About ¥ 20-30 billion lost per year caused by five major rice pests & disease in China.

- **Rice Lepidopteran 稻螟虫**
- **Rice Planthopper 稻飞虱**
- **Rice Blast 稻瘟病**
- **Rice Bacterial Leaf Blight 白叶枯病**
- **Rice Sheath Blight 纹枯病**

螟虫（rice stem borers） 稻飞虱(Brown planthopper)

稻瘟病(Rice blast) 白叶枯病(Rice bacterial blight)

The Major Rice Lepidopteran Insect Pests in China

Latin name	English name	Chinese name
Chilo suppressalis (Walker)	Striped stem borer	二化螟
Scirpophaga incertulas (Walker)	Yellow stem borer	三化螟
Cnaphalocrocis medinalis Guenee	Rice leaf folder	稻纵卷叶螟
Sesamia inferens (Walker)	Pink borer	大螟
Chilo auricillius (Dudgeon)	Dark-headed stem borer	台湾稻螟

Striped stem borer
Chilo suppressalis Walker

Lepidopteran insects has been the most important pest, it caused serious damage to rice production, but there are no lepidopteran pests resistant germplasm to be found in rice variety and related species.

Traditional Rice Breeding

Farmer spray chemical insecticide to control pests after strong earthquake in May.2008. Wenchuan city. Sichuan Province

The Candidate Genes Resistant to Lepidoptera Pest

- B.t toxic(δ -toxin) genes belong to *cry* gene family, are isolated from *Bacillus thuringiensis* and widely used genes in GM plants. One of them, *cry*1A(c) was used in this research.
- Cowpea Trypsin inhibitor(CpTI) was from the edible part of cowpea, CpTI belong to Bowmun-Birk inhibitor family and has a broad insect resistance spectrum (Lepidoptera、Coleoptera、Orthoptera).

CpTI is Safe for Human Being

- *Cowpea trypsin inhibitor（CpTI）is coming from the edible part of cowpea, which has been a food by human being for thousands years;*
- *CpTI Belongs to the Bowman-Birk Inhibitors (BBI), there is no report that BBI family is allergens or poisonous;*
- *BBI has been reported as an anticarcinogenic agent.*

Bowmun-Birk Inhibitor (BBI) was Anticarcinogenic Agent

Type of Cancers	Suppression effect	Treatment	Reference
Colon cancer (DMH induced)	100%	0.1-0.5% BBIC	Clair, 1990
Liver cancer (3-MCA induced)	66-79%	0.5% BBIC	Clair, 1990
Oral cancer (DMBA induced)	43.8%	Not indicated	Messadi, 1986
Esophageal cancer (MBNA induced)	35%	0.5% BBIC	Von Hofe, 1991
Breast cancer (MNU induced)	33-60%	Not indicated	Barnes, 1990
Lung cancer (3-MCA induced)	52%	0.5% BBIC	Witschi, 1989

DMH: Dimethylhydrazine，DMBA: 7,12-Dimethylbenanthracene，MCA: 3- methylcholanthrene，MBNA: N-nitrosomethylbenzylamine, MNU: N-methyl-N-nitrosourea, BBIC: BBI concentrate

Lot of study results showed, Bowmun-Birk inhibitors (BBI) (from soybeans）in the diet are associated with low incidence rates for many kinds of cancers, such as colon cancer, liver cancer, oral cancer, etc. BBI can prevent and suppress malignant transformation and without any toxicity effects.

(Kennedy, A. 1994, 1995)

Strategies in Developing GM rice

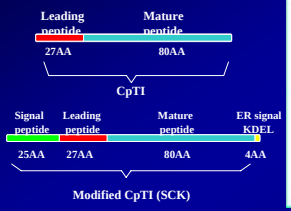
---- Progress of Study on Insect-resistant Rice

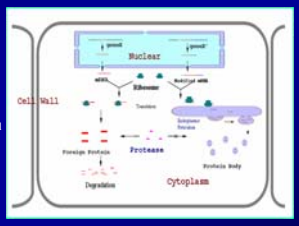
❖ ***First version of insect-resistant rice：***
Increasing insect-resistance by sub-cellular targeting of foreign insecticidal protein .

❖ ***Second version of insect-resistant rice：***
Establishment of multi insect resistant mechanism by using two different insect-resistant genes.

❖ ***Third version of insect-resistant rice：***
Stable and high level expression of foreign gene by using MAR sequence and Marker-free transgenic rice.

Version 1 of Insect-resistant Rice

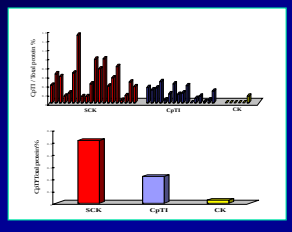




Strategy of sub-cellular targeting of foreign insecticidal protein:

Increasing insecticidal protein accumulation level in host plant cell by subcellular targeting of Cowpea trypsin inhibitor (CpTI) into endoplasmic reticulum(ER), where provide an inactive environment for foreign protein.

sck Transgenic Rice Confer High Resistance to Rice Stem Borers



- *Red columns represent SCK transgenic plant, blue columns represent CpTI transgenic plant as positive control, yellow columns represent wild type rice as negative control.*

Version 2 of Insect-resistant Rice

- Multi-mechanism Pest-resistant Rice by Transfer Two Insecticidal Genes(*sck*+*cry 1Ac*)
- Increasing resistance
- Widen insect resistant spectrum
- Delay insect development of tolerance

Showed High Resistance to Stripded Stem Borer and Leaffolder

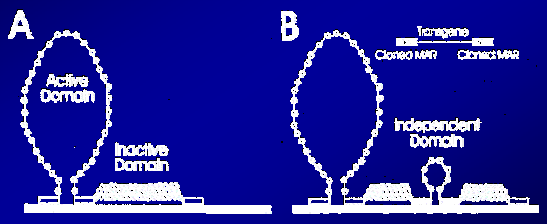
Version 3 of Insect-resistant Rice

- **Stable and high level expression**
- **Marker free transformation system**

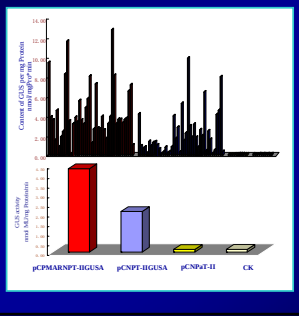
Isolation and Utilization of MAR Sequences

MARs can prevent transgene flanked it from forming herterochromatin and overcome “position effect” or “transgene inaction”.

Matrix Attachment Regions(MARs) and Chromatin Loop



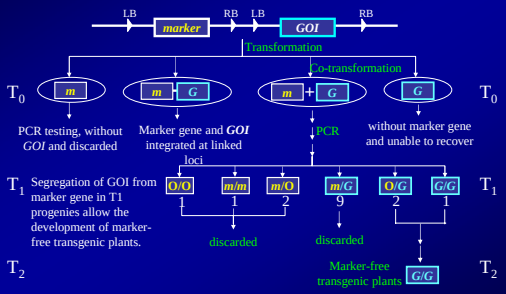
Isolation and Functional Analysis of MAR Sequence



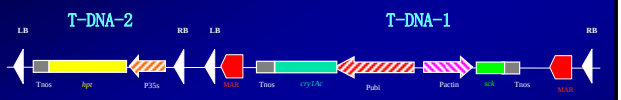
A 745 bp MAR sequence located downstream of the pea plastocyanin gene was isolated. Assays of transgenic tobacco plants showed that the MAR sequence could increase the average expression level of gusA gene by two fold and minimize the variation of gusA expression level.

Marker Free Transforming System

Marker Free Strategy by Using Double T-DNAs Vector

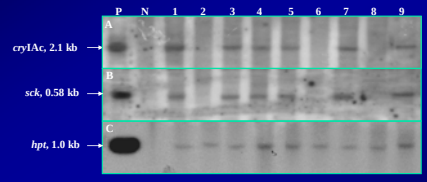


Double T-DNA Rice Transformation Vector



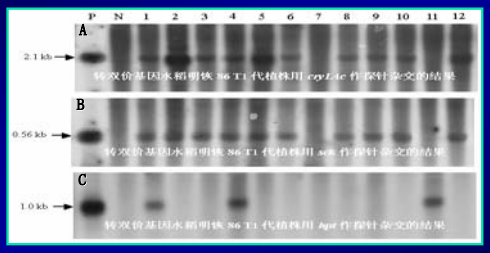
LB and RB—left and right border of T-DNA, hpt—Hygromycin phosphotranferase gene, CaMV 35S —CaMV 35S promoter, Tnos—terminal of nopaline synthase gene, MAR—matrix attachment region from plastocyanin gene, cryIAc—Bt toxin gene, sck—sck gene, Pubi—ubiquitin gene promoter and Pact—actin gene promoter

Southern Analysis of T0 Marker-free Transgenic Rice Plants



A and **B**, genomic DNA was digested with the restriction enzyme *Bam*H I and hybridizations were done with *cryIAc* or *sck* probe respectively; **C**, genomic DNA was digested with the restriction enzyme *Xho* I and hybridizations were done with *hpt* probe. **P**, positive control; **N**, non-transgenic control plant; **1-9**, T_0 pCDMARUBAC-Hyg-transformed rice plants.

Southern analysis of T1 Marker-free Transgenic Rice Plants



P: Plasmid control; N: non-transgenic control; 1-12: T_1 progenies.
A, B and C, rice total DNA probed with cryIAc, sck and hpt respectively. The result showed that sck and cryIAc were segregated from marker gene hpt. Lanes 2, 3, 5, 6, 8, 9, 10 and 12 were marker-free sck+cryIAc rice progenies.

Marker-free Insect-resistant GM Rice

(Fuzhou, Fujian Province, May and Aug. 2005)

"863" and frame are marker free transgenic rice, the other is control.

Marker free GM Rice in Field

--Fujian, 2006

Fourth version:

Breeding multi-insect-disease resistant rice using molecular marker assistant and pyramid breeding strategy

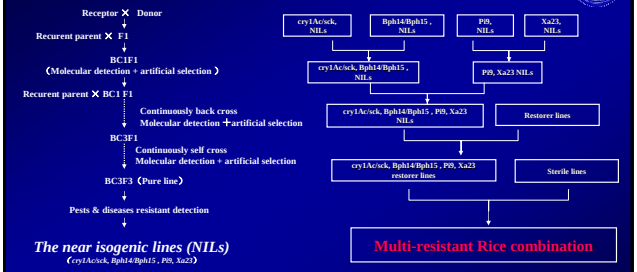
The Major Materials Will be Used in Future Research

Four kinds of genes were used in our research, *cry1Ac/sck* for Rice Lepidopteran, *Bph14/Bph15* for Rice Planthopper, *Pi9* for Rice Blast and *Xa23* for Rice Bacterial Leaf Blight. We have constructed the near isogenic lines (NILs) for these genes, the background including 93-11 and Minghui 86.

Pests & Dieases	*Gene*	*Rice variety*
Rice Lepidopteran	*cry1Ac/sck*	*KBA6343（Background: Minghui86）*
Rice Planthopper	*Bph14/Bph15*	*C60（Background: 93-11/B5）*
Rice Blast	*Pi9*	*C751（Background: 93-11/Q1347）*
Rice Bacterial Leaf Blight	*Xa23*	*C682（Background: 93-11//93-11/CBB23）*

cry1Ac/sck *Bph14/Bph15* *Pi9* *Xa23*

The Strategy of Multi-resistant Rice Breeding



The Strategy of Multi-resistant Rice Breeding, firstly, we develop the near isogenic lines for the four kinds of resistant genes, then use these lines developing dual-resistant NILs, through crossing, we will get multi-resistant NILs rice, at last, though traditional rice breeding way, we can obtain the ***Multi-resistant hybrid Rice combinations.***

Part II: Biosafety Assessment

Environment safety assessment and food safety assessment

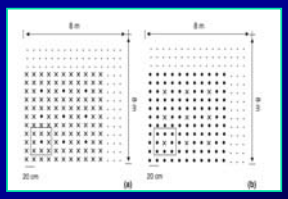
Arthropod Community Structure in Field of sck Rice at Tillering Stage *-- Fujian*

Rice line or combination	Phytophagous insects		Parasitic insects		Neutral insects		Predatory natural enemies				Total	
	No. of insect	Compare to CK（±%）	No. of insect	Compare to CK（±%）	No. of insect	Compare to CK（±%）	No. of predatory insect	No. of spider	Total	Compare to CK（±%）	No.	Compare to CK（±%）
MH86《CK》	131		21		149		2	15	17		318	
Kefeng-2	15	-88.6	9	-57.1	188	+26.2	3	19	22	+29.4	234	-26.4
TeA/Kefeng-2	17	-87.0	5	-76.2	161	+8.1	3	17	20	+17.7	203	-36.2
Kefeng-1	29	-77.9	12	-42.9	204	+36.9	11	18	29	+70.6	274	-13.8
TeA/Kefeng-1	26	-80.2	15	-28.6	175	+17.5	6	17	23	+35.3	239	-24.8

Compared with that in the non-GM field, the population densities of pests in transgenic rice field decreased greatly, and neutral insects and predatory natural enemies increase significantly.

Low Frequency of Transgene Flow From *sck+cry1Ac* Rice to Non-GM Rice

- **The result showed low frequencies (0.05–0.79%) of transgene flow from GM to non-GM rice at close spacing.**
- **The dramatic reduction in transgene frequencies with increasing distance from the GM crop, ranging from 0.28% at 0.2 m to < 0.01% at 6.2 m.**



Field layout of the transgene flow experiments in an 8 × 8 m plot, where the solid circles represent hills of non-GM rice varieties, the crosses represent hills of GM rice lines.

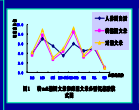
Jun Rong et al., 2005, 2007, *New Phytologist*

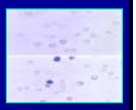
Food Safety Test on GM Rice

Test items:
- Nutritional Composition Analysis
- Nutritional Bio-availability Evaluation
- Teratogenicity Test in Rats
- Immunotoxical Assessment
- 90 days-Feeding Subchronic Toxicity test in Rats
- 60 day-Feeding test in Pigs

Results and Conclusions:
The results shows there are No differences in transgenic and non-transgenic rice, which implies substantial equivalence between GM and non-GM rice.

Tested by:
National Institute of Nutrition and Food Safety, Chinese Center for Disease Control and Prevention (CDC), and Peking University


Nutritional Composition Analysis


Toxicity Test


60 day-Feeding Studies in Pigs


Teratogenicity Test

Allergenicity Assessment for CpTI Proteins

Comparasion of amino acid sequence homology for :

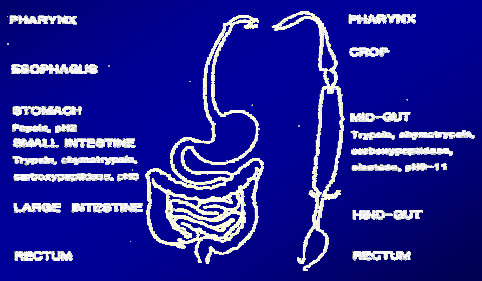
Animal allergens: egg, milk, fish, testacean etc;
Plant allergens: peanut, soybean, nut, wheat etc;
Pollen allergen
Others: dust acarid……

Search results for the important allergens:

No homology be found by comparing the amino acid sequences of CpTI with the known allergens

Digestive System of Mammalian and Insect

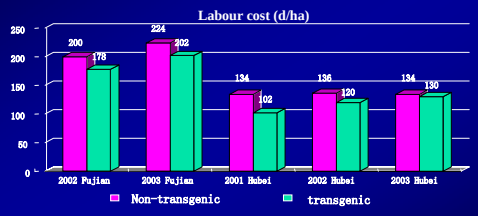
(without anti-nutritional effect)



The Industrialization Perspective

Saving Chemical Insectcides and Labor Cost

--Economical Benefit of GM Rice

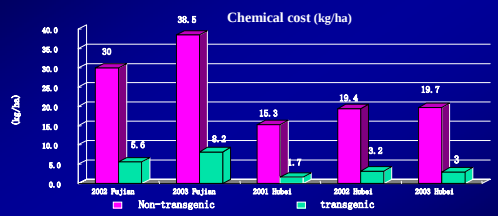


Insect-resistant GM rice reduce 3 times for spraying agrochemicals and save 9 working days/ha.

Huang et al., 2005, *Science*

Reducing Chemical Pesticide

--Economical Benefit of GM Rice

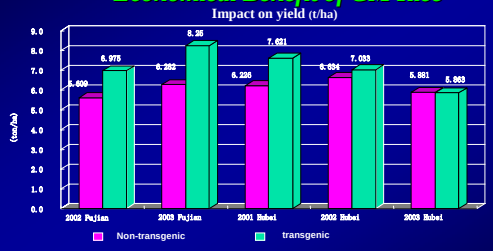


Insect-resistant rice can reduce chemical pesticide to **80%**, **15** kg/ha.

Huang et al., 2005, *Science*

Increasing Yield

--Economical Benefit of GM Rice



Insect-resistant GM rice varieties will increase yields 6-9% , the highest increase 2 tones/ha.

Huang et al., 2005, *Science*

Economical Benefit of GM Rice Totally

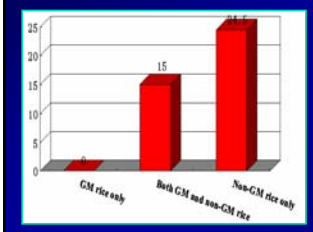
Impact of transgenic rice on economy (estimated if distributed to 50% of total acreage in China)

- Reduce chemical Pesticide: saving total 210 thousand ton/year
- Yield increasing: 6%, 24kg/ha, total increase 360 million tons.
- Reduce production lost about ¥ 200/ha. and increase farmer's income at about ¥ 570/ha.
- Increase income: ¥ 20 billion/year

If GM rice area reach 90% in China, would increase income about ¥ 34.3 billion/year

Huang et al., 2005, *Science*

Other Benefits of GM Rice



The effect of insect-resistant GM rice use on the health effects of farmers in Hubei and Fujian

- **Positive impacts of GM Rice on farmers health**

  The cases of pesticide-related incidence of morbidity were extremely decrease , if the farmer grows GM rice .

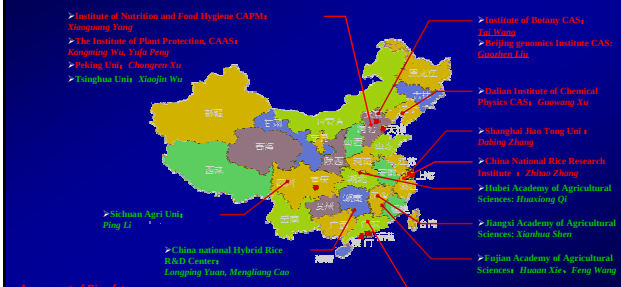
All above data were statistics by Prof. Jikun Huang and Ruifa Hu, Center for Chinese Agricultural Policy, Chinese Academy of Sciences

Collaborated with Most Famous Rice Breeding Scientists

Prof. Yuan Long-ping and Prof. Zhan Zhu(lab director)

Prof. Xie Hua-an and Prof. Zhan Zhu(lab director)

Co-operation Network in China



Thank you!