Quarantine Processing of Imported Transgenic Rice and Evaluation of Risk in Import

Shashi Bhalla[1], VC Chalam, Baleshwar Singh, Rajan, Kavita Gupta, P Firke*, N Kumar, Anju Jain, R Som, Gurinder Jit Randhawa*, Manju Lata Kapur and RK Khetarpal

Division of Plant Quarantine, National Bureau of Plant Genetic Resources, New Delhi-110012, India
**NRC on DNA Fingerprinting, National Bureau of Plant Genetic Resources, New Delhi-110012, India*

The germplasm of transgenics with desirable traits is being imported into the country for various research programmes. The National Bureau of Plant Genetic Resources (NBPGR) is the nodal agency to issue the import permit, to undertake quarantine processing of germplasm including transgenics and also to ensure absence of embryogenesis deactivator gene. During 1997-2008, a total of 7,008 samples of transgenic rice with different traits from nine countries were processed at NBPGR for quarantine clearance. Of the total samples, 1,642 samples were found infested/ infected with various pests from different countries. These comprised 1,548 from Singapore, 65 from Belgium, 16 from Vietnam, 7 from USA, 4 from China and 2 from the Philippines. The pests intercepted were insects viz., *Cryptolestes ferrugineus, C. pusillus, Liposcelides* spp., *Rhizopertha dominica, Sitophilus oryzae, Sitotroga cerealella* and *Tribolium castaneum*; nematode *viz.*, *Aphelenchoides besseyi*; and pathogens viz., *Alternaria alternata, A, padwickii, Drechslera halodes, D. rostrata, Fusarium dimerium, F. moniliforme* and *Phoma glumarum.* All the infested and infected samples were salvaged using various techniques. Also the risk associated (literature-based) with the import of rice from source countries has been evaluated.

The samples were also tested to ensure the absence of *cre recombinase* gene (the gene sequence involved in one of the steps of terminator technology) using polymerase chain reaction with the designed primer pair and *cre* gene sequence was not detected in any of the tested samples.

Key Words: Quarantine, Transgenic rice, Pest interceptions, Embryogenesis deactivator gene

Introduction

Transgenics or genetically modified crops are under cultivation in different countries including India. The germplasm of transgenics with desirable traits is being imported into the country for various research programmes. Movement of planting material poses the risk of inadvertent introduction of exotic pests, which may prove more dangerous in the new environment. This necessitates the strict implementation of plant quarantine regulations for the introduction of planting material. In India, import of transgenic planting material is permitted only for research as per the Plant Quarantine (Regulation of Import into India) Order, 2003. The National Bureau of Plant Genetic Resources (NBPGR) is the nodal agency to issue the import permit, to undertake quarantine processing of germplasm and also to ensure absence of embryogenesis deactivator gene (Khetarpal and Pandey, 2002). A number of pests have been intercepted in the introduced germplasm of transgenic and non-transgenic crops in the country (Khetarpal *et al*., 2006; Singh *et al*., 2003).

Materials and Methods

During 1997-2008, a total of 7,008 samples of transgenic rice with different traits from nine countries were processed at NBPGR for quarantine clearance (Table 1).

Quarantine Processing of Transgenic Rice

All the samples were first examined visually under higher magnification and then subjected to various techniques for detailed examination for detection of various pests (insects, mites, nematodes, fungi, bacteria and viruses) as given below:

Detection of Insect-pests: All the imported samples were examined visually either by naked eye or with the help of magnifying glass for detecting the presence of dead or actively moving larval and adult stages of insects, damaged/ deformed/ discolored seeds, plant parts, plant debris, dust, flour, webbing, presence of excreta, soil clods, etc.

Detection of Nematodes: As the paddy seeds are known/ suspected to carry seed-borne nematodes, these were soaked in water overnight to soften the seed coat and seeds were teased/ opened under the stereobinocular

[1] *Author for Correspondence: E-mail: sbhalla@nbpgr.ernet.in*

Table 1. Details of Transgenic Rice Processed for Quarantine Clearance

Transgene/trait	Source	No. of Samples
Ama1 for improved nutrition	Philippines	34
Herbicide (Basta) resistance, EPSPS-5 (enolpyruvylshikimate-3-phosphate synthase)	USA	3
cry1 Ac for resistance against lepidopteran pests	UK	14
*cry9C, cry*1*Ab* for resistance to lepidopteran pests	Belgium	86
*cry*1*Ab* for resistance to stem borer; *Xa 21* for resistance to bacterial blight; *PR* for resistance to sheath borer	Philippines	4
Phytoene synthase (Psy), bacterial phytoene desaturase (crt I) and lycopene B-cyclase (lcy)-genes for synthesis of β-carotene in rice endosperm	Switzerland	1
cry1Ab for resistance to stem borer	Belgium	75
Xa-21 for resistance to bacterial blight	Philippines	2
Psy, *crtl* and lycopene *b-Cyclase* (*Icy*) for synthesis of Beta-carotene in rice endosperm	Vietnam	21
Transposable element Ds	Singapore	6,598
"Cocodrie" capable of synthesizing β-carotene containing *Psy* (Phytoene synthase), *crtl* (Phytoene desaturase)	UK (England)	24
psy (Phytoene synthase), *crtl* (Phytoene desaturase) "Kaybonnet" capable of synthesizing β-carotene	USA	12
GFM *Cry1A* (*cry1Ab-1Ac*) Bt fusion gene Imparting insect-resistance	China	3
cry1Ab and *bar, cry1Ab, cry1Ac, PNC1* for resistance to lepidopteran insects and *bar* for resistance to glufosinate ammonium	Belgium	131
Total		7,008

microscope. "Staining technique" (Byrd *et al*., 1983; Holbrook *et al*., 1983) is used to locate the infected tissues in 20 days old seedlings. The plant parts were boiled in acid fuchsin lactophenol solution for few minutes and then destained in clear lactophenol. Nematodes, if present, retain red stain more deeply than the plant tissue and can easily be detected under stereobinocular microscope.

Detection of Fungi and Bacteria: All the samples were examined visually for detecting presence of fungal sclerotia, smut balls, malformed seeds, fungal frucitifications and symptoms on seed surface etc. Seeds were then subjected to Blotter test to detect fungal/ bacterial pathogens producing fungal and bacterial growth (Singh *et al*., 2003). Observations for the associated pathogenic fungi and bacteria were recorded on 8th day under stereobinocular microscope. Wherever needed, slides were also prepared for associated fungal spores/ conidia and for bacterial ooze and observed under compound microscope.

Post-entry Quarantine Inspection: Samples of rice grown by the indenters in Department of Biotechnology (DBT)-approved containment facilities/ glass houses were also inspected for seed-borne pests.

Ensuring the Absence of Embryogenesis Deactivator Gene Sequence

The samples were tested for ensuring the absence of embryogenesis deactivator gene. Seeds of all these samples were grown in National Containment Facility, 3-4 week old seedlings were used for DNA extraction and the residues were incinerated. DNA was extracted using protocol of Dellaporta *et al.* (1983) and Polymerase Chain Reaction analysis (PCR) was carried out using the primers designed for *cre* sequence of embryogenesis deactivator gene. Plasmid cloned with *cre* sequence was used as positive control in PCR analysis.

Risk of Pests Associated with Import of Rice from Source Countries

Available literature was screened for the associated risk of introducing exotic pests (pests not yet reported from India) with the import of rice from the source countries viz., Belgium, the Philippines, Singapore, Switzerland, UK, USA and Vietnam.

Results and Discussion

Of the total samples, 1,642 samples were found infested/ infected with various pests from different countries. These comprised 1,548 from Singapore, 65 from Belgium, 16 from Vietnam, 7 from USA, 4 from China and two from the Philippines. The pests intercepted are presented in Table 2.

The pests detected through visual examination were insect pests viz., *Cryptolestes ferrugineus, C. pusillus, Liposcelid* spp., *Rhizopertha dominica, Sitophilus oryzae, Sitotroga cerealella* and *Tribolium castaneum*. The

Table 2. Interception of Pests in Transgenic Rice

Pest	Country
Insects	
Cryptolestes ferrugineus, C. pusillus, Liposcelides spp., *Rhizopertha dominica, Sitophilus oryzae, Sitotroga cerealella, Tribolium castaneum*	Singapore
C. ferrugineus, R. dominica, S. cerealella, T. castaneum	China
Nematode	
Aphelenchoides besseyi	Singapore
Pathogens	
Fusarium moniliforme	Belgium
Alternaria padwickii	China
A. alternata, A, padwickii, Fusarium dimerium	Philippines
Drechslera halodes, D. rostrata, F. moniliforme, Phoma glumarum	Singapore
Curvularia lunata, D. oryzae, F. semitectum	Singapore, Vietnam
F. moniliforme	USA
Nigrospora oryzae, Verticillium cinnabarinum	Vietnam

soaking test revealed the presence of nematode, *Aphelenchoides besseyi*. The blotter test has led to the interception of fungi viz., *Alternaria padwickii, Drechslera halodes, D. oryzae, D. rostrata, Fusarium dimerium, F. moniliforme, F. semitectum, Nigrospora oryzae, Phoma glumarum* and *Verticillium cinnabarinum.*

Prior to release all the infested/ infected samples were salvaged and also provided prophylactic treatments by using various methods.

The infested seed lots were cleaned mechanically by removing dead/ live insects, damaged/ infested seeds, soil clods, plant debris, weeds and discoloured/ deformed seeds. Samples infested with insect pests were salvaged by fumigating with a mixture of EDCT (ethylene dichloride: carbon tetrachloride in the ratio of 1:4) mixture @ 320mg/ litre for 48 h at normal atmospheric pressure. The remaining healthy samples were also given prophylactic fumigation to safeguard against insect attack. For eliminating bacteria and nematodes, seeds were given hot water treatment at 52^0 C for 30 min. as a salvaging/ prophylactic treatment.

Post-entry Quarantine Inspection

The post-entry quarantine inspection of 137 samples grown in DBT approved containment facilities by the indenters viz., Hybrid Rice International (98) at Gurgaon and M/S Mahyco Seeds Ltd. (39) at Jalna was undertaken. Leaf and soil samples were collected and subjected to laboratory testing. The pests detected were fungi: *F. equisetum* and *N. oryzae*. However, no exotic pest of quarantine significance was detected.

Significance of Important Interceptions

Cryptolestes ferrugineus (Stephens) – (Coleoptera: Cucujidae), rusty grain beetle intercepted from Singapore can survive in sub-zero temperatures (Lee *et al*., 1992). *C. pusillus*, flat grain beetle is widespread in regions with warmer climates, can spread internationally with the movement of grain/ stored commodities. ***Liposcelides* spp.** (Psocoptera: Liposcelididae), psocids damage results in the loss of quality. Heavy infestation elicits an allergic response in susceptible people (Turner *et al*., 1996). All the stages can be transported with infested commodities. ***Rhizopertha dominica*** Fabricius (Coleoptera: Bostrychidae), lesser grain borer, a serious pest of stored products with wide host range is of quarantine significance in international trade in food products (CAB International, 2007). ***Sitophilus oryzae*** Linnaeus (Coleoptera: Dryophthoridae), rice weevil, a serious pest with adult longevity of about 7-8 months, causes loss in stored rice (unhusked). Also causes reduction in protein content of grains (CAB International, 2007). Though reported from India, it is of quarantine significance as 38 strains of three species of *Sitophilus* are reported (Grenier *et al*., 2000). ***Sitotroga cerealella*** Olivier (Lepidoptera: Gelechidae), rice grain moth is a major pest in storage and also occurs in field in tropical and subtropical countries and has 12 generations per year. An overall yield losses upto 30% have been reported (Singh and Benazet,1975). *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), red flour beetle is a secondary pest of stored material. A single larva can cause a mean dry weight loss of 12.3 mg, in laboratory tests on millet (Roorda *et al*., 1982). Larvae primarily feed on germ point and damaged grains are unfit for sowing. Adults may live for about 18 months depending on weather conditions.

Aphelenchoides besseyi (Aphelenchoididae) is widely distributed and causes damage more in lowland and deep water systems than in upland environments. Though species is reported from India, there is an inherent risk of introduction of new biotypes having wider host range (Rajan *et al*., 1990; Gokte *et al*., 2001). The seed-transmitted nematode swims vigorously in water, readily cultures on most of the fungi and can survive drying in adverse situations.

Alternaria padwickii (Ganguly) M.B. Ellis (= ***Trichoconis padwickii*** Ganguly) – a fungus causes grain discoloration alone or in combination with other

fungi, can influence the market value (Lee *et al.*, 1986). High levels of seed infection and associated seed rotting can seriously affect crop establishment in nursery beds and in the field (Ou, 1985). ***Drechslera oryzae*** (Breda de Haan) Subram. & Jain (=***Cochliobolus miyabeanus*** (Ito & Kurib.) Drechsler ex Dastur), causes brown leaf spot of rice and this was the main cause of the Great Bengal Famine of 1943 in India, which resulted in yield losses ranging from 40 to 90% (Padmanabhan, 1973). ***Fusarium moniliforme*** Sheldon (= ***Gibberella fujikuroi*** (Sawada) S. Ito) causes bakanae disease of rice, and yield losses have been reported as high as 40-50% in Japan, 15% in eastern districts of Uttar Pradesh, India, and 3.7-14.7% in northern and central Thailand (Ou, 1985). In 1993 disease incidences of 4.17 and 96.25% caused grain losses of 3.04 and 95.45%, respectively, on inoculated rice cultivar Taraori Basmati in India (Sunder *et al.*, 1997).

The samples with different transgenes (Table 1) were tested to ensure the absence of embryogenesis deactivator gene. PCR analysis was carried out using the primers designed for *cre* sequence of embryogenesis deactivator gene. Plasmid cloned with *cre* sequence was used as positive control in PCR analysis. The amplicon of 1031 bp size was amplified only in positive plasmid sample while, no amplicon of corresponding size was observed in any of tested rice samples thus ensuring the absence of embryogenesis deactivator gene (Fig. 1).

Screening of the available literature revealed that the import of rice from different countries is associated with risk of introducing pests (Table 3). These are the pests which are either not yet reported from India or requires additional declaration under Schedule V and VI of Plant Quarantine (Regulation of Import into India) Order 2003.

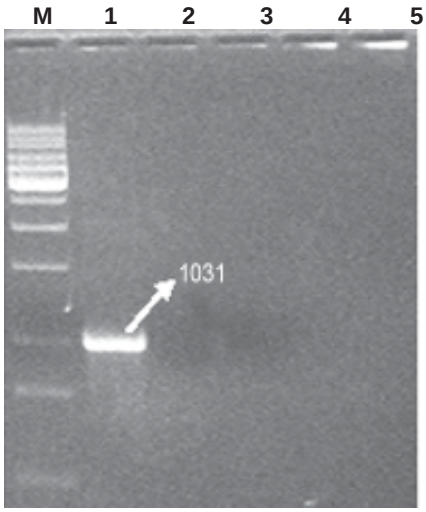

Fig. 1: Polymerase chain reaction-based detection of *cre* terminator gene sequence in transgenic rice

The number of interceptions made during quarantine of imported transgenic rice and the literature screening reveals that its import also poses the risk of introduction of various pests as their non-transgenic counterparts and highlights the significance of quarantine. The pests with races/ biotypes/ strains are of quarantine significance as more virulent strains or races may get introduced. This further requires preparedness in terms of detection and disinfestation protocols to deal with the bulk imports. Analysis of risk prior to import is also the first step in this direction. Ensuring the absence of embryogenesis deactivator gene sequence is very important for the perpetuation of the material.

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Table 3. Risk of Introducing Pests of Quarantine Significance along with Import of Rice from Different Countries

Pest	Belgium	Philippines	Singapore	Switzerland	UK	USA	Vietnam
Insects							
**Ahasversus advena*, Foreign grain beetle	–	+	+	–	+	+	–
**Haplothrips aculeatus*, Cereal thrips	–	–	–	–	–	–	+
#*Sitophilus granarius*, Grain weevil	+	–	–	–	+	+	–
**Trogoderma variabile* Grain dermestid	–	–	–	–	+	+	–
Fungus							
* *Monographella nivalis*	+	–	–	+	+	+	–
Bacteria							
*# *Burkholderia glumae* Bacterial grain rot	–	+	–	–	–	–	–
**#Pseudomonas fuscovaginae* Sheath brown rot	–	+	–	–	–	–	–

* Pest not reported from India, # Pests requiring additional declaration as per Plant Quarantine (Regulation of Import into India) Order (2003), + reported from the country, - not reported from the country.

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