

ADVISORY COMMITTEE ON NOVEL FOOD AND PROCESSES

UNAUTHORISED PRESENCE OF GM RICE (LLRICE601) IN LONG GRAIN RICE FROM THE USA

Issue

This paper provides information on LLRICE601, an unauthorised line of GM rice that has recently been identified in samples of US long grain rice. Drawing on this example, the Committee is invited to comment on the risk assessment of unauthorised GM foods

Background

1. On 18 August the US Department of Agriculture (USDA) issued a statement indicating that traces of LLRICE601 had been identified in rice samples. The US authorities considered that this GM rice poses no food or animal feed safety concerns, as indicated in a short statement issued by the Food and Drug Administration (**Annex A**).
2. In the US, two types of herbicide-tolerant GM rice (LLRICE62 and LLRICE06) have been officially cleared to be grown and used for food and feed. Although these authorisations are in place, no GM rice has so far been grown commercially in the US. LLRICE62 is currently being evaluated for use in food in the EU.
3. The company responsible for development of these GM rice lines (Bayer CropScience) notified the US authorities on 31 July that “traces” of another type of GM rice, LLRICE601, were detected in samples of commercial long grain rice and may have entered the food and feed supply. LLRICE601 is another line of GM rice developed by Bayer; it was used in field trials in 1998-2001 but was not taken forward for authorisation
4. Like LLRICE62 and LLRICE06, LLRICE601 has been genetically modified by the addition of a bar gene, which codes for the enzyme PAT (phosphinothricin acetyl transferase). The presence of PAT renders the plants tolerant to the herbicide glufosinate ammonium.
5. When the incident was first notified, Bayer informed the FSA that the level of GM seeds detected in the contaminated samples was very low, around 0.03%. Subsequent reports have confirmed that the LLRICE601 has been found in a proportion of US rice samples, in trace amounts. Bayer CropScience does not supply rice seed commercially in the US and the origin of the GM contamination has not yet been identified.

EU response

6. This is an international issue involving imports into the EU and the European Commission has therefore taken the lead and has been dealing with Bayer CropScience and with the US authorities to obtain further information. However, it quickly became clear that the US government and US rice exporters could not guarantee that future rice exports would not contain the unauthorised GM rice.
7. On 23 August the Commission therefore adopted an emergency measure that requires all consignments of US long grain rice to be tested and certified to be free from LLRICE601 before they can be imported into the EU. The Commission also passed the information provided by Bayer CropScience to the European Food Safety Authority, to obtain advice on the safety of this GM rice.

UK action

8. While the Commission has a leading role in the regulation of imports, it is the responsibility of member states to take action in relation to products that are in circulation, including rice that was imported before the incident came to light. The Food Standards Agency needed to assess quickly whether there was a need for urgent action in case LLRICE601 was present in rice that was already be on the market in the UK.
9. The Agency initially contacted the ACNFP Chairman who advised that the limited information available at that time (24 August) provided no pointers to suggest that LLRICE601 was a significant safety concern. This was on the basis that the inserted DNA contained only a small number of sequences as most of the "backbone" of the bacterial-derived vector had been removed, including the kanamycin resistance gene nptIII. The only transgenic protein introduced into the transformants is the PAT enzyme, which has already been fully evaluated in the context of maize lines Bt11 and T25 and found not to present any concerns. A more detailed assessment of LLRICE601 would be required to confirm the identity of the expressed protein and to examine any unintended effects. The Chairman also noted that there was no evidence that the GM rice was in circulation in the UK at that time.
10. When it became apparent that EFSA's assessment would not be available before the next meeting of its GMO Panel on 13-14 September, the Agency sought further advice from the ACNFP Chairman and another Committee member. This advice was sought on the basis of a dossier of information on LLRICE601 prepared by Bayer, and circulated by the Commission to Member States on 25 August (**Annex B**). This dossier contains information on the nature of the genetic modification, the transgenic protein and general characteristics of the transformed plants. The members were also informed that LLRICE601 might be present in some shipments of long grain rice at levels around 0.1%.
11. Based on this information, the two members agreed that the presence of low levels of LLRICE601 in the food supply did not present a safety concern. The Agency subsequently published a Press Release relaying this advice to the public (**Annex C**). The Agency would conduct a survey at the levels of UK rice

mills, to ensure that further batches of contaminated rice do not enter the food supply chain. The majority (>90%) of US long grain rice imported into the UK is un-milled and requires processing by millers before the rice can be sold to consumers or used in food manufacture.

EFSA risk assessment

12. Bayer subsequently provided an updated version of this dossier, which was evaluated by EFSA's GMO Panel on 13-14 September. The Panel's opinion is attached (**Annex D**) and concluded that, while the data are insufficient to conduct the full risk assessment that would be required for authorisation of LLRICE601, "the consumption of imported long grain rice containing trace levels of LLRICE601 is not likely to pose an imminent safety concern to humans or animals".

Correspondence from Genetic Food Alert UK

13. The attached letter (**Annex E**) questions the basis for the initial advice offered by ACNFP members, asking whether this was on the basis that (a) there were sufficient data to prove the safety of the GM rice, or (b) there were insufficient data to prove it was harmful. This specific question will be dealt with by the Secretariat in consultation with the individual members who provided the advice. It does however highlight the more general question of how to conduct urgent risk assessments of unauthorised GM material that is adventitiously present in food.

Risk assessment of unauthorised GM foods

14. It is possible that this situation could arise in several scenarios, including imported food from countries where GM crops are grown that are not authorised in the EU; accidental planting of unauthorised GM seeds (as in the case of Bt10 maize, discovered in 2005); or low-level presence of unauthorised GM events in other varieties due to the impurity of seed used by farmers.
15. Under EU regulations, unauthorised GM material in food or animal feed is illegal, at any level, and should not be on the market. Enforcement of food law is based on the principle of proportionality and the action to be taken in cases of non-compliance is driven by the risk. Hence there is a need to obtain urgent risk assessment advice in all food incidents, including those involving GM food and feed.
16. Parallel situations occur from time to time in relation to other substances found to be present in food, such as unauthorised food additives or environmental contaminants. Where there is no existing risk assessment at UK or EU level the Agency will obtain advice quickly from in-house scientists and/or external experts, including Committee members. The purpose of these assessments is to determine the immediate risk to health and in many cases the judgements must be made on the basis of incomplete data.

17. The present guidelines for assessment of GM food are intended to ensure the maximum assurance of safety, so that the food from GM crop varieties can be marketed and consumed on a permanent basis. If an unauthorised event is found to be present in the food supply, it is unlikely that all of the data needed for a full assessment will be available. Depending on the source of the unauthorised material, it may be present in the food supply at low levels and for a limited period.
18. The adventitious presence of GM material is one of the issues due to be discussed at the Codex ad hoc Task Force on Foods Derived from Biotechnology in November 2006, and drawing on the current incident it would be useful to have the Committee's comments on possible approaches to the risk assessment of unauthorised GM material in food.

Committee action required

19. The Committee is invited to

- note the information on LLRICE601; and
- consider whether it would be possible and useful to develop guidelines for the identification of immediate risks to health associated with unauthorised GM food.

**Secretariat
September 2006**

Annexes attached:

Annex A: Statements from the US Department of Agriculture and the Food and Drug Administration (18 August 2006)

Annex C: Food Standards Agency Press Release (1 September 2006)

Annex D: EFFO GMO Panel opinion on LLRICE601 (15 September 2006)

Annex E: Letter from Robert Vint, Genetic Food Alert UK (18 September 2006)

Available on request:

Annex B: Bayer CropScience application for deregulation of LLRICE601 (17 August 2006) - an updated version of this document can be found at:
http://www.aphis.usda.gov/brs/aphisdocs/06_23401p.pdf

**STATEMENTS FROM THE US DEPARTMENT OF AGRICULTURE AND THE
FOOD AND DRUG ADMINISTRATION (18 AUGUST 2006)**

Documents published at

<http://www.usda.gov/2006/08/0307.xml> and
<http://www.cfsan.fda.gov/%7Elrd/biorice.html>

**BAYER CROPSCIENCE APPLICATION FOR DEREGULATION OF LLRICE601
(DATED AUGUST 2006)**

An updated version of this document can be found at:
http://www.aphis.usda.gov/brs/aphisdocs/06_23401p.pdf

FOOD STANDARDS AGENCY PRESS RELEASE (1 SEPTEMBER 2006)

Document published at

<http://www.foodstandards.gov.uk/news/pressreleases/2006/sep/gmricetesting>

EFSA GMO PANEL OPINION ON LLRICE601(15 SEPTEMBER 2006)

Document published at

http://www.efsa.europa.eu/en/science/gmo/gmo_statements0.html

LETTER FROM GENETIC FOOD ALERT UK

18 September 2006

TO: The ACNFP

FROM: Robert Vint, Director, Genetic Food Alert UK

RE: Safety Assessment of LLRICE601

Dear Sirs,

In relation to the widespread contamination of long grain rice imports with illegal LLRICE601 the EFSA has recently stated that there is "insufficient data to provide a full risk assessment in accordance with EFSA's GM guidance".

In the absence of such data it is unable to state categorically either that LLRICE601 is hazardous or that LLRICE601 is safe. I understand that the ACNFP is advising the FSA on this matter. The ACNFP could:

a) assume, in accord with the Precautionary Principle, that LLRICE601 "may be a risk" because there is inadequate proof of safety, and therefore should be removed from sale

or

b) assume, according to what one might call the USA's 'Inverse Precautionary Principle', that LLRICE601 "may be safe" because there is inadequate proof of danger, and therefore can continue to be sold.

In the absence of adequate safety data is the ACNFP adopting the precautionary principle or is it assuming that rice contaminated with LL601 can continue to be sold because it "may be safe"? Which is the most appropriate response to the absence of adequate data?

I look forward to your response to this urgent matter at the earliest opportunity.

Yours faithfully,

Robert Vint

Director

Genetic Food Alert UK



**Application for an Extension of the Determination of Nonregulated
Status for Glufosinate-Tolerant Rice (98-329-01p):**

**Transformation Event LLRICE601
(amended)**

The undersigned submits this petition under 7 CFR 340.6 to request that the Director, Scientific Services, makes a determination that the article should not be regulated under 7 CFR 340.

Submitted by:

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August 17, 2006



COMPANY NAMES

On June 3, 2002, Bayer CropScience was formed by the acquisition of Aventis CropScience by Bayer AG. From this date, Bayer CropScience is the agricultural business unit of Bayer that is engaged in the research, development, and marketing of crop protection, seed technology, turf and ornamentals, professional pest and vector control, and home and garden products.

On December 15, 1999, Aventis S.A. was formed by the completion of the merger between Hoechst AG and Rhône-Poulenc S.A. Hoechst AG was the parent company of AgrEvo USA Company.

Some of the activities described in this report were undertaken before the merger and acquisition. Consequently, the names Aventis CropScience, AgrEvo USA Company, AgrEvo, and Hoechst Schering AgrEvo GmbH may appear throughout this report. However, all inquiries regarding this report and the data contained herein should be addressed to: Bayer CropScience, P. O. Box 12014, 2 T. W. Alexander Drive, Research Triangle Park, North Carolina, 27709.



SUMMARY

Bayer CropScience requests a determination from APHIS that rice with glufosinate herbicide tolerance event LLRICE601 and any progeny derived from crosses of this event with traditional rice varieties, and any progeny derived from crosses of this event with transgenic rice varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340, and that APHIS consider this document as an extension to petition 98-329-01p.

Glufosinate-tolerant rice based upon the transformation event LLRICE601 was produced by the introduction of a chimeric 35S/*bar* gene construct using *Agrobacterium*-mediated gene transfer. The rice events described in petition 98-329-01p were transformed by direct gene transfer of a chimeric 35S/*bar* gene construct. All events produce the same protein, the enzyme phosphinothricin acetyltransferase (PAT), which confers resistance to the herbicide glufosinate.

Agronomic evaluation has demonstrated that there were no morphological, beneficial organism, disease susceptibility or pest susceptibility differences observed when comparing the events to cultivated rice.

Regulatory status of glufosinate-tolerant rice in the USA:

- 1) USDA. 1999. Determination of non-regulated status for rice genetically engineered for glufosinate herbicide tolerance. Federal Register 64:22595-22596. Environmental Assessment and Finding of No Significant Impact <www.aphis.usda.gov/biotech/dec_docs/9832901p_det_ea.html>.
- 2) FDA, Center for Food Safety and Applied Nutrition, Office of Pre-Market Approval. 2000. Biotechnology Consultation Note to the File BFN No. 000063 <www.cfsan.fda.gov/~rdb/bfnm063.html>



CERTIFICATION

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which is unfavorable to the petition.

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ACRONYMS, SYNONYMS AND SCIENTIFIC TERMS

APHIS – Animal and Plant Health Inspection Service
bar – Phosphinothricin Acetyltransferase Gene - bialaphos resistance gene
BLAST -Basic Local Alignment Search Tool
bp – base pairs
CaMV – Cauliflower Mosaic Virus
cm - centimeter
DNA – Deoxyribo-Nucleic Acid
ELISA - Enzyme Linked Immunosorbent Assay
FDA – Federal Drug Administration
germ - germination
ID - identification
LB - Left Border
lbs - pounds
LibertyLink® – Bayer CropScience trade name for events that are glufosinate (Liberty®) tolerant
LOD – Limit of Detection
LSU – Louisiana State University
mm – millimeter
mM - millimolar
MW – molecular weight
ng - nanogram
n.s. – not significant
ND - Not Detectable: Below the limit of detection.
nm – nanometers
nos – nopaline synthase
NT- Non-transgenic
ORF – Open Reading Frame
PAT – Phosphinothricin Acetyltransferase Protein
PCR – Polymerase Chain Reaction
PD1, PD2, etc – Planting Date
PVP – Plant Variety Protection
RB – Right Border
SD - Standard Deviation
 $T_1, T_2, \text{ etc}$ – generations after T_0 (transformation)
T-DNA – transfer DNA from *Agrobacterium*
US – United States
USA - United States of America
USDA – United States Department of Agriculture
vir – virulence
WT – wild type

States and territories:

LA - Louisiana
TX - Texas

MS - Mississippi
AR - Arkansas
PR – Puerto Rico



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Statement of Grounds for Nonregulated Status

I. Rationale for Submission of Request for Extension

There are no changes in the rationale from the previously approved petition; 98-329-01p, entitled “Petition for Determination of Nonregulated Status: LibertyLink® Rice Transformation Events LLRICE06 and LLRICE62.” The specific differences between LLRICE601 and its progeny, and the events in the previous petition are discussed in the appropriate sections. (see also Table 1).

The new event to be considered under this extension is LLRICE601.

Table 1. Comparison of events LLRICE62 and LLRICE06 with LLRICE601

Characteristic	LLRICE601	Events LLRICE06 and LLRICE62
Crop	Rice	Rice
Genus species name	<i>Oryza sativa</i> L.	<i>Oryza sativa</i> L.
Parent Line	Cocodrie	M202 and Bengal
Transformation Method	<i>Agrobacterium tumefaciens</i> mediated transformation	Direct gene transfer
Trait	Tolerance to glufosinate herbicide	Tolerance to glufosinate herbicide
Gene product	phosphinothricin acetyltransferase (PAT)	phosphinothricin acetyltransferase (PAT)
Vector	pGSV71	pB5/35Sbar
Gene /Donor	Phosphinothricin acetyltransferase (<i>bar</i>) gene/ <i>Streptomyces hygroscopicus</i>	Phosphinothricin acetyltransferase (<i>bar</i>) gene/ <i>Streptomyces hygroscopicus</i>
Promoter/Donor	35S promoter (P35S) / Cauliflower Mosaic Virus	35S promoter (P35S) / Cauliflower Mosaic Virus
Terminator/Donor	3' untranslated end of the nopaline synthase gene / <i>Agrobacterium tumefaciens</i>	35S terminator (T35S) / Cauliflower Mosaic Virus



II. The Rice Family

There are no changes from the previously approved petition submission.

III. The Transformation System

The LLRICE601 event was obtained using a different approach to insert the *bar* gene into rice. The transformation system used was *Agrobacterium*-based and the parent line was Cocodrie, a widely grown long grain rice variety. Neither the use of a different transformation system nor the use of a different parent variety changes the rationale for determination of nonregulated status. **The *bar* gene and the 35S promoter are common genetic elements used in the current and previous events.**

A. Transformation System

For transformation of plants, the vector system as described by Deblaere *et al.* (1985, 1987) is used. The vector system consists of an *Agrobacterium* strain and two plasmid components: 1) an intermediate cloning vector, plasmid pGSV71, and 2) a non-oncogenic Ti-plasmid.

The *Agrobacterium* is co-cultivated with the small rice tissues and then removed. Transformed rice cells are selected by addition of glufosinate ammonium (with phosphinothricin 5 mg/L) to the rice tissue culture medium. Calli growing on glufosinate ammonium are transferred to regeneration medium. When plantlets with roots and shoots develop, they are transferred to soil, and placed in the greenhouse.

The transformation is confirmed by phosphinothricin acetyl transferase activity assay, by glufosinate ammonium application to leaves, and by PCR and Southern blot analysis.

B. Parent Line

The parent line used for the transformation was Cocodrie, a long grain rice variety with broad adaptation for the Southern US (Linscombe, *et al.* 2000).

C. Construction of the Plasmid Used for Transformation

The plasmid pGSV71 was derived from pGSC1700 (Cornelissen and Vandewiele, 1989). It contains an artificial T-region consisting of the left and right border sequences of the TL-DNA from pTiB6S3 and multilinker cloning sites allowing the insertion of chimeric genes between the T-DNA border repeats. There are no residual T-DNA sequences present between the border repeats. In pGSV71, the gene of interest, inserted between the T-DNA border repeats, is *P35S-bar-3'nos*.



The acceptor *Agrobacterium* strain carries a non-oncogenic (disarmed) Ti plasmid from which the T-region has been deleted. This Ti plasmid carries the necessary *vir* gene functions that are required for transfer of the T-DNA region of the plasmid pGSV71 to the plant genome. It also has a homology region that allows co-integrate formation with pGSV71.

Plasmid pGSV71 is constructed in *Escherichia coli*. It is transferred to the acceptor *Agrobacterium tumefaciens* strain via a triparental mating involving an *E. coli* strain that carries a mobilization helper plasmid (Van Haute *et al.*, 1983, Deblaere *et al.*, 1987). The structure of the T-DNA in the resulting *Agrobacterium* strain is confirmed by Southern blot hybridization (Deblaere *et al.*, 1985). *Agrobacterium*-mediated gene transfer of pGSV71 results in transfer to the plant genome of the DNA fragment between the T-DNA border repeats.

D. Open Reading Frames and Associated Regulatory Regions in pGSV71

The chimeric *bar* gene construct used in LLRICE601 contains the 35S promoter from the Cauliflower Mosaic Virus (CaMV), followed by the 3' untranslated region of the nopaline synthase (*nos*) gene. The transforming DNA fragment was derived from plasmid pGSV71, which contains no other genes expressed in plants. A map of the vector pGSV71 is shown in Figure 1. A description of the DNA elements between the right and left border containing *P35S-bar-3'nos* is provided in Table 2.

CaMV 35S promoter

The 35S promoter sequence are derived from CaMV and control expression of the *bar* gene. CaMV is a doublestranded DNA cauliflower mosaic virus with a host range restricted primarily to cruciferous plants. The 35S promoter directs high level constitutive expression and is widely used as a promoter for high expression of genes. The CaMV sequences, as used in the LibertyLink® Rice, do not cause the rice to become a plant pest as determined in the LLRICE62 petition.

bar gene

The *bar* gene was isolated from *Streptomyces hygrosopicus*, strain HP632 (Thompson *et al.*, 1987). It encodes the enzyme phosphinothricin acetyltransferase (PAT), which confers resistance to the phytotoxic activity of glufosinate ammonium, the active ingredient of Liberty® Herbicide.

3' nos terminator

A 260bp *TaqI* fragment from the 3' nontranslated region of the nopaline synthase gene (3' *nos*) from the T-DNA of pTiT37 was isolated from *Agrobacterium tumefaciens* (Depicker *et al.*, 1982). The 3' *nos* terminator controls the expression of the *bar* gene due to its role in transcription termination and polyadenylation (Depicker *et al.*, 1982).

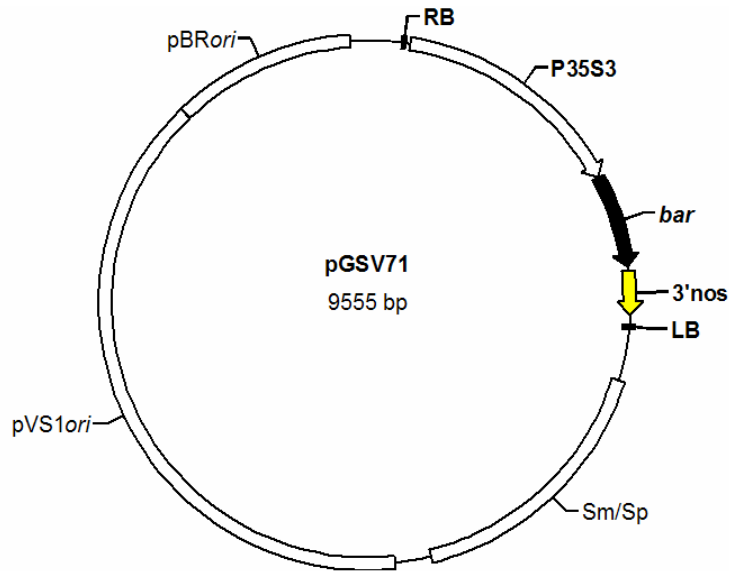


Figure 1. Map of the vector pGSV71

Table 2. Genetic Elements of the plasmid pGSV71

Position in Vector	Genetic Element and Function
198–222	Right border repeat from the TL-DNA from pTiB6S3 (Gielen <i>et al.</i> 1984)
223–249	Polylinker derived sequences
250–1634	<i>P35S3</i> : promoter region from the Cauliflower Mosaic Virus 35S transcript (Odell <i>et al.</i> 1985)
1635–2186	The coding sequence of the bialaphos resistance gene (<i>bar</i>) of <i>Streptomyces hygrosopicus</i> (Thompson <i>et al.</i> 1987). The N-terminal two codons of the wild type <i>bar</i> coding region have been substituted for the codons ATG and GAC respectively.
2187–2205	Polylinker derived sequences
2206–2465	A 260 bp <i>TaqI</i> fragment from the 3' untranslated end of the nopaline synthase gene (3' <i>nos</i>) from the T-DNA of pTiT37 (Depicker <i>et al.</i> 1982)
2466–2519	Polylinker derived sequences
2520–2544	Left border repeat from the TL-DNA from pTiB6S3 (Gielen <i>et al.</i> 1984)

IV. Genetic Characterization of LLRICE601

A. Description, History and Mendelian Inheritance

The early generation observations of LLRICE601 were conducted in small field plots. The first field test was in a winter nursery setting in Puerto Rico (winter of 1998-99). Subsequent field activities allowed the evaluation of the material to assess the stability and performance of the introduced trait and the agronomic characteristics of the event. The parent variety, Cocodrie is widely grown in the Southern US states of Arkansas, Louisiana, Mississippi and Texas. Table 3 presents a summary of the field trials and associated authorization permits.

Table 3. Summary of field activities under USDA permits for event LLRICE601.

USDA Authorization	Planting dates	Number of locations	Type of Trial	Location
98-254-02n (LLF-8B)	Dec 1998	1	Breeding, T ₁ generation	Puerto Rico
99-019-06n (LL2-9C)	May 1999	1	Breeding, T ₂ generation	LA
99-266-05n (LL-9F)	Nov 1999	1	Breeding, T ₃ generation	Puerto Rico
00-049-12n (LL-0A)	May 2000	7	Breeding, T ₄ and agronomic evaluation	LA, MS
00-076-06n (LL-0D)	May 2000	4	Nutritional composition testing	LA, MS, AR
00-124-05n (LL-0G)	June 2000	3	Agronomic evaluation	AR, LA
00-243-02n (LL-0L)	Nov 2000	1	Seed increase	Puerto Rico
01-071-04n (LL-1A)	May 2001	8	Agronomic evaluation	AR, LA, MS
01-110-01n (LL-1C)	May 2001	1	Agronomic evaluation	TX

Copies of the termination reports for these field trials are provided in Appendix 1.

T1 seed harvested from self-pollinated T0 plants surviving a glufosinate herbicide greenhouse screen were planted in December 1998 in Puerto Rico. T1 plants were selected for survival following glufosinate herbicide application. Panicles were harvested from individual plants and T2 panicle rows were planted in May 1999 at Louisiana State University (LSU) for evaluation. Each row was planted with the seed of a single panicle.



Application of glufosinate herbicide was used to score the rows for segregation of the herbicide tolerant phenotype (Table 4). Rows containing no sensitive plants were considered to be homozygous for the *bar* gene, while the partially resistant rows were considered hemizygous. In this situation, Mendelian inheritance for a single gene locus would predict one fully resistant row for every two partially resistant rows. The expected ratio was achieved with a high degree of certainty (see Table 4 for Chi² value). The fully resistant rows were harvested as independent populations for advanced variety evaluation. Panicles of the fully resistant rows were taken to winter nursery in Puerto Rico in 1999 for seed increase to supply the multi-state evaluations conducted in 2000. Each panicle-row was increased as an independent line and best lines were selected for further evaluation. A schematic graphic of the breeding process is illustrated in Figure 2. All plants are self pollinated.

Table 4. Segregation Analysis of Event LLRICE601

T₂ Panicle Rows

Fully Resistance Rows	Partially Resistant Rows	Total Rows	Expected Ratio	Chi² Value*
48	96	144	1:2	0.005

*No significant difference for the Chi square goodness of fit test for the hypothesis of 1:2 segregation. Significance test level at p=0.05 for Chi² values greater than 3.84, with one degree of freedom.

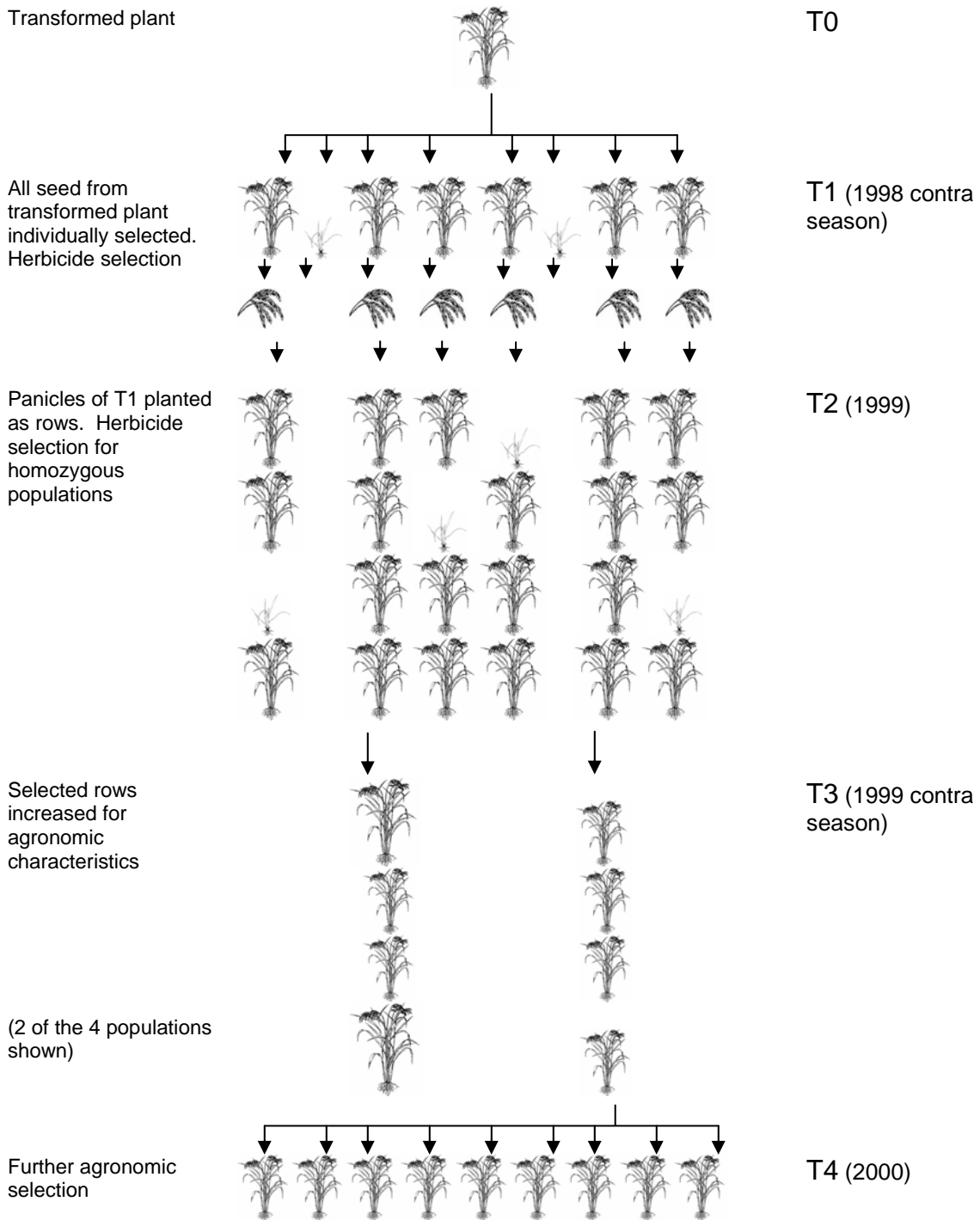


Figure 2. Plant selection diagram



B. Genetic Analysis of Event LLRICE601

Molecular characterization of the insertion event, LLRICE601 has confirmed the presence of one copy of the *bar* gene. Southern blot hybridization data with genomic DNA cut with different restriction enzymes demonstrate that the event LLRICE601 contains only one copy of the gene of interest (*bar*) (see Appendix 2). This is supported by the analysis of Mendelian inheritance.

In addition, it has been found that a second copy of the P35S promoter or part of it, is also present, but not the *bar* gene, therefore, the event is a single gene insert. The random insertion of an extra 35S promoter, or part of it, in the rice genome is unlikely to have any consequence as the effectiveness of the promoter is dependent on its full insertion and inserting close enough to DNA encoding a functional gene. In the unlikely case that a full promoter was inserted, at a low frequency, it could potentially insert near enough to another gene to alter the expression of a native rice gene. Alternatively, it could integrate within a native rice gene and disrupt its function. As submitted phenotypic and compositional data revealed no differences between LLRICE601 and the parent variety, if there are any changes in gene expression, those changes do not appear to pose a plant pest risk.

The *bar* gene was used as the selectable marker, therefore the same gene of interest acts as a marker. No other marker genes were present.

No bacterial origin of replication is transferred with the *Agrobacterium* mediated transformation system. The inserted DNA within event LLRICE601 will not add a bacterial origin of replication to the wild type *Oryza sativa* genome as a result of the transformation.

Southern blot hybridization between genomic DNA of the event LLRICE601 and the vector DNA demonstrate the absence of any coding sequences from the vector used for the transformation, including the spectinomycin gene, integrated into the rice genome. (see Appendix 3)

In addition, the stability of the insert over generations was demonstrated by Southern blot analysis (Appendix 4) and was supported by the Mendelian inheritance of the tolerance to glufosinate.

The transgene can be characterized by the location and the configuration at the site of incorporation of the recombinant DNA molecule in the plant genome. The site in the plant genome where a transgene has been inserted is also referred to as the "insertion site" or "target site". A flanking region or sequence refers to a sequence of at least 20 bp (up to 5000 bp) of the plant genome which is located either immediately upstream and/or downstream of and contiguous with the transgene.



Transformation procedures leading to random integration will result in transformants with unique flanking sequences, that will not be altered by conventional crossing. The query sequences were subjected to a BLASTn similarities search in order to map the site of integration of *Oryza sativa* event LLRICE601 on the rice genome and to find similarities between plant flanking sequences and known genes. Sequence alignment between 5-prime and 3-prime query sequences against different databases located the site of integration in *Oryza sativa* elite event LLRICE601 on chromosome 12.

Due to the insertion of the *P35S3-bar-3'nos* gene cassette in rice, a 5' and 3' junction, where rice genomic DNA and inserted T-DNA are fused, was created. The flanking regions were analyzed to confirm that no important rice genes were interrupted and that no chimeric proteins would be expressed due to this insertion.

Open reading frame (ORF) and gene search tools were applied to predict the presence of potential newly created coding sequences in the 5-prime flanking genomic/insert DNA junction region and in the 3-prime flanking insert/genomic DNA junction region. Several bioinformatics tools were applied to look for regulatory elements to see if these newly created ORFs could be putatively active. Alignment of the 5-prime and 3-prime flanking sequences with a fragment of wild-type chromosome twelve containing homologous sequences confirmed the presence of the target site deletion of 19 bp in the transgenic locus of *Oryza sativa* event LLRICE601. No homology was found with known genes, mRNA, cDNA or ESTs in the flanking rice genomic DNA. From these analyses we can conclude that no known rice genes were interrupted due to the insertion of the *P35S3-bar-3'nos* gene cassette into the rice genome and the probability of an expression of newly created proteins coming from the 5' or 3' junction region is also highly unlikely. (see Appendix 5)

No information obtained in the molecular characterization of the inserted DNA has changed the rationale for determination of nonregulated status.

C. Gene Expression of Event LLRICE601

The field performance criteria for LibertyLink® rice varieties requires plants to be tolerant to the herbicide, glufosinate ammonium (tradenname, Liberty®) in the vegetative stages of rice plant development, spanning the rice plant growth stages of first leaf to panicle initiation. Liberty® herbicide applications are recommended for the rice plant growth stages 2-4 leaf and first tiller. The leaves (blade and sheath) of the rice plant are the principle plant parts exposed to herbicide applications. Commercial-level herbicide tolerance depends upon the function of PAT enzyme in the leaves. The event selection process employed by Bayer CropScience targets a commercial crop tolerance and transformation event, LLRICE601 meets this criteria.



The content of phosphinothricin acetyltransferase (PAT) protein, encoded by the bar gene, was determined in rice grain by an Enzyme Linked Immunosorbent Assay (ELISA). Polyclonal antibodies recognizing PAT protein were used in the ELISA.

PAT protein constitutes 119 ng/g fresh weight of grain of LLRICE601. This corresponds 0.000034% of the crude protein in grain of rice event LLRICE601. For comparison, the amount of PAT protein measured in the grain of LLRICE62 was reported to be 12 µg/g fresh weight of grain or 0.0012% of the crude protein in the grain.

These differences in the amount of PAT protein do not change the rationale for determination of nonregulated status. The PAT protein represents only a small component of the total protein in the current and previous events and PAT has been demonstrated safe for consumption and the environment.

a. Expression of the PAT protein

The expression of the PAT protein was evaluated in grain produced in the 1999 season at the Louisiana State University Agricultural Center in Crowley, Louisiana. Rice grain from harvested at maturity (Table 5) were analyzed for PAT protein content by quantitative ELISA. PAT protein constitutes 119 ng/g fresh weight of grain. This corresponds 0.000034% of the crude protein in grain of rice event LLRICE601. PAT was not found in the control grain.

The limit of detection (LOD) is determined using the average standard curve and the concentration derived from the background optical density (OD) of the negative control samples. The LOD is the concentration corresponding to an OD value three standard deviations above the mean background OD. The LOD for this ELISA method was thus estimated to be 9.4 ng/g fresh weight of grain.

The limit of quantitation (LOQ) is given by the lowest concentration of the standard that meets the criteria for the LOQ. Validity criteria are a) analyte recoveries from fortified matrix samples are 60 % and 130 % and b) the coefficient of variance (relative standard deviation) is less than 25%. When a lower recovery is caused by the nature of the specific matrix, the lowest concentration of the standard that gives a smaller coefficient of variance than 25% is used as the LOQ. The limit of quantitation (LOQ) was estimated to lie between 19 and 75 ng/g fresh weight.

Table 5. PAT Protein in Grain (Rough Rice) of Transgenic Rice event LLRICE601

<i>Matrix</i>	<i>Treatment</i>	<i>Sample Number</i>	<i>Laboratory Sample ID</i>	<i>PAT (ng/g sample)</i>	<i>SD^a (ng/g sample)</i>
Grain (rough rice)	Transgenic	BK99B006-18	319D	141	5.2
		BK99B006-19	319E	113	15.8
		BK99B006-20	319F	105	22.1
			Average	119.4	18.7

^a Standard Deviation (Each data point is the average of two assays each performed on two subsamples).

Expression of the PAT protein has also been demonstrated in leaf material as indicated in Appendix 6. The semi quantitative method indicates that the leaf expression levels in LLRICE601 is between the levels of the other approved rice events LLRICE62 and LLRICE06.

b. Expression of other parts of the insert

There is no expression of other genes of the insert since the inserted sequence consists only of the *bar* gene. The absence of any additional DNA from the vector used for the transformation has been documented in Appendix 3.

c. Equivalence of the expressed protein

In order to determine the substance equivalence between plant produced PAT protein and bacterial produced PAT protein, SDS-PAGE and Western Blotting tests were performed. There is no significant difference in molecular weight based on Western analysis between bacterial and plant produced PAT protein (encoded by the *bar* gene). In addition, the PAT protein expressed in event LLRICE601 was compared to the PAT protein expressed in events LLRICE62 and LLRICE06. All proteins did show a molecular weight of approximately 22-24 kDa and can be considered equivalent. (see Appendix 6)

d. PAT protein safety

The PAT (phosphinothricin-acetyl-transferase) enzyme, encoded by the *bar* gene is the same protein that is in Bayer CropScience LLCotton25, LLRICE62, LLRICE06, OSR MS8/Rf3 among others. EPA has determined that PAT and the genetic material necessary for its production in plants are also exempt from the requirement of a tolerance.



40 CFR Part 180, Sec. 180.1151

Phosphinothricin Acetyltransferase (PAT) and the genetic material necessary for its production all plants; exemption from the requirement of a tolerance.

Phosphinothricin Acetyltransferase (PAT) and the genetic material necessary for its production in all plants are exempt from the requirement of a tolerance when used as plant-pesticide inert ingredients in all plant raw agricultural commodities. "Genetic material necessary for its production" means the genetic material which comprise genetic material encoding the PAT protein and its regulatory regions. "Regulatory regions" are the genetic material that control the expression of the genetic material encoding the PAT protein, such as promoters, terminators, and enhancers.

Detailed information regarding the toxicology and safety of the PAT enzyme encoded by the *bar* gene is contained in the reports listed below. In addition, an extensive overview of the evaluation of the safety of the PAT protein is available in a 2005 article published in Regulatory Toxicology and Pharmacology (H rouet, et al., 2005).

The results of studies show that the PAT protein has no homology with any known allergens or toxins. It has no glycosylation sites, which can often be present on food allergens. It is not stable in an acidic environment. It is quickly degraded and denatured in gastric and intestinal fluids of domestic animals and humans. The PAT enzyme is highly substrate specific. There were no effects found in the acute mouse test, even at a high dose level of the PAT protein. Based on this information, there is a reasonable certainty of no harm resulting from the inclusion of the PAT protein in food and feed. **The safety of the PAT protein has been further confirmed and is no finding that would change the rationale for determination of nonregulated status.**

D. Conclusions

In summary, the event LLRICE601 contains the same genetic elements as the deregulated events with the exception of the terminator sequences of the expression cassette. Although the transformation method employed was different, the genetic elements of the *bar* gene expression cassette are similar and both the deregulated events and LLRICE601 event, produce an equivalent protein.

No information developed in the genetic characterization of Event LLRICE601 has changed the rationale for determination of nonregulated status.

V. Agronomic Performance of Event LLRICE601

Agronomic observations were taken during the field trials, as well as evaluating the tolerance of the herbicide glufosinate. Agronomic performance data was collected in multiple locations. Other field activities allowed the evaluation of the material to assess the stability and performance of the introduced trait and the agronomic characteristics of the LLRICE601 event. The parent variety, Cocodrie is widely grown in the Southern US states of Arkansas, Louisiana, Mississippi and Texas.

In addition, disease resistance data was obtained for sheath blight, panicle blight, and rotten neck blast. No significant difference between LLRICE601 and the control Cocodrie were found. Morphological parameters necessary for the filing of a Plant Variety Protection (PVP) certificate were measured. These parameters are described in the objective description of the variety for rice and involve grain characteristics, maturity, morphology of the panicle, disease resistance, among others. Evaluations of seed dormancy and shattering were conducted as these can be potential weedy characteristics in rice. In all of the above, LLRICE601 and Cocodrie were found to be similar and without plant pest characteristics.

A. Field Tests of Event LLRICE601

Seed of four T₃ generation lines was increased in the winter nursery (1999-2000 season). In the summer of 2000, these 4 lines of LLRICE601 were tested by replicated yield evaluations (Louisiana and Mississippi) and similar tests in 2001 (Arkansas, Louisiana and Mississippi) were conducted to identify best adaptation for the Southern long grain market. Field evaluations included the testing of conventional and glufosinate herbicide systems for weed control. Summary of findings is provided for 2000 in Table 6 and for 2001 in Table 7. In both tables, selected entries from the tests highlight results of Cocodrie and LLRICE601-line 5201.

Table 6. Summary for 2000 season field tests in Louisiana and Mississippi, comparing LLRICE601-line 5201 with Cocodrie.

<i>Entry / Herbicide</i>	<i>Yield lbs/acre</i>	<i>Height cm</i>	<i>Maturity days</i>	<i>Lodging 1 to 9</i>	<i>Milling, Whole</i>	<i>Milling, Total</i>
601-5201 / Glufosinate	6219	86	83	1	64	72
601-5201/ Conventional	6021	86	83	1	65	72
Cocodrie / Conventional	6389	95	83	1	62	71
Cypress / Conventional	5403	82	86	2	63	70

Yield is reported as lbs per acre at 12% grain moisture.

Maturity is reported as days from emergence to 50% heading.

Lodging score of 1 indicates erect straw strength.

Milling, Whole indicates % by weight of unbroken milled grains.

Milling, Total indicates % by weight of all milled grain (broken and whole).

ANOVA analysis of data from 10 entries, four locations in Louisiana found differences in yield with an LSD of 319 lbs/acre for glufosinate herbicide and LSD of 329 lbs/acre for the conventional herbicide system. Similar tests in Mississippi had LSD of 502 lbs/acre for glufosinate herbicide and 556 lbs/acre for the conventional herbicide system. Mean yield of LLRICE601-line 5201 across all the sites was 6219 lbs/acre with glufosinate and 6021 lbs/acre using conventional herbicide. In the conventional herbicide system, Cocodrie produced 6389 lbs/acre and Cypress produced 5403 lbs/acre. Although Cocodrie had a higher yield, the difference between it and the LLRICE601 yield does not exceed the LSD value.



Table 7. Summary of 2001 season field tests in Arkansas and Louisiana, comparing four lines of LLRICE601 with Cocodrie.

Location: Stuttgart, AR

<i>Entry / Herbicide</i>	<i>Yield lbs/acre</i>	<i>Crop injury</i>	<i>Height cm</i>	<i>Maturity days</i>	<i>Sheath blight</i>
601- 5001 / Glufosinate	8988	0	97	83	2.8
601- 5201 / Glufosinate	8598	0	97	82	3
601- 5401 / Glufosinate	8670	0	97	85	2.5
601- 5601 / Glufosinate	8520	0	97	82	3.3
Cocodrie / Conventional	9720		94.3	83	3.5
Cypress / Conventional	8130		96.3	86	4.5
LSD (0.05) glufosinate	908				
LSD (0.05) conventional	1081				

Yield is reported as lbs per acre at 12% grain moisture.

Crop injury observed following glufosinate application is given in % of damage.

Maturity is reported as days from emergence to 50% heading.

Sheath blight rating of 0 indicates no disease development, 9 indicates maximum.

Location: Tillar, AR

<i>Entry / Herbicide</i>	<i>Yield lbs/acre</i>	<i>Crop injury</i>	<i>Height cm</i>	<i>Maturity days</i>	<i>Lodging 1 to 9</i>
601- 5001 / Glufosinate	7860	0	98.8	86.5	1
601- 5201 / Glufosinate	7980	0	96.3	86.5	2.8
601- 5401 / Glufosinate	8040	0	96.3	87.5	2
601- 5601 / Glufosinate	8040	0	98.8	87.5	1
Cocodrie / Conventional	8040		92.8	82	1
Cypress / Conventional	7380		92	85	1
LSD (0.05) glufosinate	1014				
LSD (0.05) conventional	1224				

Yield is reported as lbs per acre at 12% grain moisture.

Crop injury observed following glufosinate application is given in % of damage.

Maturity is reported as days from emergence to 50% heading.

Lodging score of 1 indicates erect straw strength.

Location: Acadia, LA

<i>Entry / Herbicide</i>	<i>Yield lbs/acre</i>	<i>Height in</i>	<i>Maturity days</i>	<i>Milling, Whole</i>	<i>Milling, Total</i>
601- 5001 / Conventional	6926	34	78	67	71
601- 5201 / Conventional	7204	36	79	64	70
601- 5401 / Conventional	7071	34	78	66	71
601- 5601 / Conventional	6948	33	77	65	70
Cocodrie / Conventional	7721	35	77	65	70
Cypress / Conventional	6729	34	83	65	68
C.V. %	7.6	4.0	1.2	2.2	1.1
LSD (0.05)	871.2	1.5	2.4	2.8	1.6

Yield is reported as lbs per acre at 12% grain moisture.

Maturity is reported as days from emergence to 50% heading.

Milling, Whole indicates % by weight of unbroken milled grains.

Milling, Total indicates % by weight of all milled grain (broken and whole).



Summary:

7 locations in Louisiana, herbicide treatments compared

<i>Entry / Herbicide</i>	<i>Yield lbs/acre</i>
601- 5001 / Glufosinate	6892
601- 5201 / Glufosinate	6796
601- 5401 / Glufosinate	7323
601- 5601 / Glufosinate	7376
Cocodrie / Conventional	7314
Cypress / Conventional	6828
LSD (0.05) glufosinate	1014
LSD (0.05) conventional	1224

Yield is reported as lbs per acre at 12% grain moisture.

Conclusion.

No crop injury or decrease in yield was observed following application of glufosinate herbicide at any location. Commercial level tolerance to the herbicide, glufosinate was demonstrated by all LLRICE601 lines at all locations and all years.

Findings across the locations show that LLRICE601 and Cocodrie are similar for maturity (the days from emergence to 50% maturity) and yield. Both have an erect straw strength indicating resistance to lodging. Milling yield may be slightly improved over Cocodrie. LLRICE601 does not display any significant reduction in any agronomic parameter compared to its parental line, Cocodrie.

Finally, LLRICE601 is no different from its parent comparator Cocodrie. LLRICE62 and LLRICE06 were no different from their parent comparators Bengal and M202 respectively. Following this rationale, there is no evidence that would suggest that LLRICE601 and LLRICE62-LLRICE06 would behave differently nor pose a risk as a plant pest.

B. Agronomic Characteristics

Objective Variety Description

At three locations in 2000, all the parameters necessary for plant variety protection application were collected for LLRICE601-line 5201 (USDA PVP Objective Variety Description for rice - see Appendix 7). The advanced line 5201 of LLRICE601 was found to be phenotypically similar to Cocodrie. Like Cocodrie, LLRICE601-line 5201 is an early maturing, semi-dwarf, long grain rice variety with adaptation for the southern USA rice growing area. The plant height of LLRICE601-line 5201 was shorter (86 cm) than Cocodrie (95cm). Maturity, the days from emergence to heading was the same, 83 days. The leaves are dark green, erect and glabrous. No pubescence is observed on the lemma and palea. Kernels have purple apiculus at heading and straw colored hulls and apiculi at maturity. This is similar to Cocodrie,



which has a purple apiculus at heading, which fades at grain maturation. Both have endosperm that is non-glutinous, non-aromatic and light brown pericarp. The grain type of Cocodrie and LLRICE601-line 5201 are identical. Grain parameters measured in side-by-side fields grown in Mississippi with three planting dates are provided in Table 8.

Table 8. Grain parameters compared LLRICE601-line 5201 and Cocodrie

Grain measurements in mm (mean of 20 grains)

Variety	Length	Thickness	Width	Shape
Cocodrie, Paddy	9.27	1.95	2.37	3.9
LLRICE601, Paddy	9.1	1.85	2.37	3.8
Cocodrie, Brown	7.17	1.59	2.03	3.5
LLRICE601, Brown	7.01	1.57	2.09	3.36
Cocodrie, Milled	6.81	1.64	2.09	3.3
LLRICE601, Milled	6.88	1.56	2.04	3.37

1000 grain weight in grams of milled rice from three planting dates

	PD1	PD2	PD3
Cocodrie	19.3	17.6	16.9
LLRICE601	18.7	16.9	17.21

Conclusion.

LLRICE601-line 5201 is similar from Cocodrie for all parameters considered by Objective Variety Description. The plant height of LLRICE601-line 5201 is shorter, reflecting the breeder’s preference, however remaining within the guidelines for semi-dwarf height classification, the same category of Cocodrie. In addition, in the 2001 trials there was no height difference (see Table 7). The major difference between Cocodrie and LLRICE601 is the addition of the *bar* gene giving tolerance to glufosinate herbicide in LLRICE601.

C. Disease and Pest Characteristics

Event LLRICE601 was observed to have the same disease susceptibility profile as its parent variety, Cocodrie. No changes were observed in the seed characteristics of dormancy or shattering that would indicate a reason to change the rationale for determination of nonregulated status.

a. Disease screen

The response of LLRICE601 to common rice pathogens was assessed in 2000 by LSU rice pathology staff (Table 9). Three lines of LLRICE601 were compared to two standard varieties, Bengal and Cypress (which are used as the resistant and susceptible reference varieties respectively for sheath blight screening). Event LLRICE601 has the same disease susceptibility profile as Cypress. This result is expected given the shared lineage of both Cypress and LLRICE601 to Cocodrie.



Disease ratings were made using a scale of 0 to 9, where 0 indicates no disease development and 9 indicates the maximum disease possible. The test nursery was inoculated with *Rhizoctonia solani*, the causal organism for sheath blight. Incidence of rotten neck blast (*Pyricularia grisea oryzae*) and panicle blight (causal agent unknown) were naturally occurring and moderate in severity during the 2000 season. Sheath blight severity was high.

Table 9. Rice disease evaluation.

Entry	Sheath Blight	Rotten Neck Blast	Panicle Blight
Bengal	4.8	0.5	3.5
Cypress	7.3	1.0	4.5
LLRICE601-5001	7.0	1.1	3.7
LLRICE601-5401	7.3	1.7	4.2
LLRICE601-5601	7.2	1.0	4.2
LSD	0.63	0.87	n.s.

Conclusion.

LLRICE601 did not display any significant change in disease susceptibility profile or response to plant pathogens compared to the profile expected.

b. Seed Dormancy Evaluation

Laboratory tests were completed to assess the seed characteristics that contribute to the weediness of red rice including seed dormancy and panicle shattering. LLRICE601 and Cocodrie had identical results in these tests. The laboratory protocol recommended to screen for seed dormancy¹ was followed.

Protocol: Three panicles were hand harvested from each of four replicate plots at the time of physiological maturity. The plots represented three advanced lines of LLRICE601 in Cocodrie background and the variety, Cocodrie.

Using care not to disturb the seed coat, individual dispersal units (seeds) were removed from the panicle by hand. Seed samples were transferred to containers for dry after-ripening. A subsample of 15-20 randomly selected seeds was removed from each sample and tested for germination and dormancy.

Germination dishes were prepared with Anchor Standard brown germination paper and 8-10 ml of 0.01% Diathane or 0.005% Chlorothalonil fungicide diluted with deionized water. Seeds were incubated at 30°C, high humidity and no illumination. Germination was scored at 5 and 7 days. Data collected at each evaluation included 1) number of seeds in dish; 2) number of seeds germinating

¹ Dr. Marc Cohen- red rice seed expert at LSU

(at least 1 cm of root or shoot emerged from seed coat); 3) number of seeds not germinating; and 4) number of non-germinating seeds that are firm. Firmness was determined by a gentle touch with forceps across the breadth of the endosperm. If the seed yields, it was considered soft, and likely non-viable as the endosperm is degenerating in the absence of germination. Germinated seed were removed from the dish at each evaluation.

Any seeds that have not germinated but remain firm after 14 days of imbibition at 30°C, were transferred into glass vials for a survival stress test. Such seeds were completely submerged in deionized water and returned to the 30°C chamber. Seeds were evaluated after 2 days for firmness. If seeds remained firm, the incubation continued for 3 weeks and tested for firmness again. Seeds surviving this extended test in warm incubation were considered to be dormant.

Panicles of three advanced lines LLRICE601 and Cocodrie were harvested from the Aventis CropScience field station in Leland, MS (2000 season). Three panicles were bulked per replication, four replications were prepared per line (Table 10). All firm seed germinated within 7 days. All soft seed decomposed. Thus no dormant seeds were observed and no survival stress tests were necessary.

Table 10. Germination of three lines of LLRICE601 compared to Cocodrie

Rice Lines	5 day germination		7 day germination		Number of seeds tested	Percent soft seed
	Mean germ	Std dev	Mean germ	Std dev		
601-5000	88.1%	0.1	96.7%	0.1	75	4%
601-5200	80.8%	0.1	96.6%	0.2	75	1.3%
601-5400	78.2%	0.1	92.5%	0.1	66	4.5%
Cocodrie	74.8%	0.1	98.8%	0.0	80	1.3%

Conclusion

All seed germinated or were dead (soft seed) within 7 days. No seed dormancy was observed. No requirement for dry after ripening was observed in this test. No evidence of seed dormancy that would be characteristic of weedy, red rice was observed. No difference in the shattering of grain from mature panicle was observed.

D. Composition analysis

Field trials were established in typical long-grain rice-producing areas of the southern United States of America. The plants were grown under conditions typical of production practices in MS, LA and AR. Each test site consisted of non-transgenic (Cocodrie) plots and transgenic plots. Transgenic plots consisted of treatments sprayed with glufosinate-ammonium (Liberty® Herbicide) at a nominal application rate



of 0.45 lbs ai/A, and plots managed with conventional herbicides. Samples of grain, also known as rough rice, were obtained from each field plot for composition analysis.

Pairwise t-tests were performed for each treatment against each other treatment (2-tailed, 15 datapoints per treatment). None of the pairwise comparisons showed a significant difference ($p >> 5\%$). Thus it can be concluded that the non-transgenic and the two transgenic treatments are not significantly different for any of the composition parameters tested. From examination of the composition data, the transgenic LLRICE601 rice (both sprayed and unsprayed) has a composition which is almost identical to that of the non-transgenic rice.

E. Conclusions

It can be concluded that there are no substantial differences in the agronomic characteristics when LLRICE601 and the parent variety, Cocodrie are compared. The transformation event, LLRICE601 provides stable and commercial level of tolerance to the glufosinate herbicide.

Comparison of the characteristics recommended by the Plant Variety Protection office of the USDA find LLRICE601-line 5201 to be similar to Cocodrie in all characters with two distinct exceptions. The plant height of LLRICE601-line 5201 is shorter, reflecting the breeder's preference, however remaining within the guidelines for semi-dwarf height classification. The LLRICE601-line 5201 and Cocodrie differ by the addition of the *bar* gene giving tolerance to glufosinate herbicide in LLRICE601.

The profile of LLRICE601 for disease resistance and response and seed dormancy were not changed by the transformation process or the addition of the *bar* gene, from what would be expected to be associated with Cocodrie-derived lines.

USDA-APHIS has previously issued determinations of nonregulated status to other genetically engineered glufosinate-tolerant rice (98-329-01p) with similar genetic constructs as those used in LLRICE601 rice. No adverse impacts on agricultural practices associated with the cultivation of these events have been observed.

Finally, since the determinations of nonregulated status to other genetically engineered glufosinate-tolerant rice LLRICE62 and LLRICE06 (98-329-01p), no new information has come to Bayer CropScience's attention that would indicate that there is an increased plant pest risk due to the incorporation of the *bar* gene in rice

VI. Potential for Environmental Impact from Non-contained Use of Event LLRICE601

Event LLRICE601 has the same potential for environmental impact as other cultivated rice. **No observations of event LLRICE601 have changed the rationale for determination of nonregulated status.**



A. Potential for Gene Transfer

Event LLRICE601 has the same reproductive nature as other cultivated rice. The potential for gene transfer is the same as the previously approved petition submission. **No observations concerning the reproductive biology of event LLRICE601 have changed the rationale for determination of nonregulated status.**

B. Weediness Potential of LLRICE601

There are no changes concerning weediness potential from the assessment of the previously approved petition submission. Like LLRICE62, LLRICE601 is sensitive to herbicides registered for pre-plant and pre-emergence use for weed control in rice. Volunteer rice can also be controlled with pre-plant burndown applications of paraquat (Gramoxone Extra) and glyphosate (Roundup Ultra or Roundup WeatherMax). LLRICE601 is also sensitive to the herbicides used in the Clearfield system, including imazethapyr (Newpath) and imazamox (Beyond). Volunteer rice is usually treated with a post-emergence grass soybean herbicide such as quizalofop (Assure II), fluazifop (Fusilade), sethoxydim (Poast), or glyphosate in Roundup Ready® soybeans. These products are also widely used for post treatments of annual grasses.²

C. Effects on Non-target Organisms

There are no changes from the previously approved petition submission. The FDA issued a finding of “No Concern” for glufosinate tolerant rice. As the presence of the PAT protein is the only difference found in LLRICE601 that is not found in conventional rice, LLRICE601 and its progeny should have no indirect or direct plant pest effects.

D. Indirect Effects on other Agricultural Products

With the exception of herbicide tolerance to glufosinate, LLRICE601 has the same agronomic properties as other cultivated rice. No interactions in agriculture have changed from the previously considered glufosinate tolerant rice events.

E. Conclusion of Environmental Impact Assessment

There were no differences, apart from the intended changes, demonstrated in field tests of event LLRICE601 compared with a non-transgenic variety. No morphological, beneficial organisms, disease or pest differences between event LLRICE601 and the previously considered glufosinate tolerant rice events were noted. There is no reason to think cultivation of event LLRICE601 will have environmental effects different from

² Recommendations for weed control in rice from LSU may be found on the LSU Agcenter web site: http://text.lsuagcenter.com/en/crops_livestock/livestock/pasture_forage/Weed+Control/Louisianas+Suggested+Chemical+Weed+Control+Guide_seriespage-2.htm



cultivation of glufosinate tolerant rice events previously considered by APHIS. No adverse consequences from the introduction of event LLRICE601 are expected.

VII. Statement of Grounds Unfavorable

No unfavorable information and data have been demonstrated for the glufosinate herbicide tolerant transformation event LLRICE601.



VIII. References

Linscombe, et al. (2000) Registration of 'Cocodrie' Rice. *Crop Sci.* 40:294

USDA. 1999. Determination of non-regulated status for rice genetically engineered for glufosinate herbicide tolerance. *Federal Register* 64:22595-22596. Environmental Assessment and Finding of No Significant Impact <www.aphis.usda.gov/biotech/dec_docs/9832901p_det_ea.html>.

FDA, Center for Food Safety and Applied Nutrition, Office of Pre-Market Approval. 2000. Biotechnology Consultation Note to the File BFN No. 000063 <www.cfsan.fda.gov/~rdb/bfnm063.html> accessed on 4-17-2003



IX. Appendix 1. Termination reports for USDA notification permits



Field Trial Termination Report for LibertyLink® rice Transformation Events

Date of Report: May 26, 2000
Notification Numbers: 98-254-02n
Dates of Release: December 1998 to June 1999
Dates of Termination: May 1999
Number of States and Sites: Puerto Rico (1)

Purpose of Release

Evaluation of rice plants containing the *bar* gene, LibertyLink® rice, for tolerance to Liberty® herbicide (glufosinate-ammonium).

Results

Glufosinate tolerant lines of rice were identified for advancement in the Liberty Link™ rice breeding program.

Locations and Events

Planting at University of Puerto Rico field station
December 26, 1998 – T₁ events (601, 602, 604)
Harvest May 3-4, 1999

Observations

The plots were visited on at least a weekly basis during the duration of the release. Seed were harvested from T₁ plants that survived Liberty herbicide treatment and panicle rows were planted in the next season for evaluation.

Herbicide Tolerance

Transgenic rice plants exhibited tolerance to glufosinate herbicide.

Insect Susceptibility

The primary insect pests of rice grown in Puerto Rico are rice water weevil and stem borer. We observed a slight infestation of stem borer in both the transgenic and non-transgenic rice.

Disease Susceptibility

Infestation of rice blast was expected to occur within the genetic background, which are susceptible to the fungus. As expected, we did observe some disease symptoms, however, applications of fungicide held the disease in check.

Weather Related Conditions

The weather was typical for the fall-winter season at the Puerto Rico breeding station.

Physical Characteristics

Rice plants were observed from emergence through maturity. No differences were observed between transgenic and non-transgenic rice in emergence, seedling vigor, and stand establishment, and in other casual observations. The various genetic backgrounds performed as expected under the tropical conditions of the Puerto Rico winter season.

Weediness Characteristics



Growth rate and habit were identical in both transgenic and non-transgenic plants. Weediness characteristics such as excessive vegetative growth or seed shattering were not observed.

Means of Plant Disposition

Panicles were hand harvested from the plants selected for advancement in the breeding program. Bulk harvest of rows selected for subsequent agronomic trials were accomplished by hand harvest. Following harvest, any remaining seed in the field were destroyed by cultivation.

Time / Method of Monitoring for Volunteers

The site was visited at least monthly, especially when rainfall of sufficient amount to germinate rice seed was experienced. As expected, flushes of germination were observed after the first two rainfalls. The site was maintained as fallow ploughed land. Visual inspection for volunteer rice plants was made and volunteers were destroyed before panicles emerged from the boot.



Field Trial Termination Report for LibertyLink® rice Transformation Events

Date of Report: May 26, 2000
Notification Numbers: 98-254-02n, 99-019-06n, 99-266-05n

Dates of Release: May 1999 to May 2000
Dates of Terminations: September 1999, April 2000
Number of States and Sites: Louisiana (1), Puerto Rico (1)

Purpose:

Evaluation of rice plants containing the bar gene, LibertyLink rice, for tolerance to Liberty herbicide (glufosinate-ammonium).

Locations and Events

98-254-02n, 99-019-06n
Planting at LSU, Rice Research Station
May 19, 1999, T2 events (601, 602, 604)
May 6 and 10, 1999, T1 (additional events)
Harvest September 1999

99-266-05n
Planting at University of Puerto Rico field station
November 10, 1999, T3 (601, 604)
Harvest April 4, 2000
Destruction, April 6, 2000

Results:

Application of Liberty™ herbicide has been used to score the T2 rows for segregation for the PAT phenotype. Rows considered to be homozygous (no sensitive plants) were harvested as independent populations. In the T3 generation, populations were evaluated for Liberty tolerance and plant breeding characteristics. Superior selections were advanced evaluation.

Observations:

The plots were visited on at least a weekly basis during the duration of the release.

T2 generation seed were planted as panicle rows to advance the lines and to score for segregation of the herbicide tolerance trait. Each row represented up to 60 seed from a single panicle. Herbicide application was used to score the rows for segregation of glufosinate resistance. The goal was to identify lines within each event that were homozygous for the inserted gene locus. Homozygous populations were identified. The homozygous rows were evaluated by the plant breeders for uniformity, maturity, heading quality, plant type and general vigor. Rows considered to be homozygous (no sensitive plants) were harvested as independent populations. This T3 generation seed was advanced on generation in Puerto Rico to provide seed for evaluation and variety advancement.

Also planted were T1 seed of additional lines. Plants were evaluated for agronomic characteristics and tolerance to herbicide application in the LSU breeding nursery.



Liberty Herbicide Tolerance:

Transgenic rice plants treated with Liberty Herbicide exhibited tolerance to the herbicide.

Insect Susceptibility:

The primary insect pest in Louisiana is the rice water weevil. Control measures were in place to prevent infestations, however, slight numbers of rice water weevil were observed in both the transgenic and non-transgenic rice plots.

The primary insect pests of rice grown in Puerto Rico are rice water weevil and stem borer. No evidence of insect damage was observed.

Disease Susceptibility:

The same ranges of diseases were noted in both parent and transgenic; panicle blight, sheath blight and stem rot.

Weather Related Conditions:

It was a typical season for southwest Louisiana. The weather was typical for the fall-winter season at the Puerto Rico breeding station.

Physical Characteristics:

Rice plants were observed from emergence through maturity. Within the panicle rows the plant breeder observed a range of somaclonal variation typical in his experience with rice in the early generations following regeneration from tissue culture. Variation was observed for stature, maturity, grain type and leaf width. A 15 day span in maturity was noted for the transgenic rows. When compared to the non-transgenic parent plots, the transgenic rows spanned a range of 10 days later to 5 days earlier in maturity than the parent. The overall vigor of the parent and transgenic rows was equivalent. As expected, variation for plant height, and leaf width and length were observed.

Weediness Characteristics:

Growth rate and habit were identical in both transgenic and non-transgenic plants. Weediness characteristics such as excessive vegetative growth or seed shattering were not observed.

Means of Plant Disposition:

Samples were harvested and treated as specified by the protocols. Following harvest, any remaining seed in the field was destroyed by cultivation.

Time / Method of Monitoring for Volunteers:

The sites will be visited at least monthly, especially when rainfall of sufficient amount to germinate rice seed is experienced. Monitoring will be continued until no volunteer plants have been observed for two visitations. Volunteers will be destroyed before panicles emerged from the boot.

1999 USDA Termination Report for Transgenic Rice Lines 6001-699
Aventis CropScience

Trials Conducted by State and County

99-019-06n: LA; Acadia Parish
99-266-05n (=99-293-03n, duplicate): PR; Lajas District

Trials Not Planted by State and County

99-019-06n: CA; Sutter, Yolo
99-266-05n: TX; Brazoria (2)

Planting Dates

May 5, 10, 19, 1999 (Acadia Parish, LA) through November 10, 1999 (Lajas District, PR)

Purpose

Field plots were established for breeding and residue studies. Transgenic plants contained the bar gene, which expresses the PAT enzyme, conferring tolerance to the herbicide glufosinate-ammonium.

General Field Observations

These plots were observed and maintained by personnel experienced and qualified in rice cultivation. Recorded observations were made of obvious differences between transgenic and control plots for pre-tiller through harvest growth stages.

Insect and disease pressure were low. At the Acadia Parish, LA site, pest insects recorded were the rice water weevil (*Sitophilus oryzae*), stem borer (*Languria sp.*) and stinkbugs (Pentatomidae: unspecified taxa). Diseases at this site consisted of the blights associated with *Rhizoctonia sp.* No differences were noted between the respective plots in these observations. In addition, no beneficial insect species were noted in either of the plot types.

The only phenotypic difference noted between the plots was the levels of tolerance to Liberty[®] herbicide treatment.

Harvest and plot destruction occurred on September 16, 17, and 18, 1999 at the Acadia, LA site.

Post Trial Monitoring

Planting areas were scouted for volunteer rice through June of 2000 with no plants found.



USDA 2000 Termination Report for Liberty Link Rice
Aventis CropScience USA, LP

Trials Conducted by State and County

00-049-12n: LA: Acadia, Calcasieu, Jefferson Davis, Vermillion, East Carroll, Catahoula Parishes
MS: Washington
00-042-06n: CA: Sutter
00-076-06n: AR: Crittenden
MS: Washington
LA: St. Landry Parish (two sites)
00-074-17n: MS: Washington
00-124-05n: AR: Crittenden, Jackson
LA: St. Landry Parish
00-243-02n: PR: Lajas District
00-292-16n: PR: Lajas District

Trials Not Conducted

00-049-12n: LA: Davis Parish, Morehouse Parish, Rapides Parish
TX: Brazos
00-049-10n: FL: Santa Rosa
00-076-06n: CA: Sutter
MS: Washington
00-074-17n: MS: Washington
00-243-02n: PR: Juana Diaz District

Planting Dates

March 17, 2000 (Acadia Parish, LA) through November 28, 2000 (Lajas District, PR)

Harvest/Plot Destruction Dates

July 20, 2000 (Jefferson Davis Parish, LA) through January 5, 2001 (Lajas District, PR)

Purpose

Field trials were conducted to test the efficacy of transgenic herbicide-tolerant rice, for breeding purposes, tissue analyses, and seed increase. Aventis Liberty Link rice contains the bar gene which expresses the PAT enzyme conferring tolerance to the broad-spectrum herbicide glufosinate-ammonium.

General Field Observations

Experienced personnel qualified in rice cultivation performed all plot observations. Recorded observations for transgenic and non-transgenic control plots were provided from emergence through harvest.

Germination counts taken at 15 days post-emergence ranged from 80% to 95%. Plant vigor was described as good with uniform growth in both plot types. Final transgenic stand counts taken post Liberty® treatment of the plots ranged from 75% to 95%. Non-transgenic control plants were destroyed by the herbicide treatment.

Insect pest species recorded were stinkbug (Hemiptera: *Pentatomidae*), armyworm (Lepidoptera: *Spodoptera* sp.), and rice water weevil (*Sitophilus oryzae*). No beneficial insect species were recorded. Sheath blight (*Rhizoctonia* sp.) was the only pathogen observed (Acadia Parish, LA).

Weather at the sites was described as drier and hotter than normal.

No morphological differences were noted between transgenic and non-transgenic plants. The only phenotypic difference observed between the two plant types was their respective levels of tolerance to glufosinate-ammonium.

Final Disposition

Plant materials remaining at the termination of the studies were shredded and disced under.

Post-season volunteer plants of transgenic rice numbering less than ten/plot were found at the Louisiana study sites in Acadia Parish, Calcasieu Parish, and Jefferson Davis Parish. In addition, over 50 volunteers were found at the Lajas District site in Puerto Rico. All volunteer plant materials were mechanically destroyed.

USDA 2001 Termination Report for Liberty Link® Rice
Bayer CropScience LP

Trials Conducted: State (County)

01-071-04n: AR (Arkansas, Drew), LA (Acadia Parish, Catahoula Parish, East Carroll Parish, Jefferson Davis Parish, Morehouse Parish, Vermillion Parish), MS (Washington)
01-039-01n: CA (Sutter)
01-110-01n: TX (Colorado)

Trials Not Conducted: State (County)

01-071-04n: AR(Drew), CA (Yolo), PR (Lajas)
01-297-04n: PR (Lajas)

Planting and Plot Destruction Dates

Field plots were established from March 19, 2001 (Jefferson Davis Parish, LA) through May 31, 2001 (Colorado Co., TX). Harvest and crop destruction occurred from September 12, 2001 (Colorado Co., TX) through October 27, 2001 (Drew Co., AR). Methods of plant material destruction included under-tilling followed by RoundUp® plot treatment as well as mowing and incineration. Volunteer monitoring reports indicated their presence only in Colorado Co., TX with plants shredded and plots treated with RoundUp®. Volunteer-free plots were verified for this site by Nov. 2, 2001. No reports of red rice were received.

General Observations

Rice field trials were established for efficacy studies, breeding, and seed increase. Plant phenotypes were classified as HT and PQ/HT. All constructs contained the bar gene inferring tolerance to the herbicide glufosinate-ammonium through the action of the PAT enzyme. In addition to this trait, some constructs also expressed an altered carbohydrate metabolism (gene(s) listed under CBI status). All plots compared transgenic experimental lines to non-transgenic control lines. Personnel well experienced in rice agriculture conducted all studies.

With the exception of limited lines of Cocodrie that failed to emerge beyond the 50% level (Arkansas Co., AR), germination and emergence patterns were otherwise reported as normal throughout all sites. In Texas, both transgenic and non-transgenic medium grain rice exhibited slightly superior percent emergence than long grained rice plantings. Final stand counts over all sites ranged from 75 to 90%. The only phenotypic difference noted between the experimental and control plants was the degree of tolerance to glufosinate-ammonium with the majority of transgenic plants showing no effect secondary to this herbicide treatment while non-transgenic control lines were completely destroyed.

Insect species categorized as pests and beneficials were noted among the plots. The only pest taxon recorded was stinkbug (Hemiptera: *Pentatomidae*). Beneficial groups observed included "spiders and wasps" (further diagnostic information absent).



The only phytopathology found was sheath blight (*Rhizoctonia* sp.) with path-damage ratings made at three sites in Louisiana. There were no differences in susceptibility noted between transgenic and non-transgenic plants.

Weather notations indicated most sites experienced typical climatic conditions. Mississippi (Washington Co.) had a very wet growing season and Texas (Colorado Co.) recorded the last third of the season as unusually wet.



X. Appendix 2. Insert characterization



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XI. Appendix 3. Vector backbone analysis



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XII. Appendix 4. Stability of the insert



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XIII. Appendix 5. Bioinformatics analysis

BIOINFORMATICS ANALYSIS

The flanking regions of the inserted sequence

Rice plants transformed using *Agrobacterium tumefaciens*-mediated transformation inserting the T-DNA from vector pGSV71 into the rice genome generated the event LLRICE601. Due to the insertion of the P35S3-bar-3'nos gene cassette in rice, a 5-prime and 3-prime junction, where rice genomic DNA and inserted T-DNA are fused, was created. The junction regions were analyzed to confirm that no important rice genes were interrupted and that no chimeric proteins would get expressed due to this insertion. (Figure A5-1)

Open reading frame (ORF) and gene search tools were applied to predict the presence of potential newly created coding sequences in the 5-prime flanking genomic/insert DNA junction region and in the 3-prime flanking insert/genomic DNA junction region. Five ORFs were found, two that span the 5-prime junction and three that span the 3-prime junction. Several bioinformatics tools were applied to look for regulatory elements such as core promoters, polyadenylation (polyA) signals and ribosome binding sites (RBS) to see if these newly created ORFs could be putatively active.

- ORF-1 at the 5-prime junction (sense strand): no homology was found with CART- and TATA-boxes, polyA signal and RBS which are consensus sequences of respectively initiation of transcription, termination of transcription and initiation of translation.
- ORF-2 at the 3-prime junction (sense strand): no homology was found with CART- and TATA-boxes, polyA signal and RBS.
- ORF-3 at the 3-prime junction (reverse sense strand): no homology was found with CART- and TATA-boxes, polyA signal and RBS.
- ORF-4 at the 3-prime junction (reverse sense strand): no homology was found with CART- and TATA-boxes or RBS. Homology was found with a polyA signal.
- ORF-5 at the 5-prime junction (reverse sense strand): no homology was found with CART- and TATA-boxes and RBS. Homology was found with a polyA signal.

Since no transcriptional elements were found around ORF-1, ORF-2 or ORF-3, the detected ORFs are considered to be transcriptionally and translationally not active. The CART- and TATA-box of the core promoter, where the RNA polymerase will bind and initiate transcription, is not present at the 5-prime end of ORF-4 and ORF-5. Also a RBS, where the translation machinery initiates translation, is not present and ORF-4 and ORF-5 can be considered as transcriptionally and translationally not active. Therefore the similarities with the polyA and translational signal sequences are not relevant and the probability of an appearance of a newly created protein is highly unlikely.

From these analyses we can conclude that no known rice genes were interrupted due to the insertion of the P35S3-bar-3'nos gene cassette into the rice genome and the probability of an expression of newly created proteins coming from the 5-prime or 3-prime junction region is also highly unlikely.

CBI figure

Figure A5-1: Schematic overview of the transgenic locus of the LLRICE601 event

The bioinformatics tools that were used for this analysis focus on the localization of gene elements on the query sequences. In this approach the presence of ORFs was analysed and a homology search was performed to compare specific patterns of known genes, such as promoter sequences and regulatory elements, to similar patterns in the analyzed sequences. The bioinformatics analysis of newly expressed fusion proteins in the LLRICE601 event, followed the strategy:

- An analysis was performed to detect endogenous rice genes located in the 5-prime plant genomic flanking sequence and in the 3-prime genomic flanking sequence. For this a BLASTn similarity search was performed to locate and identify the genes.
- To find ORFs at the 5-prime and 3-prime junction gene and ORF search tools were applied.
- In order to identify regulatory DNA motifs, which could be involved in regulation of the expression of the putative chimeric ORFs, sequence that contains the 5-prime plant genomic flanking sequence, the inserted DNA and in the 3-prime genomic flanking sequence of the LLRICE601 event was subjected to several bioinformatics tools. These allowed us to search for homology with consensus sequences of core promoter motifs (CART- and TATA-boxes), polyA and initiation of translation signals (RBS). Analysis of the composition and localization of the identified regulatory motifs allows to predict whether investigated sequences are transcriptionally and translationally active.

Right and left border integration fragment

The DNA sequence of several hundred base pairs at and next to the integration site has been determined. The sequences include plant and insert DNA.

The border integration fragment was amplified using the Genome Walker protocol. The template DNA was *Stul* digested and purified. After adaptor ligation, the integration fragment was amplified. In a primary PCR reaction, an insert specific primer and an adaptor primer were used. The obtained amplicon of the secondary nested PCR reaction was amplified again. The obtained amplicon was sequenced.

BLASTn similarity search

To identify the presence of endogenous genes located near the 5-prime and 3-prime junctions of the LLRICE601 event, a BLASTn similarity search was performed. The query sequence was subjected to a sequence similarity search using the BLAST algorithm. This release of BLAST implements version 2.0 of BLAST from the National Center for Biotechnology Information (NCBI) described in Altschul et al. (1997). BLAST is known as "gapped BLAST" because it allows for gapped alignments between query and database sequences. The BLASTn similarity search compares a nucleotide sequence with sequences in nucleotide databases. Table A5-1 shows an overview of the databases used.

Table A5-1. Overview of the BLASTn database versions

<i>Database</i>	<i>Posted date of database</i>	<i>Date of analysis</i>	<i>Number of sequences in database</i>
Non Redundant; Non Plant	July 30, 2006	August 3, 2006	1,353,223
Plant	August 1, 2006	August 3, 2006	8,575,936
Refseq	May 29, 2006	August 3, 2006	773,972
Rice	July 30, 2006	August 3, 2006	1,501,072
TIGR_Rice_v3	April 25, 2006	August 3, 2006	12
TIGR_Rice_cDNA_v3	April 25, 2006	August 3, 2006	61,250
TIGR_Rice_cds_v3	April 25, 2006	August 3, 2006	61,250
TIGR_Rice_genes_v3	April 25, 2006	August 3, 2006	57,892
TIGR_Rice_cds_v4	April 25, 2006	August 3, 2006	12
TIGR_Rice_cDNA_v4	April 25, 2006	August 3, 2006	62,827
TIGR_Rice_cds_v4	April 25, 2006	August 3, 2006	62,827
TIGR_Rice_genes_v4	April 25, 2006	August 3, 2006	55,890
BGI_Rice_Chromosome	April 14, 2006	August 3, 2006	12
Rice_BAC_RGP	April 17, 2006	August 3, 2006	3,455
Rice_EST	July 30, 2006	August 3, 2006	1,187,543
Rice_mRNA	May 21, 2006	August 3, 2006	35,490



Sequence alignment between 5-prime and 3-prime query sequences against different databases located the site of integration on chromosome twelve. Alignment of the 5-prime and 3-prime flanking sequences with a fragment of wild-type chromosome twelve containing homologous sequences confirmed the presence of the target site deletion of 19 bp in the transgenic locus of *Oryza sativa* event LLRICE601. No homology was found with known genes, mRNA, cDNA or ESTs in the flanking rice genomic DNA.

Gene prediction and open reading frame search

FGENESH search

FGENESH is used for gene structure prediction (Softberry Inc.). It allows multiple gene finding on both strands. It predicts exons, introns by statistical sequence analysis and polyA signals by homology search with known consensus sequences from monocotyledon plants, dicotyledon plants or *Nicotiana tabacum*. As rice is a monocotyledon plant, this database of consensus sequences was used. Only the genes with sequence that spans the 5-prime or 3-prime DNA junction and which would therefore give rise to chimeric proteins, were taken into consideration. For optimal analysis, the sequence (3140 bp) of event LLRICE601 was used to perform the FGENESH search. Using FGENESH, no genes containing exons and introns were found which span the 5-prime or 3-prime DNA junctions.

GetORF search

The ORF search was performed by means of the ORF search program GetORF from the EMBOSS (European Molecular Biology Open Software Suite) tools. Standard codon usage was selected for the start and stop codon. The ORFs were defined as regions between START (ATG) and STOP (TAA, TAG, TGA) translation codons with a minimum size of eight amino acids (AA). Nucleic sequences (minimum size of 24 nucleotides, stop codon not included) and the translation of these regions between start and stop codon were noted. In all cases the six reading frames were examined. The potential newly created chimeric ORFs formed in the 5-prime or 3-prime DNA junction region of the LLRICE601 event were analyzed. Only the ORFs that span the 5-prime or 3-prime junctions were taken into consideration.

When using GetORF, which looks for ORFs that could code for eight AA or more, two ORFs were found that span the 5-prime rice genomic/insert DNA junction and three ORFs that span the 3-prime insert/rice genomic DNA junction. (See Figure A5-2)

CBI figure

Figure A5-2: Schematic overview of the newly created ORF in the 5-prime and 3-prime DNA junction regions of the LLRICE601 event

Prediction of regulatory elements

Prediction of core promoter sequences

Gene expression begins with the binding of multiple protein factors to promoter and enhancer sequences. These factors facilitate the formation of the transcription initiation complex, which includes the enzyme RNA polymerase and polymerase-associated proteins. The CART- and TATA-box, which are regulatory sequences that make up the core promoter, occur generally within 200 bp upstream of the ATG codon. Enhancer sequences can be located at variable distances upstream of the transcription start site.

To detect such expression signals, the query sequence is analyzed by the bioinformatics tool TSSP. TSSP predicts plant promoters using the RegSite Database (version 4, Softberry Inc.). The TSSP search tool, is a pattern-finding tool used to search for core promoter (TATA- and CART-boxes) and enhancer sequences.

The TSSP search program did not find sequence similarities with known consensus promoter sequences in the vicinity of 100-200 bp upstream of the chimeric ORFs. The CART- and TATA-box of the core promoter, where the RNA polymerase respectively will bind and initiate transcription, are not present at the 5-prime end of any of the predicted chimeric ORFs. Therefore it is unlikely that any of the potential newly created chimeric ORFs will be transcribed in the event LLRICE601.



Prediction of a polyadenylation site

The addition of a polyA tail at the 3-prime end of an mRNA is an important step in the expression of eukaryotic genes. The polyA tail protects the mRNA from degradation and thus plays an important role in the stability of the mRNA. Plant 3-prime untranslated regions are generally up to 300 bp long and have a consensus polyA signal sequence (AATAAA) (Li and Hunt, 1997) or related sequences (Berghman, 2005) at the 3-prime end.

The search revealed 100% homology with a related polyA signal sequence (ATGAAA) in the vicinity downstream of the putative chimeric ORF-4. For ORF-5 five out of six nucleotides showed homology with a related polyA signal sequence (TAAATA instead of AAAATA). If there would be an initiation of transcription of ORF-4 or ORF-5, the mRNA would contain a polyA signal sequence and would be processed resulting in an addition of a polyA tail.

Prediction of the putative ribosome binding site

Based on a bioinformatics analysis of nucleotide frequencies at positions flanking the translation start codon of dicotyledon and monocotyledon plant genes a consensus sequence has been determined (Joshi et al., 1997). This sequence (aaaaaaaA(A/C)aATGGCtacta(c/t)ta) has been shown to be important for initiation and efficiency of translation (Gallie et al., 1987). By comparison of the ATG context sequences, present in analyzed DNA fragments, with the consensus sequence it is possible to predict whether the first ATG codon of the putative ORF is a potential start of translation. The -3 and +4 positions (where the A of ATG is +1) are considered as the most important in determining a favorable context of initiator ATG. The putative ribosome binding sequence around the ATG of the newly created ORFs shows low to no homology with the consensus sequence for initiation of translation and this for all five ORFs. If one of these ORFs would be transcribed there will most likely be no translation as the translation complex (ribosomes) will not bind to the mRNA due to the low homology with the consensus ribosome binding site.



XIV. Appendix 6. Protein equivalency

PROTEIN EQUIVALENCY DEMONSTRATED BY WESTERN BLOT ANALYSIS

Isolation of protein produced in plants.

Proteins were purified from frozen leaves of rice, events LLRICE601, LLRICE06 and LLRICE62 by the Bayer CropScience MBAS laboratory (Gent, Belgium). Fresh leaves were harvested, placed in aluminum foil and placed directly on dry ice. The frozen leaves were then stored at -10 °C or lower until grinding. The sample for analysis was ground with mortar and pestle prechilled with liquid nitrogen. Small amounts of liquid nitrogen were added to the mortar periodically to ensure the sample remained frozen during preparation. The ground sample was stored on ice before extraction.

The PAT/*bar* protein was extracted by mixing ground plant leaves using a ratio of 0.3 gram of ground leaves to 0.9 mL of extraction buffer in a 1.5 mL eppendorf centrifuge tube. The extraction buffer contained SEB (50 mM Tris, pH 7.5, 100 mM KCl, 5% Glycerol, 10 mM EDTA and 10 mM EGTA), with the addition of 1 µg/ml leupeptin, 1 mM phenyl-methane-sulfonyl-fluoride (PMSF), 1 mM benzamidine HCl and 1 µg/mL antipain. The tube was placed at 4 °C on a rocking platform for 15 minutes. The extract was clarified by centrifugation for 15 minutes.

The amount of total extractable protein was estimated with the Bradford analysis, a colorimetric method for protein quantitation. When coomassie dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant color change from brown to blue. To perform the assay, a small amount of protein sample is combined with the assay reagent, mixed well, incubated briefly and the absorbance is measured at 595 nm. Protein concentrations are estimated by reference to absorbances obtained from a series of albumin from bovine serum as standard protein dilutions, which are assayed alongside the unknown samples. The protein concentration is calculated by extrapolation.

Analysis by western blotting

The PAT/*bar* protein purified from *E. coli* and the proteins from rice, events LLRICE06, LLRICE601 and LLRICE62 were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The proteins from the plants and the corresponding protein from *E. coli* were denatured and separated by electrophoresis on a denaturing polyacrylamide gel where mobility is directly related to molecular weight. Standards on the gel were a series of other proteins of known molecular weight.

SDS-PAGE was performed using a Pierce 4-20% polyacrylamide gradient minigel (product number 25224) and a Tris-HEPES-SDS running buffer according to the manufacturer's instructions. Approximately, 50 micrograms of total plant protein were present per lane and 1 microgram of purified bacterial protein.

Western blotting was performed after the electrophoresis system and the gel was blotted to PVDF membranes (BioRad, product number 162-0177) according to the instructions provided by the manufacturer. The proteins in the gel were transferred out of the gel perpendicular to the direction of the first electrophoresis. They were adsorbed to a

membrane giving an exact replica of the positions of all the proteins in the gel. The membrane was then exposed to an antibody to the PAT/*bar* protein and through a series of additional steps a tag was attached to the bound antibody to reveal the position of the protein of interest. Rabbit polyclonal antibodies produced by Bayer CropScience to the PAT/*bar* protein were used at a dilution of 1:1000. The second antibody was alkaline phosphatase (AP) linked anti-rabbit antibody. All developing reagents were obtained from BioRad as an AP color reagent (product number 170-5018).

The results of the western blot are shown in Figure A6-1. The electrophoretic mobilities of the PAT/*bar* protein produced in *E. coli* and rice, events LLRICE601 and LLRICE62, are indistinguishable. The results show a comparable immunoreactivity of the PAT/*bar* protein produced in *E. coli* and rice, events LLRICE601 and LLRICE62. The immunoreactive bands have a molecular weight which matches the molecular weight of the PAT/*bar* protein produced in *E. coli*. The level of expression in the rice event LLRICE06 was too low to be detected. The degree of expression by the rice events is sustained by data obtained by lateral flow strip analysis.

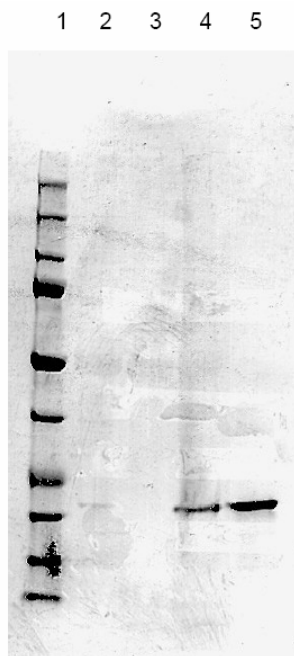


Figure A6-1: Comparison of the PAT/*bar* protein from *E. coli* with the PAT/*bar* protein isolated from leaves of transgenic rice, events LLRICE06, LLRICE601 and LLRICE62

Lanes 1 contains molecular weight markers of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa. Lanes 2, 3 and 4 contain approximately 100 µg of total protein extracted from rice, events LLRICE601, LLRICE06 and LLRICE62, respectively. Lane 5 contains approximately 1 µg of the PAT/*bar* protein from *E. coli*.



The analytical test offers a multi-directional approach to demonstrate equivalence of the PAT/*bar* protein produced in *E. coli* and rice, events LLRICE601 and LLRICE62. The results show that the PAT/*bar* protein produced in *E. coli* is representative of the PAT/*bar* protein produced in rice, events: LLRICE601, LLRICE62 and that the safety data obtained for the PAT/*bar* protein produced in *E. coli* can be used to support the safety of the PAT/*bar* protein produced in rice, event: LLRICE601 and LLRICE62.



XV. Appendix 7. USDA PVP Objective Variety Description for rice



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