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

Transformation Event LLRICE601

OECD Unique Identifier BCS-OSØØ3-7

(Revised)

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.

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August 17, 2006
Amended August 22, 2006
Revised August 27, 2006

CONTAINS CONFIDENTIAL BUSINESS INFORMATION

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>

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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, 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	*Oryza sativa* L.	*Oryza sativa* L.
Parent Line	Cocodrie	M202 and Bengal
Transformation Method	*Agrobacterium tumefaciens* 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 (*bar*) gene/*Streptomyces hygroscopicus*	Phosphinothricin acetyltransferase (*bar*) gene/*Streptomyces hygroscopicus*
Promoter/Donor	35S promoter (P35S) / Cauliflower Mosaic Virus	35S promoter (P35S) / Cauliflower Mosaic Virus
Terminator/Donor	3' untranslated end of the nopaline synthase gene / *Agrobaterium tumefaciens*	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 event and the previous events of the petition 98-329-01p.

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 generally 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 hygroscopicus*, 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 *Taq*I 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).

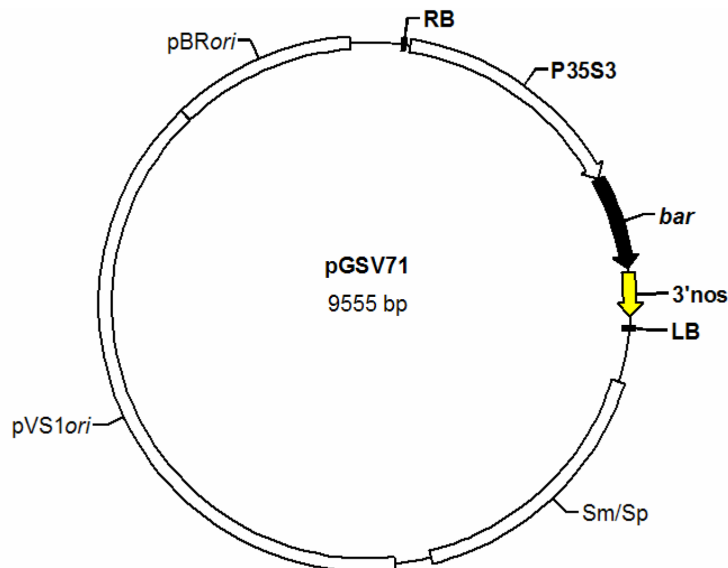


Figure 1. Map of the vector pGSV71

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

T_1 seed harvested from self-pollinated T_0 plants surviving a glufosinate herbicide greenhouse screen were planted in December 1998 in Puerto Rico. T_1 plants were selected for survival following glufosinate herbicide application. Panicles were harvested from individual plants and T_2 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 Chi^2 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

T_2 Panicle Rows

Fully Resistance Rows	*Partially Resistant Rows*	*Total Rows*	*Expected Ratio*	*Chi^2 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 Chi^2 values greater than 3.84, with one degree of freedom.

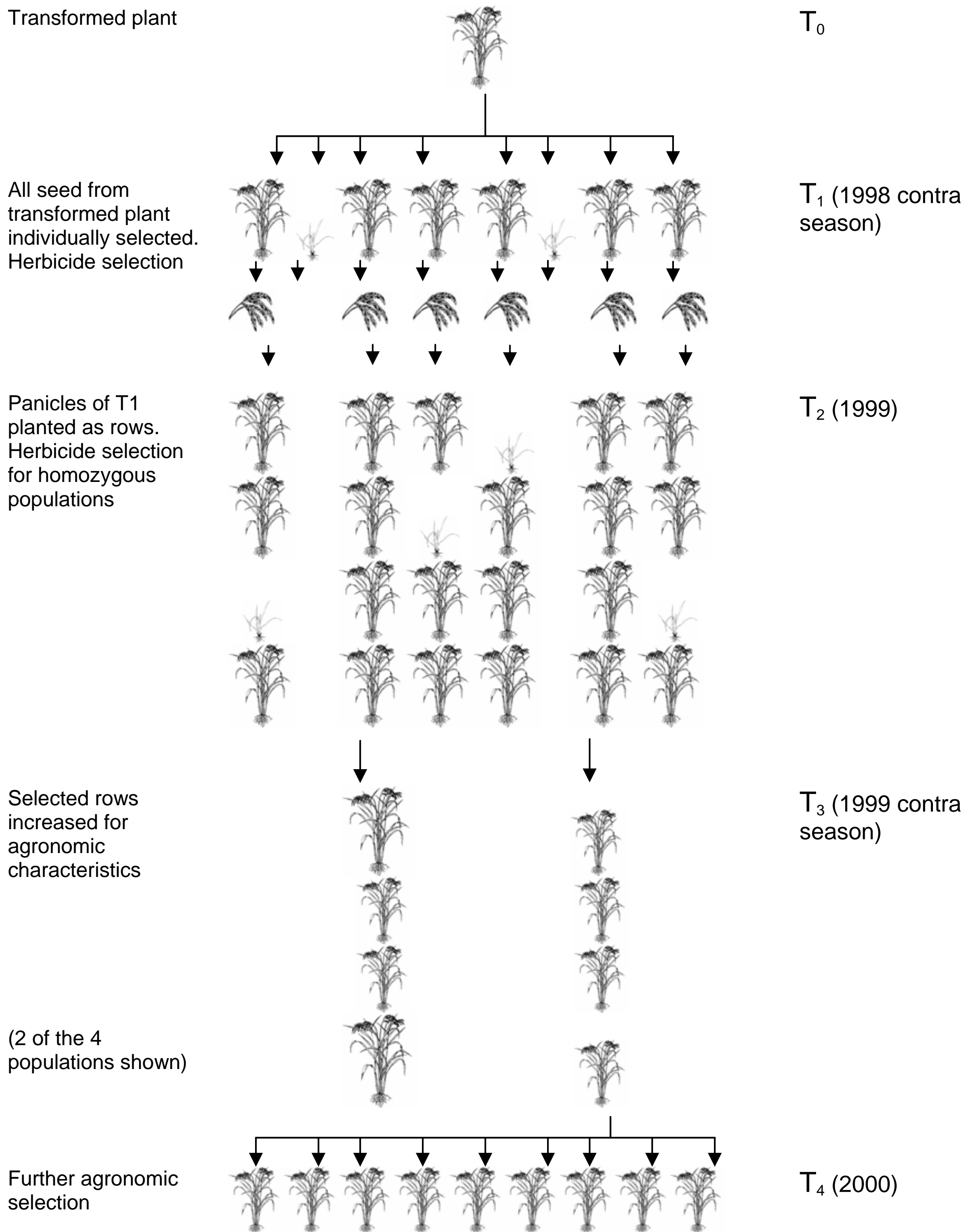


Figure 2. Plant selection diagram

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.

Southern blot data also suggests that the nos terminator is truncated. The function of *3'nos* terminator is to start the polyadenylation after transcription of the gene and to stabilize the mRNA. There are additional polyadenylation sites in the region, so the gene would use the next active site available. This could have an influence on the stability of the produced mRNA, which could lead to low expression of the *bar* gene. Since there are data on the expression levels of the PAT protein, we can conclude that the presence of part of the *3'nos* terminator is sufficient to allow expression of the PAT protein in LLRICE601 at levels that confer herbicide tolerance.

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.

Agrobacterium mediated transformation transfers the DNA known as T-DNA located between the left border and the right border regions. It is not expected that other parts of the vector outside the T-DNA region are transferred. This was supported by the absence of signals in DNA probed with overlapping segments of the vector backbone, however, a faint band was present with the 3'pVS1 ori probe. This is a very rich AT region and could be non specific. Even if it was part of the *ori*, it is likely incomplete and therefore unable to function. The If any portion of the bacterial origin of replication was inserted, phenotypic and compositional data revealed no differences between LLRICE601 and the parent variety, and therefore do not appear to pose a plant pest risk.

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)

These findings show that LLRICE601 behave similar to the antecedent organisms LLRICE62 and LLRICE06, therefore the rationale that allows the latter events to be achieve the non-regulated status should be the same.

C. Gene Expression of Event LLRICE601

The field performance criteria for LibertyLink® rice varieties requires plants to be tolerant to the herbicide, glufosinate ammonium (tradename, 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 affect the tolerance to the herbicide when compared to the antecedent organisms. The event LLRICE601 is similar to the events LLRICE06 and LLRICE62, and therefore should be extended 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 quantative 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

Matrix	*Treatment*	*Sample Number*	*Laboratory Sample ID*	*PAT (ng/g sample)*	*SD[a] (ng/g sample)*
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 coding sequence consists only of the *bar* gene.

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 (Hérouet, 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. These findings regarding the safety of the PAT protein are the same for event LLRICE601 and the antecedent organisms LLRICE62 and LLRICE06, that have already achieved a 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.

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, and was used as the comparator in these tests.

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.

Similar tests were conducted for the antecedent organisms LLRICE62 and LLRICE06 and their parent varieties. The results are summarized the tables and/or referenced back to the original petition 98-329-01p. The results are in the normal range for rice varieties.

A. Field Tests of Event LLRICE601

Seed of four T_3 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, entries from the tests highlight results of Cocodrie and LLRICE601-line 5201. Note that the LLRICE601-line 5201 and Cocodrie were only two of 20 other lines (including transgenic and conventional lines) included in these tests. The other lines are not relevant subject matter for this petition and were not included in the table.

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

Entry / Herbicide	*Yield lbs/acre*	*Height cm*	*Maturity days*	*Lodging 1 to 9*	*Milling, Whole*	*Milling, Total*
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

Entry / Herbicide	*Yield lbs/acre*	*Crop injury*	*Height cm*	*Maturity days*	*Sheath blight*
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

Entry / Herbicide	*Yield lbs/acre*	*Crop injury*	*Height cm*	*Maturity days*	*Lodging 1 to 9*
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

Entry / Herbicide	*Yield lbs/acre*	*Height in*	*Maturity days*	*Milling, Whole*	*Milling, Total*
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

Entry / Herbicide	*Yield lbs/acre*
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 as illustrated in Table 8 and the antecedent petition (98-329-01p, Section V.B “Agronomic Characteristics”). 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.

Table 8. Agronomic findings summary for LL rice events and their respective comparators.

Ranges reported for T_3 and/or T_4 generation homozygous lines in replicated trials.

	LLRICE06	***M202***	***LLRICE62***	***Bengal***	***LLRICE601***	***Cocodrie***
Height	97	94	88	95	86	65
Yield	8100	8500	7200	7800	6200	6400
Maturity	80	78	81	81	83	83
Emergence	81%	79%			85%-90%	85%-90%
Vigor	5	5	3	3	4	4
Lodging	erect	erect	erect	erect	erect	erect

Source:

LLRICE06/M202 – 1998, 7 locations in California (98-329-01p pages 45-46)

LLRICE62/Bengal – 1999, 13 locations in Louisiana, Texas, Arkansas and Mississippi (BCS Internal report OS25, pages 7-15)

LLRICE601/Cocodrie – 2000, 5 locations in Louisiana and Mississippi (Table 6 page 20)

Description of parameters measured:

Height in cm from the base to the tip of the flag leaf measured at heading

Grain Yield is presented as calculated lbs/acre at 12% moisture

Days to 50% heading (number of days difference between emergence and heading)

Emergence estimated 12 to 21 days post planting

Vigor rating of 1-5 assessed at 2-4 leaf stage, 5 is best score

Lodging rated at maturity

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 9.

Table 9. 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. LLRICE62 and LLRICE06 were no different from their parent comparators Bengal and M202 respectively (98-329-01p, Section V.B "Agronomic Characteristics")

Table 10. Variety description of LL rice events and their respective comparators

	LLRICE06	*M202*	*LLRICE62*	*Bengal*	*LLRICE601*	*Cocodrie*
Maturity Class	early	early	early	early	early	early
Height Class	semi-dwarf	semi-dwarf	short	short	semi-dwarf	semi-dwarf
Grain type	medium	medium	medium	medium	long	long

Following the classification according to the Objective Variety Description for Rice of the USDA, Plant Variety Protection Office, Table 10 shows that each LLRICE event was found to be similar to its comparator variety.

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, demonstrating that LLRICE601 behaves in a similar manner to its comparators LLRICE62 and LLRICE06 and their parent varieties. These findings support the similarity to the antecedent organism, and the nonregulated status should be extended to LLRICE601.

a. Disease screen

The response of LLRICE601 to common rice pathogens was assessed in 2000 by LSU rice pathology staff (Table 11). 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 11. 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.
LLRICE62 and LLRICE06 are equally susceptible to disease and insect pests as its nontransgenic counterparts (98-329-01p, Section V.D. "Disease and Pest Characteristics" and Appendix 1).

Table 12. Disease evaluation of LL rice events and their respective comparators.

Disease	*LLRICE06*	*M202*	*LLRICE62*	*Bengal*	*LLRICE601*	*Cocodrie*
leaf blast			6.5-7.0	6.5	2.5	3.5
narrow brown spot			0-0.5	0.0	2-2.8	1.5
sheath blight	No difference	No difference	4.5-5.0	5.0	7.3-8.0	7.3
leaf smut			1-2.5	2.0	0.5-1.5	1.0

LLRICE62 and LLRICE601 were tested in 2001 (LSU rice experiment station report page 327-328]
Evaluation in 2001 included natural infestations of *P.grisea* (leaf blast), *Sphaerulina oryzina (Cerospora*-narrow brown spot), *Burkholderia glumae* (panicle blight) and inoculation of *Thanatephorus cucumeris (Rhizcotonia*-sheath blight). Disease reaction rating 1-10 where 10 is the greatest severity of disease symptoms. LLRICE06 was evaluated in California. The 1998 field studies some disease symptoms were observed, however applications of fungicide held the disease in check and there were no differences between the LLRICE06 and its comparator observed.

Table 12 provides a summary of disease evaluations in LLRICE601 and the antecedent organisms. In our observations, the LLRICE events resemble their comparators when evaluated for disease susceptibility.

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 dormancy[1] 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.

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

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 (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 13). 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 13. 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%

<u>Conclusion</u>
All LLRICE601 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.

LLRICE62 and LLRICE06 continue to be an annual plant which produces panicles that do not shatter and disperse their seed. Seed of LLRICE06 and LLRICE62 germinate uniformly without extended seed dormancy (98-329-01p, Section V.C. "Seed Characteristics").

Table 14. Seed characters of LL rice events and their respective comparators

	LLRICE06	*M202*	*LLRICE62*	*Bengal*	*LLRICE601*	*Cocodrie*
Shattering	1	1	1	1	5	3
Short term dormancy -1 wk	100%	100%	51%	47%	97%	99%
Short term dormancy – 4 wks			71%	64%		
Long term dormancy	0%	0%	0%	0%	0%	0%

Panicle shattering score (as defined by PVP)
1 = Very low (less than 1%); 3 = Low (1-5%); 5 = Moderate (6-25%); 7 = Moderately high (26-50%); 9 = High (more than 50%)
Short term dormancy– % germination after one week of Dry after ripening
Long term dormancy - % of seed that remain firm and do not germinate in the test

Table 14 shows that, as it can be expected for cultivated rice, no long term dormancy was noted. When similar testing is completed for weedy red rice, the shattering score is 9 and the long term dormancy has been reported for several years. None of the LLRICE events have seed characteristics like red rice. LLRICE601 behaves similar to its parent variety. The antecedent organisms also exhibit this characteristic, therefore the nonregulated status should be extended to LLRICE601.

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. A similar conclusion was reached for LLRICE62 and LLRICE06 (98-329-01p, Section V.D. “Composition analysis”).

Table 15. Composition of LL rice events and their respective comparators

%w/w	*LLRICE06*	*M202*	*LLRICE62*	*Bengal*	*LLRICE601*	*Cocodrie*	*Literature ranges*
Fat/Oil	2.2	2.6	2.4	3.5	3.0	3.0	1.9-2.7
Protein	7.1	6.4	8.7	10.0	8.5	8.8	6.7-8.4
Ash	5.6	5.4	4.1	6.54	5.9	5.8	3.4-6.0
Carbohydrate	85	86	85	80	83	82	85
TDF	15	19	19	23	16	16	19
Crude Fiber	9	11	14	18	16	16	8-12
Calcium	0.03	0.05	0.03	0.03	0.02	0.02	0.03-0.07
Phosphorus	0.3	0.2	0.3	0.3	0.3	0.3	0.27-0.36
Iron	0.007	0.005	0.007	0.008	0.004	0.04	0.0016-0.0045

All data adjusted to dry weight basis and reported as %w/w.
Fat (Crude) or Ether Extract in Animal Feed, AOAC Official Methods of Analysis (1990), 920.39
Modified Kjeldahl Method, AOCS Official Method (1991), Ba 4d-90
Ash of Animal Feed, AOAC Official Methods of Analysis (1990), 942.05
By calculation: % carbohydrate = 100% - (% protein + % moisture + % fat + % ash)
TDF = Total Detergent Fiber and Lignin in Animal Feed, AOAC Official Methods of Analysis (1990), 973.18

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 indicate any difference between the behavior of the event and the antecedent organisms LLRICE62 and LLRICE06, and therefore should be extended 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 (98-329-01p, Section V1.A "Potential for Gene Transfer" page 51-57). No observations concerning the reproductive biology of event LLRICE601 indicate any difference between the behavior of the event and the antecedent organisms, therefore the rationale for determination of nonregulated status should be the same.

B. Weediness Potential of LLRICE601

There are no changes concerning weediness potential from the assessment of the previously approved petition submission (98-329-01p, Section V1.B "Weediness Potential" page 57-59). Like LLRICE06 and 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]

[2] Recommendations for weed contol 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

C. Effects on Non-target Organisms

There are no changes from the previously approved petition submission (98-329-01p, Section V1.C "Effects on Non-Target Organisms" page 59-60). 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 (98-329-01p, Section V1.D "Indirect Effects on other Agricultural Products" page 60).

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 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.

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 – T_1 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 T_1 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.

CONTAINS CONFIDENTIAL BUSINESS INFORMATION

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 Distrist

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

DESCRIPTION OF THE INSERTED/DELETED DNA SEQUENCE IN THE PLANT

Characterization of the sequence(s) inserted into the plant genome

The size and structure of the inserted DNA sequence

Only the chimeric *P35S-bar-3'nos* gene from plasmid pGSV71 would be transferred to the plant genome. After analyzing the inserted DNA in the event LLRICE601, it was determined that the Right Border and *P35S-bar-* was integrated in the plant genome, but a deletion was found in part of the *nos* terminator. This insertion resulted in a single copy of the *bar* gene in the event LLRICE601. In addition, it has been found that a second copy of the *P35S* promoter is also present, but not the *bar* gene, therefore, the event is a single gene insert.

The function of 3'*nos* terminator is to start the polyadenylation after transcription of the gene. A further search for the presence of a polyadenylation site at the 3' flanking sequences of LLRICE601, found two polyadenylation sites: AATAA: bp 12 → bp 16 and ATAAAA: bp 13 → bp 18. If these polyadenylation sites were not active, transcription would not stop until the next active polyadenylation site was reached. This could have an influence on the stability of the produced mRNA, which could lead to low expression of the *bar* gene. Since there are data on the expression levels of the PAT protein, we can conclude that the presence of part of the *3'nos* terminator is sufficient to allow expression of the PAT protein in LLRICE601 at levels that confer herbicide tolerance.

Selected transformants showed the expected phenotype of glufosinate tolerance, which is evidence of the functional expression of the inserted *bar* gene.

The determination of inserted sequences in event LLRICE601 confirmed the presence of one copy of the *bar* gene.

Verification of the insert and determination of an extra P35S fragment

To determine the copy number and the insert characterization, an array of restriction enzymes were utilized. The genomic DNA was then hybridized with different probes in order to explain the insertion. As an example, complete T-DNA probes were used, as well as probes for the *bar* gene and P35S promoter.

To understand how the different probes hybridize in the LLRICE601 insert, see Figure A2-1.
Probes used:
Probe 69: complete T-DNA: pGSV71 *Hind*III-*Eco*RI
Probe 70: bar: pDE110 *Nco*I-*Bgl*II or
Probe 59: bar-3'nos: pDE110 *Nco*I-*Hind*III
Probe 19: P35S: pDE110 *Eco*RI-*Nco*I
Probe 71: 3'P35S: pDE110 *Bgl*II-*Nco*I
Probe 72: 5'P35S: pDE110 *Eco*RI-*Stu*I

T-DNA sequences of pGSV71 and PDE110 are the same. For example, Probe 72 is prepared on plasmid pDE110, using restriction enzymes *Eco*RI and *Stu*I. These enzymes cut plasmid pDE110 on positions 396 and 945. However, these enzymes are also present in plasmid pGSV71, on positions 250 (*Eco*RI) and 799 (*Stu*I). To demonstrate that both sequences are completely identical, a sequence alignment of both sequences was made.

Hybridization with the *bar*-probe resulted in the expected hybridization patterns. No extra fragments were observed. Hybridization with all other probes show extra fragments. Summary of the results are presented in Tables A2-1 and A2-2. The Figures A2-2 to A2-6. correspond to the data.

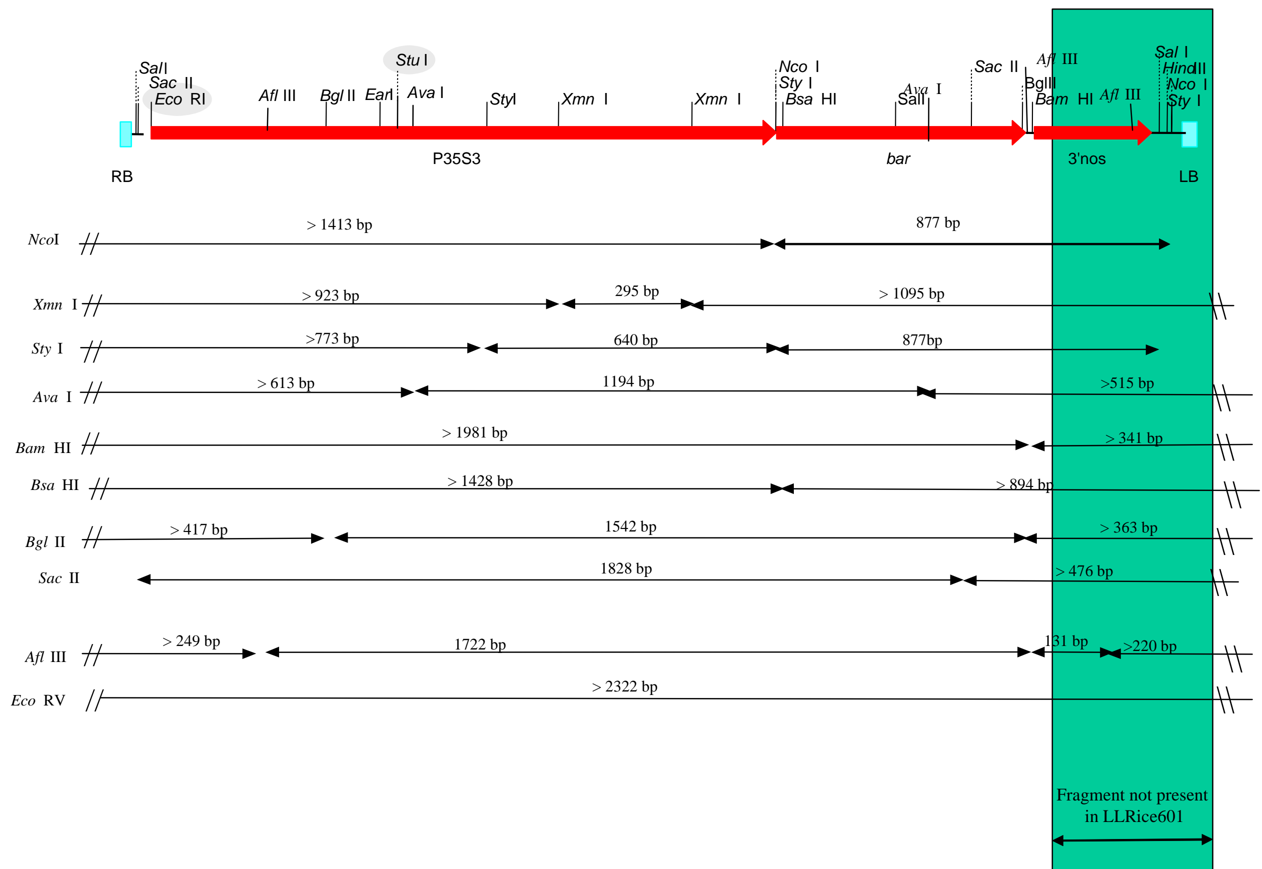


Figure A2-1. Schematic drawing of the LLRICE601 insert and hybridization fragments.

Table A2-1. Hybridization results – Insert characterization LLRICE601

Digest	*P35S3-probe*		*5'P35S-probe*		*3'P35S-probe*	
	Expected fragments	*Observed fragments*	*Expected fragments*	*Observed fragments*	*Expected fragments*	*Observed fragments*
*Nco*I	5'- border: >1413 bp	± 14000 bp ± 9000 bp	5'- border: >1413 bp	± 14000 bp ± 9000 bp	5'-border: > 1413bp	±14000 bp ± 9000 bp
*Xmn*I	3'-border:>1095 bp 5'-border:>923 bp Internal: 295 bp	3'-border: ± 3600 bp ±14000 bp ±1600 bp ± 1000 bp (weak) ± 640 bp Internal: 295 bp	5'-border: >923bp	±14000 bp ± 1600 bp ± 1000 bp ± 640 bp	3'-border: > 1095 bp 5'-border: >923 bp internal: 295 bp	3'-border: ± 3600 bp ±14000 bp ± 1600 bp ± 600 bp internal: 295 bp
*Sty*I	5'-border: >773 bp Internal: 640 bp	± 2000 bp ±3900 bp Internal: 640 bp	5'-border: > 773 bp	± 2000 bp ±3900 bp ±1200 bp(very weak.non specific)	5'- border: >773 bp internal: 640 bp	±4000 bp(very weak) ±2000 bp internal: 640 bp
*Ava*I	5'-border:>613 bp internal: 1194 bp	± 1600bp ± 8000 bp ± 7000 bp (very weak) ± 5097 bp (very weak) internal: 1194 bp	5'-border: > 613 bp	8000 bp ± 7000 bp (very weak) ± 5097 bp (very weak) ±1600 bp	5'-border: >613 bp internal: 1194 bp	± 1600bp ± 8000 bp ± 7000 bp (very weak) ± 5097 bp (very weak) internal: 1194 bp
*BamH*I	5'- border: >1981 bp	5'-border: ± 2700 bp ± 7000 bp	5'-border: > 1981 bp	5'-border: ±2700 bp	5'-border: >1981 bp	± 7000 bp (very weak) 5'- border: ±2700 bp
*BsaH*I	5'- border:> 1428 bp	± 9000 bp ± 5097 bp	5'-border: >1428 bp	± 9000 bp ± 5097 bp	5'-border: >1428 bp	±5097 bp ±9000 bp
*Bgl*II	5'-border:>417 bp Internal: 1542 bp	5'-border: 4400 bp Internal: 1542 bp	5'-border:>417 bp Internal: 1542 bp	5'-border: 4400 bp Internal: 1542 bp	internal 1542 bp	internal: 1542 bp
*Sac*II	Internal: 1828 bp	Internal: 1828 bp	Internal: 1828 bp	Internal: 1828 bp	internal 1828 bp	internal: 1828 bp
*Afl*III	5'-border: >249 bp Internal: 1722 bp	5'-border: ± 1400 bp Internal: 1722 bp ± 3800 bp (very weak)	5'-border: > 249 bp Internal: 1722 bp	5'-border: 4400 bp Internal: 1722 bp ± 3800 bp(very weak)	internal: 1722 bp	Internal: 1722 bp
EcoRV	> 2322 bp	± 14000 bp ± 6000 bp (weak)	> 2322 bp	± 14000 bp ± 6000 bp (weak)	>2322 bp	± 14000 bp ± 6000 bp (weak)
Undigest	>2322 bp	>14057 bp	>2322 bp	>14057 bp	>2322 bp	> 14057 bp
*WT Cocodrie Nco*I	\	\	\	\	\	\
*WT Rice Cocodrie +1c pGSV71 Nco*I	877 bp 8678 bp	877 bp 8678 bp	877 bp	877 bp	8678 bp	8678 bp

*: Unexpected fragments derived from an extra insertion of a P35S fragment

Table A2-2. Hybridization results – Insert characterization LLRICE601

Digest	*Total T-DNA probe*		*Bar probe*	
	Expected fragments	*Observed fragments*	*Expected fragments*	*Observed fragments*
*Nco*I	5'-border: > 1413bp 3' border: 875 bp	±14000 bp ± 9000 bp 3' border: 875 bp	3' border: 875 bp	3' border: 875 bp
*Xmn*I	5'-border: >923 bp 3'-border: >1095 bp internal: 295 bp	3'-Border: ±3600 bp ± 14000 bp ± 1600 bp ± 1000bp (very weak) ± 640 bp (very weak) 1 internal: 295 bp	3'-border: >1095 bp	3'-border: : ±3600 bp
*Sty*I	5'- border: >773 bp internal: 640 bp 3' border: 875 bp	±3900 bp ±2000 bp nternal: 640 bp 3' border: 875 bp	3' border: 875 bp	3' border: 875 bp
*Ava*I	3'-border: >515 bp 5'-border: >613 bp internal: 1194 bp	3'-border: 345 bp ± 8000 bp ± 7000 bp(very weak) ± 5097 bp (very weak) ±1600 bp internal: 1194 bp	3'-border: >515 bp internal: 1194 bp	3'-border: 345 bp internal: 1194 bp
*BamH*I	5'-border: >1981 bp 3'-border: >341 bp	5'- border: ±2700 bp	5'-border: >1981 bp	5'-border: ± 2700bp
*BsaH*I	3'-border: >894 bp 5'-border: >1428 bp	3'-border: ±1000 bp ±9000 bp ±5097 bp(2ble fragm.)	3'-border: >894 bp	3'-border: ±1000bp
*Bgl*II	5'-border: >417 bp 3'-border: >363 bp internal 1542 bp	5'-border: ± 4400 bp internal: 1542 bp	3'-border:>363bp internal: 1542 bp	internal: 1542 bp
*Sac*II	3'-border: >476 bp internal 1828 bp	3'-border: ± 980 bp (very weak) internal: 1828 bp	3'-border: >476 bp internal: 1828 bp	**3'-border: ±980 bp (very weak)** internal: 1828 bp
*Afl*III	5'-border: >249 bp 3'-border:>220 bp 2 internal: 1722 bp, 131 bp	5'-border: ±1400 bp internal: 1722 bp ± 3800 bp (very weak)	3'-border: >220bp 2 internal: 1722 bp, 131 bp	internal: 1722 bp
EcoRV	>2322 bp	±14000 bp ± 6000 bp (very weak)	>2322 bp	±14000 bp
undigested	>2322 bp	> 14057 bp	>2322 bp	> 14057 bp
*WT Cocodrie Nco*I	\	\	\	\
*WT Cocodrie + 1c pGSV71 Nco*I	877 bp 8678 bp	877 bp 8678 bp	877 bp	877 bp

*: Extra (unexpected) fragments due to an extra insertion of a P35S fragment

*: Absence of fragments or very weak fragments due to the deletion of 3'nos in LLRICE601.

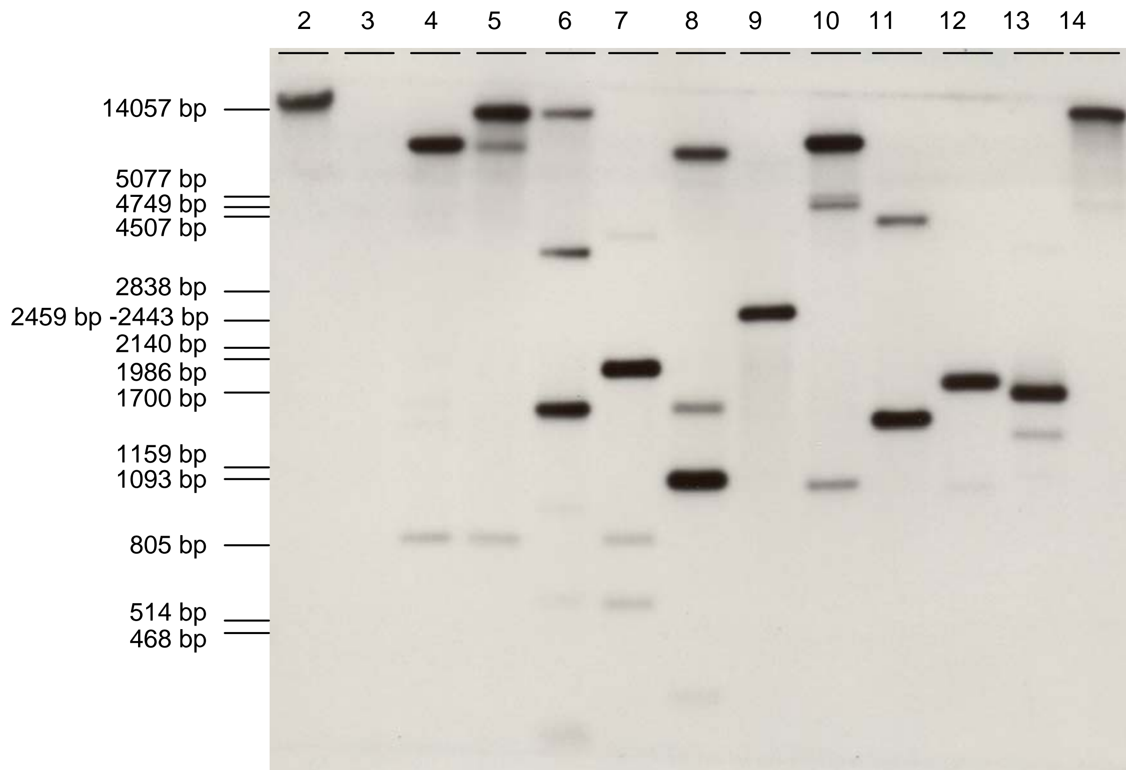


Figure A2-2. LLRICE601 – verification of the insert – probe: T-DNA (pGSV71 *Hin*dIII-*Eco*RV)
DNA was isolated from LLRICE601 plants and the non-transgenic counterpart. Genomic DNA was digested with different restriction enzymes.

Lane 2: LLRICE601 undigested
Lane 3: WT Rice Cocodrie *Nco*I digested
Lane 4: WT Rice Cocodrie *Nco*I digested + 1 copy pGSV71 *Nco*I digested
Lane 5: LLRICE601 – *Nco*I digested
Lane 6: LLRICE601 – *Xmn*I digested
Lane 7: LLRICE601 – *Sty*I digested
Lane 8: LLRICE601 – *Ava*I digested
Lane 9: LLRICE601 – BamHI digested
Lane 10: LLRICE601 – BsaHI digested
Lane 11: LLRICE601 – *Bgl*II digested
Lane 12: LLRICE601 – *Sac*II digested
Lane 13: LLRICE601 – *Afl*III digested
Lane 14: LLRICE601 – *Eco*RV digested

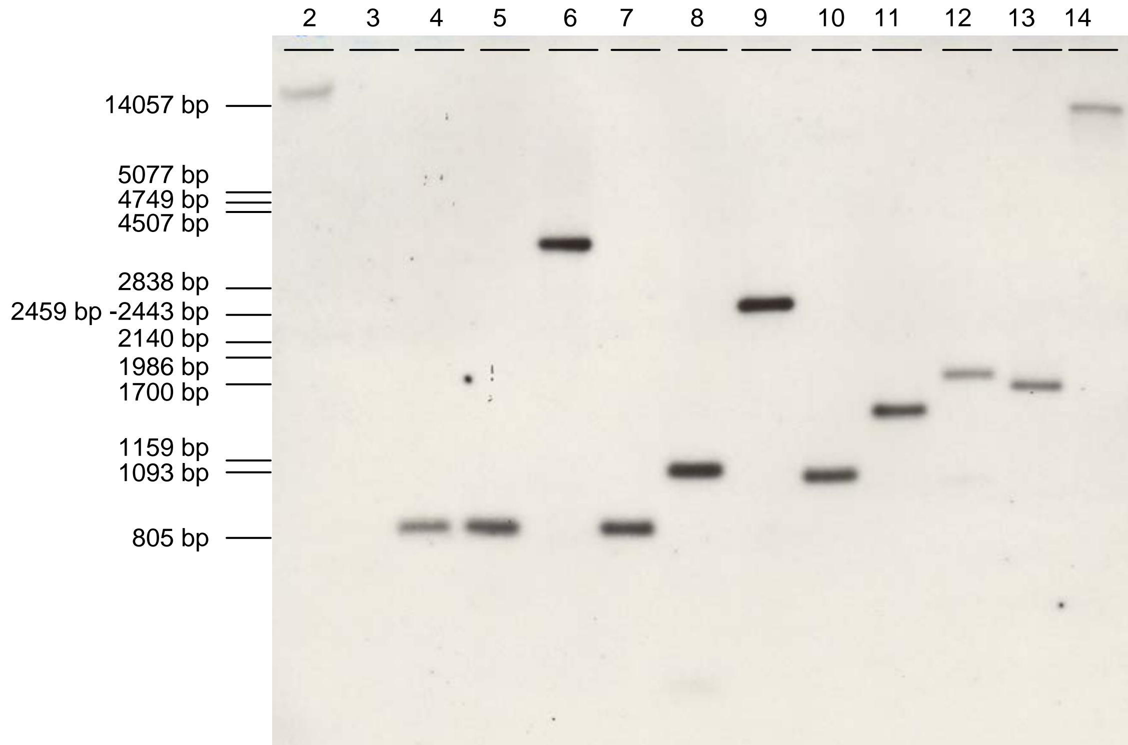


Figure A2-3. LLRICE601 – verification of the insert – probe: *bar* (pDE110 *Nco*I – *Bgl*II)
DNA was isolated from LLRICE601 plants and the non-transgenic counterpart. Genomic DNA was digested with different restriction enzymes.

Lane 2: LLRICE601 undigested
Lane 3: WT Rice Cocodrie *Nco*I digested
Lane 4: WT Rice Cocodrie *Nco*I digested + 1 copy pGSV71 *Nco*I digested
Lane 5: LLRICE601 – *Nco*I digested
Lane 6: LLRICE601 – *Xmn*I digested
Lane 7: LLRICE601 – *Sty*I digested
Lane 8: LLRICE601 – *Ava*I digested
Lane 9: LLRICE601 – BamHI digested
Lane 10: LLRICE601 – BsaHI digested
Lane 11: LLRICE601 – *Bgl*II digested
Lane 12: LLRICE601 – *Sac*II digested
Lane 13: LLRICE601 – *Afl*III digested
Lane 14: LLRICE601 – *Eco*RV digested

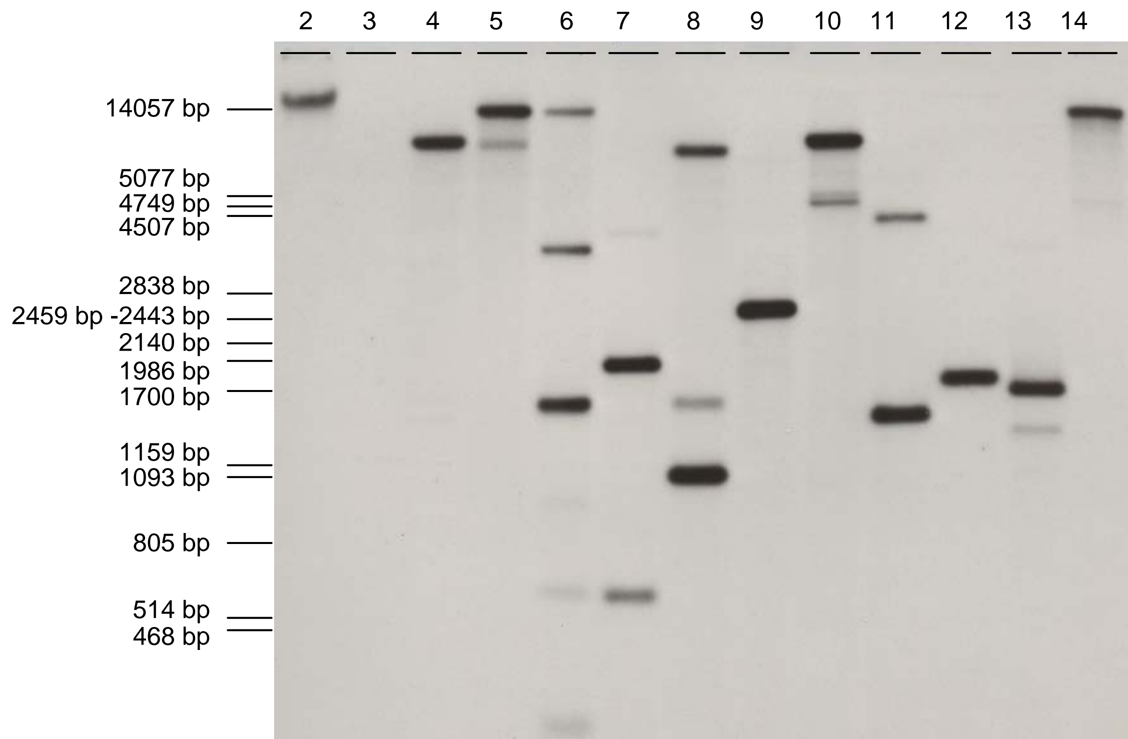


Figure A2-4. LLRICE601 – verification of the insert – probe: P35S (pDE110 *Eco*RI – *Nco*I)
DNA was isolated from LLRICE601 plants and the non-transgenic counterpart. Genomic DNA was digested with different restriction enzymes.

Lane 2: LLRICE601 undigested
Lane 3: WT Rice Cocodrie *Nco*I digested
Lane 4: WT Rice Cocodrie *Nco*I digested + 1 copy pGSV71 *Nco*I digested
Lane 5: LLRICE601 – *Nco*I digested
Lane 6: LLRICE601 – *Xmn*I digested
Lane 7: LLRICE601 – *Sty*I digested
Lane 8: LLRICE601 – *Ava*I digested
Lane 9: LLRICE601 – BamHI digested
Lane 10: LLRICE601 – BsaHI digested
Lane 11: LLRICE601 – *Bgl*II digested
Lane 12: LLRICE601 – *Sac*II digested
Lane 13: LLRICE601 – *Afl*III digested
Lane 14: LLRICE601 – *Eco*RV digested

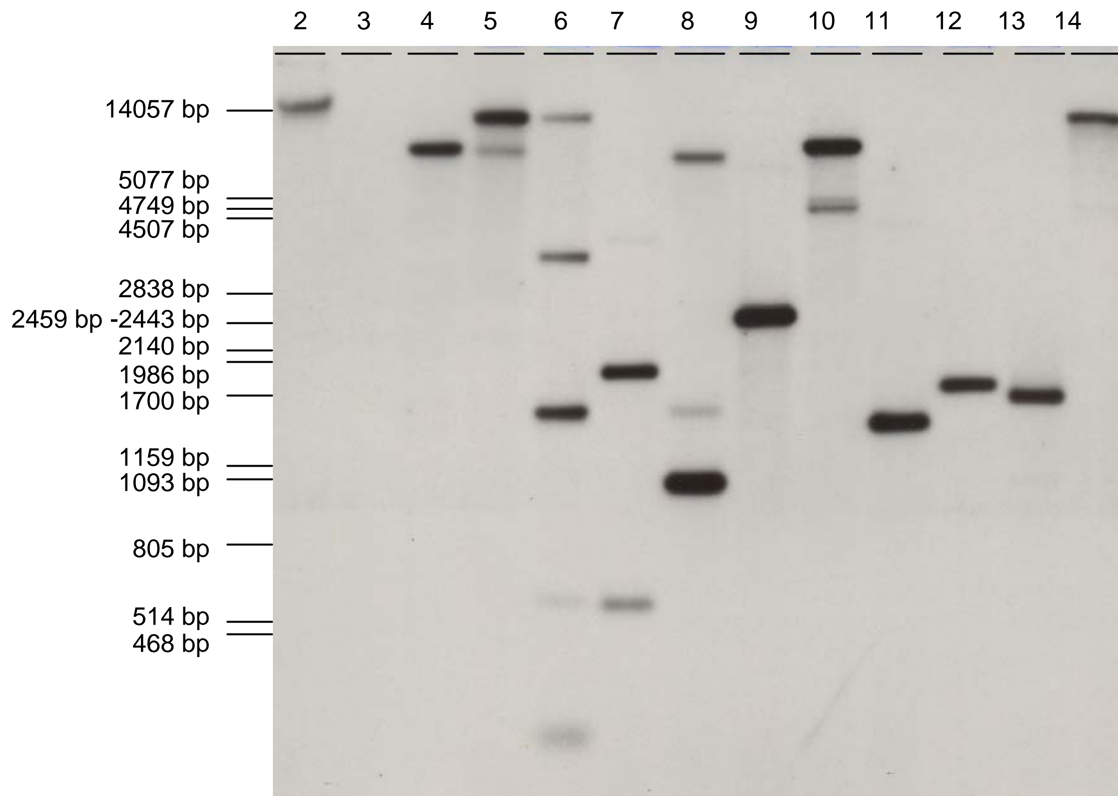


Figure A2-5. LLRICE601 – verification of the insert – probe: 3' P35S (pDE110 *Bgl*II – *Nco*I)
DNA was isolated from LLRICE601 plants and the non-transgenic counterpart. Genomic DNA was digested with different restriction enzymes.

Lane 2: LLRICE601 undigested
Lane 3: WT Rice Cocodrie *Nco*I digested
Lane 4: WT Rice Cocodrie *Nco*I digested + 1 copy pGSV71 *Nco*I digested
Lane 5: LLRICE601 – *Nco*I digested
Lane 6: LLRICE601 – *Xmn*I digested
Lane 7: LLRICE601 – *Sty*I digested
Lane 8: LLRICE601 – *Ava*I digested
Lane 9: LLRICE601 – BamHI digested
Lane 10: LLRICE601 – BsaHI digested
Lane 11: LLRICE601 – *Bgl*II digested
Lane 12: LLRICE601 – *Sac*II digested
Lane 13: LLRICE601 – *Afl*III digested
Lane 14: LLRICE601 – *Eco*RV digested

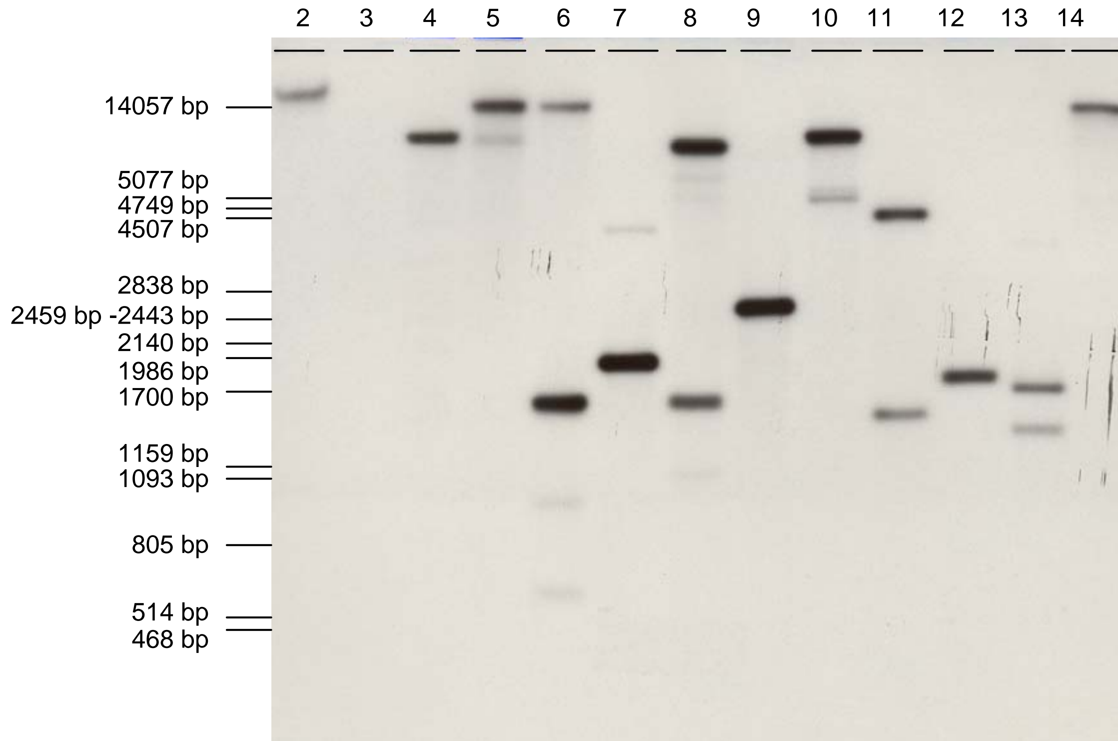


Figure A2-6. LLRICE601 – verification of the insert – probe: 5' P35S (pDE110 *Eco*RI – *Stu*I)
DNA was isolated from LLRICE601 plants and the non-transgenic counterpart. Genomic DNA was digested with different restriction enzymes.

Lane 2: LLRICE601 undigested
Lane 3: WT Rice Cocodrie *Nco*I digested
Lane 4: WT Rice Cocodrie *Nco*I digested + 1 copy pGSV71 *Nco*I digested
Lane 5: LLRICE601 – *Nco*I digested
Lane 6: LLRICE601 – *Xmn*I digested
Lane 7: LLRICE601 – *Sty*I digested
Lane 8: LLRICE601 – *Ava*I digested
Lane 9: LLRICE601 – BamHI digested
Lane 10: LLRICE601 – BsaHI digested
Lane 11: LLRICE601 – *Bgl*II digested
Lane 12: LLRICE601 – *Sac*II digested
Lane 13: LLRICE601 – *Afl*III digested
Lane 14: LLRICE601 – *Eco*RV digested

XI. Appendix 3. Vector backbone analysis

DESCRIPTION OF THE ANALYSIS OF THE ABSENCE OF VECTOR BACKBONE

[For the molecular verification of absence of pGSV71 vector backbone sequences, Southern blot analysis was performed. Five µg genomic LLRICE601 DNA was digested with *Afl*III and *Bgl*II, applying concentration, buffer and temperature according to the conditions proposed by the manufacturer. Upon termination of digestion, DNA fragments were separated by agarose gel-electrophoresis. The separated DNA fragments were transferred upon denaturation, through capillary force from the agarose gel to a Nylon membrane]. **CBI**

[The blots were hybridized with a number of overlapping probes (see Figure A3-1). The hybridization results presented in Figure A3-2 to A3-5 show that the DNA negative and the DNA positive controls gave the expected results.]

[Each gel used for the analysis had: **CBI**

- one DNA negative control in which the template DNA provided was genomic DNA prepared from a non-transgenic plant. This negative control was used to confirm the absence of background hybridization. This control was negative with every probe used (see Figure A3-2 to A3-5 lane 4).
- DNA positive control: digested genomic DNA prepared from a non-transgenic plant, supplemented with approximately a tenth, one and ten copies of *Bgl*II digested transforming plasmid. This control was used to demonstrate that the hybridization was performed under conditions allowing hybridization of the probe with target sequences. With every probe used, a *Bgl*II fragment of the expected length hybridized (see Figure A3-2 to A3-5).
- as a molecular weight standard Phage Lambda DNA digested with *Pst*I. This molecular weight standard covers an appropriate size range for the fragments that were expected to be detected on the Southern blots.]

[*Sm/Sp probe (MLD13-MLD39):* **CBI**
In the DNA positive control we observed the expected 8013 bp *Bgl*II fragment. No hybridisation signals could be observed in the transgenic LLRICE601 sample nor in the wild type DNA (negative control)(see Figure A3-2).

5' pVS1 ori probe (MLD40-MLD41):
In the DNA positive control we observed the expected 8013 bp *Bgl*II fragment. No hybridization signals could be observed in the transgenic LLRICE601 sample nor in the wild type DNA (negative control)(see Figure A3-3).

3' pVS1 ori probe (VH029-MLD42):
In the DNA positive control we observed the expected 8013 bp fragment. A very weak hybridization signal could be observed in the transgenic LLRICE601 sample. No hybridization signals could be observed in the wild type DNA (negative control)(see Figure A3-4).]

[*pVS1 ori* is a broad host-range *ori* for low copy, stable replication in *Agrobacterium* and other gene transfer competent bacteria. The unintended presence of bacterial origin of replication in transgenic plants as a result of the transformation process has occurred in the past and those plants have a history of safe use. Even if a functional *ori* were to have been transferred, it will not cause the plant to become a plant pest. **CBI**

ColEI probe (VH031-SVH042):
In the DNA positive control we observed the expected 8013 bp fragment. No hybridization signals could be observed in the transgenic LLRICE601 sample nor in the wild type DNA (negative control)(see Figure A3-5).

With the performed Southern blot analysis, using overlapping probes covering almost the complete pGSV71 vector backbone sequences.]

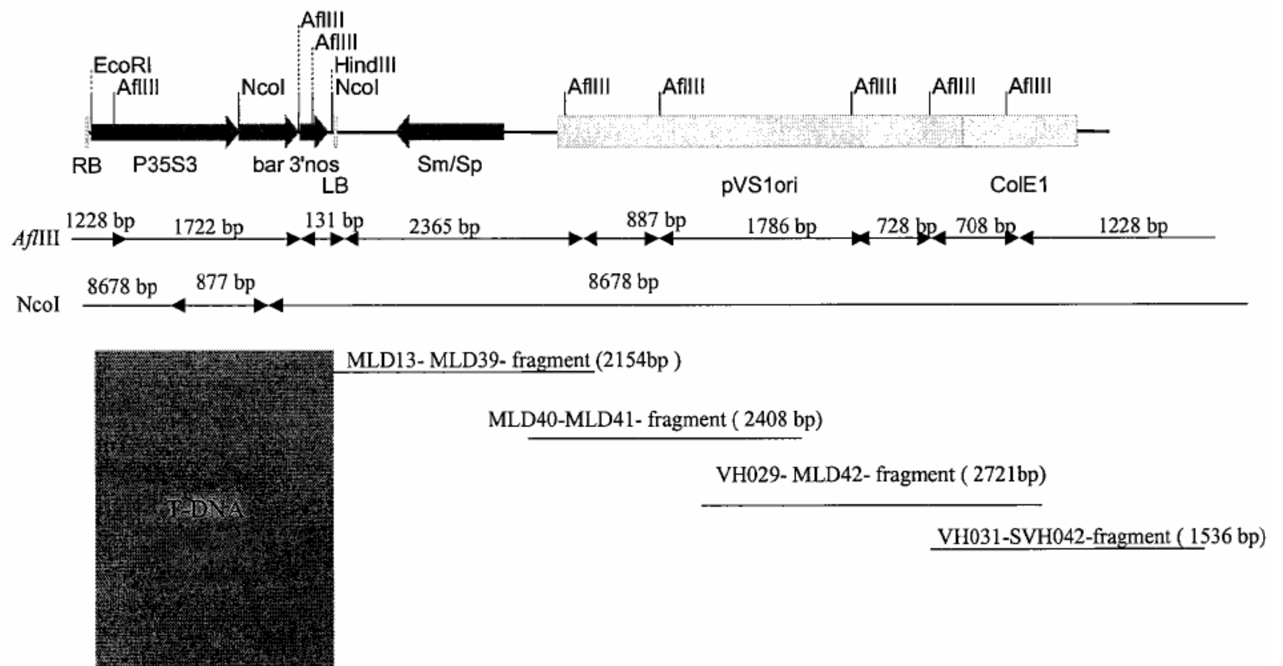


Figure A3-1. Schematic drawing of the position of probes used to verify the absence of vector backbone

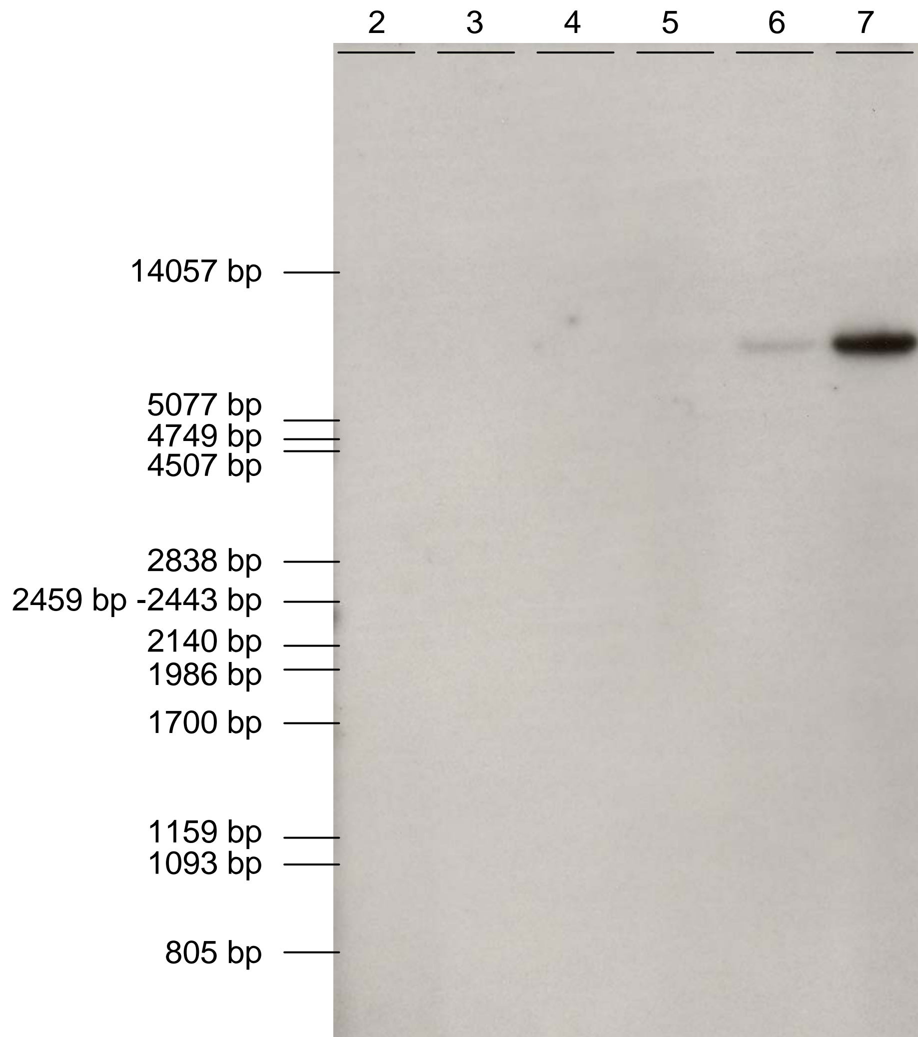


Figure A3-2. Absence of vector backbone – probe Sm/Sp

DNA was isolated from LLRICE601 plants and the non-transgenic counterpart.
5 µg DNA was digested with *Bgl*II and *Afl*III.
Gel: SB01 probe: Sm/Sp (MLD13-MLD39)
Lane 2: LLRICE601 *Bgl*II digested
Lane 3: LLRICE601 *Afl*III digested
Lane 4: WT Rice Cocodrie *Bgl*II digested
Lane 5: WT Rice Cocodrie *Bgl*II digested + 0.1 copy pGSV71 *Bgl*II digested
Lane 6: WT Rice Cocodrie *Bgl*II digested + 1 copy pGSV71 *Bgl*II digested
Lane 7: WT Rice Cocodrie *Bgl*II digested + 10 copies pGSV71 *Bgl*II digested

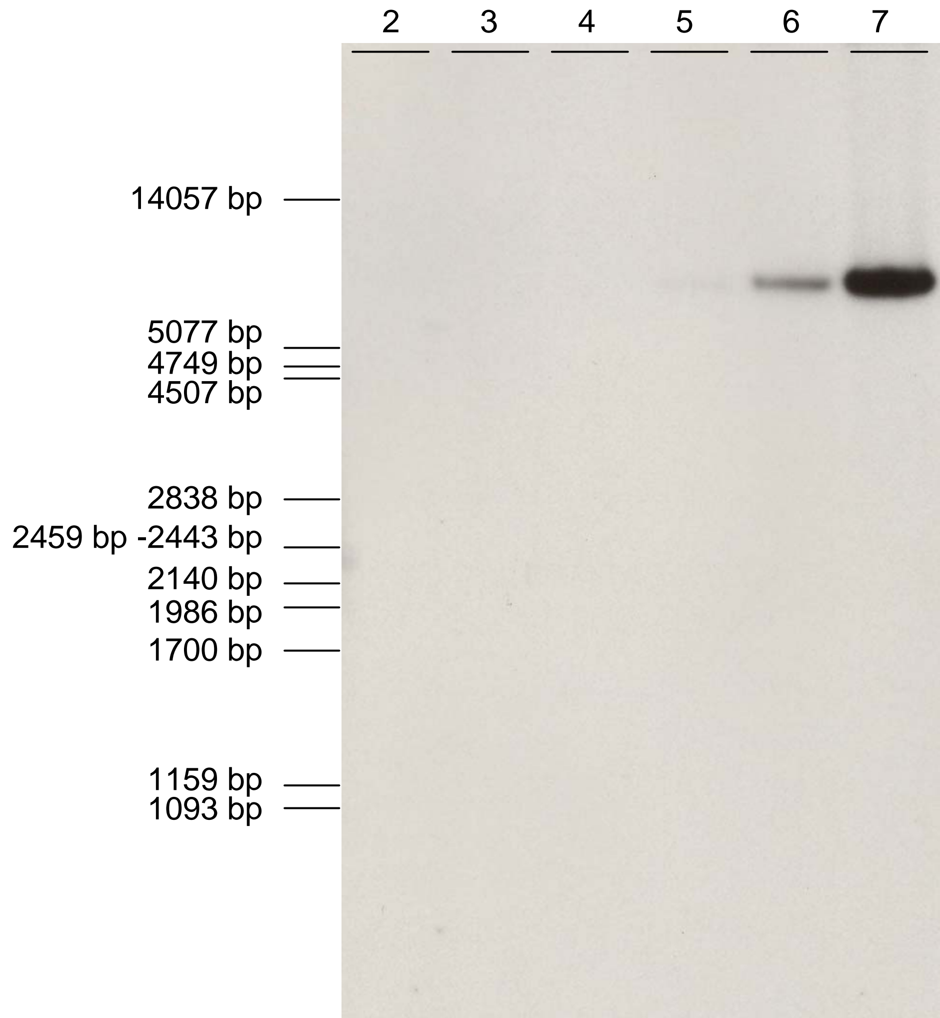


Figure A3-3. Absence of vector backbone – probe 5' pVS1 ori

DNA was isolated from LLRICE601 plants and the non-transgenic counterpart.
5 µg DNA was digested with *Bgl*II and *Afl*III.
Gel: SB02 probe: 5' pVS1 ori (MLD40-MLD41)
Lane 2: LLRICE601 *Bgl*II digested
Lane 3: LLRICE601 *Afl*III digested
Lane 4: WT Rice Cocodrie *Bgl*II digested
Lane 5: WT Rice Cocodrie *Bgl*II digested + 0.1 copy pGSV71 *Bgl*II digested
Lane 6: WT Rice Cocodrie *Bgl*II digested + 1 copy pGSV71 *Bgl*II digested
Lane 7: WT Rice Cocodrie *Bgl*II digested + 10 copies pGSV71 *Bgl*II digested

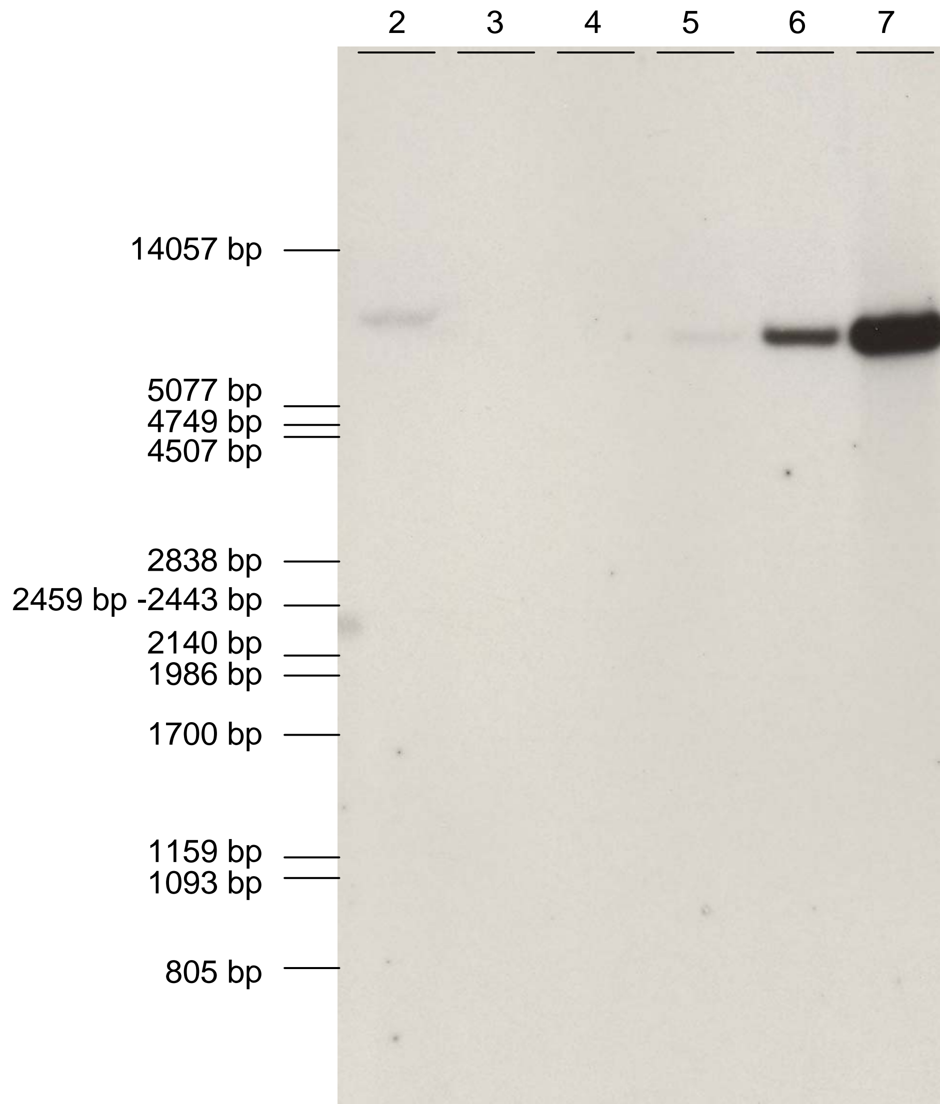


Figure A3-4: Absence of vector backbone – probe 3' pVS1 ori

DNA was isolated from LLRICE601 plants and the non-transgenic counterpart.
5 µg DNA was digested with *Bgl*II and *Afl*III.
Gel: SB03 probe: 3' pSV1 ori (VH029-MLD42)
Lane 2: LLRICE601 *Bgl*II digested
Lane 3: LLRICE601 *Afl*III digested
Lane 4: WT Rice Cocodrie *Bgl*II digested
Lane 5: WT Rice Cocodrie *Bgl*II digested + 0.1 copy pGSV71 *Bgl*II digested
Lane 6: WT Rice Cocodrie *Bgl*II digested + 1 copy pGSV71 *Bgl*II digested
Lane 7: WT Rice Cocodrie *Bgl*II digested + 10 copies pGSV71 *Bgl*II digested

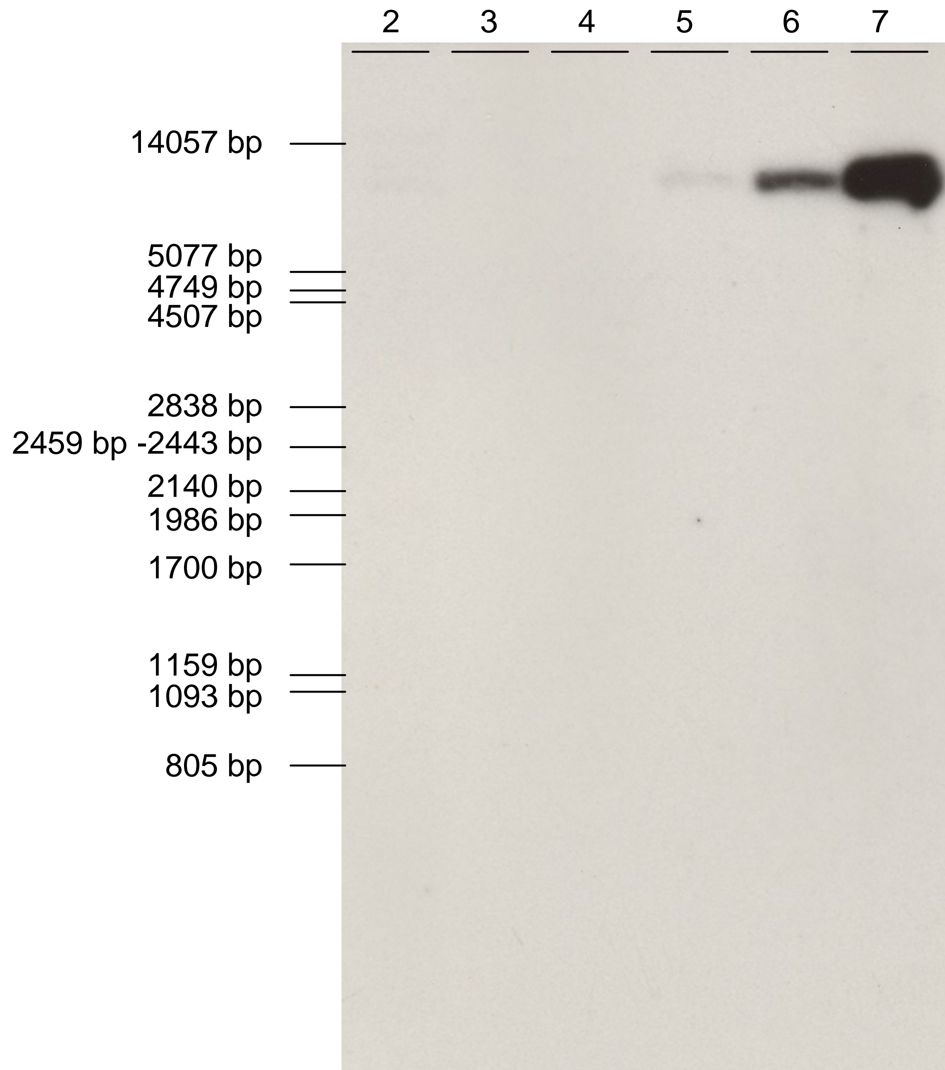


Figure A3-5. Absence of vector backbone – probe ColE1

DNA was isolated from LLRICE601 plants and the non-transgenic counterpart.
5 µg DNA was digested with *Bgl*II and *Afl*III.
Gel: SB04 probe: ColE1 (VH031-SVH042)
Lane 2: LLRICE601 *Bgl*II digested
Lane 3: LLRICE601 *Afl*III digested
Lane 4: WT Rice Cocodrie *Bgl*II digested
Lane 5: WT Rice Cocodrie *Bgl*II digested + 0.1 copy pGSV71 *Bgl*II digested
Lane 6: WT Rice Cocodrie *Bgl*II digested + 1 copy pGSV71 *Bgl*II digested
Lane 7: WT Rice Cocodrie *Bgl*II digested + 10 copies pGSV71 *Bgl*II digested

XII. Appendix 4. Stability of the insert

CONTAINS CONFIDENTIAL BUSINESS INFORMATION

STABILITY OF THE INSERTED DNA OVER SEVERAL GENERATIONS

The stability of the integration of LLRICE601 over different generations was demonstrated by means of a Southern blot analysis.
Genomic DNA was prepared from 14 LLRICE601 plants (of generations T_3, T_4, T_5). Five µg of each DNA sample were digested with *Bgl*II and the DNA fragments were then separated by agarose gel-electrophoresis. The separated fragments were transferred upon denaturation, through capillary force from agarose gel to a Nylon membrane. The blots were hybridized with the complete T-DNA probe. (pGSV71 *EcoR*I-*Hind*III fragment (2250 bp)).

The expected hybridization fragments are presented in Table A4-1 and Figure A4-1.

Table A4-1. Demonstration of the stability of the integration of LLRICE601 over different generations – Hybridization results.

	T-DNA probe (pGSV71 – EcoRI-HindIII fragment)	
	Expected fragments	***Obtained fragments***
LLRICE601 – *Bgl*II digested	1542 bp LB fragment > 363 bp RB fragment > 417bp	1542 bp ± 4500 bp (RB fragment) ± 6000 bp (partial digest)
WT Rice Cocodrie – *Bgl*II digested	No hybridization	No hybridization
WT Rice Cocodrie + 1 copy pGSV71 – *Bgl*II digested	1542 bp 8013 bp	1542 bp 8013 bp 9500 bp (partial digest)

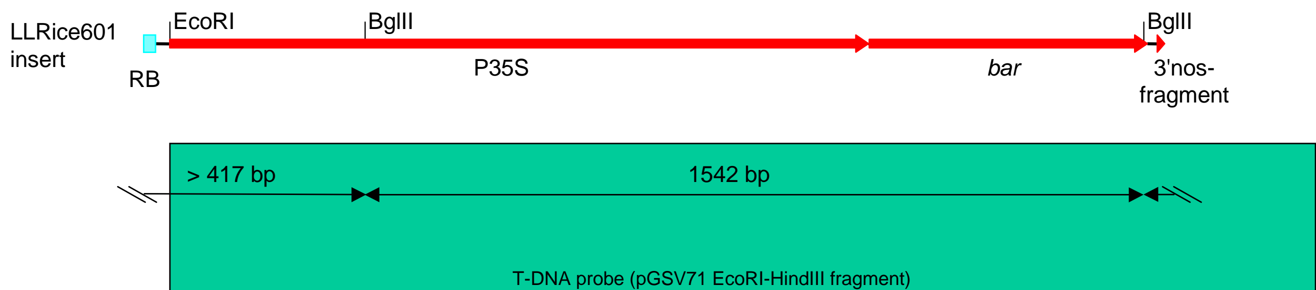


Figure A4-1. Schematic drawing of the LLRICE601 insert.

[The hybridization results are presented in Figure A4-2. The gel used for the analysis had one DNA negative control in which the template DNA provided was genomic DNA prepared from a non-transgenic Cocodrie plant. This negative control was used to confirm the absence of background hybridization.] **CBI**

[The DNA positive control (*Bgl*II digested genomic DNA prepared from a non-transgenic Cocodrie plant, supplemented with approximately one copy of *Bgl*II digested transforming plasmid) was used to demonstrate that the hybridization was performed under conditions allowing hybridization of the probe with target sequences. Next to two *Bgl*II fragments of the expected length (1542 bp, 8013 bp), a weaker third fragment of ± 9500 bp is visible. This fragment is the result of a partial digestion of the plasmid with *Bgl*II (8013 bp + 1542 bp = 9555 bp).] **CBI**

[After hybridization with the complete T-DNA probe, all examined plants show two of the expected fragments: the internal fragment of 1542 bp and a ± 4500 bp RB fragment. The LB integration fragment is not visible. From other analysis, we know that there is a deletion of about 322 bp of T-DNA sequences at the LB. Therefore, the homology between the integration fragment of LLRICE601 and the T-DNA probe is only 41 bp, which is too little to result in a hybridization fragment. In plant 2 of generation T_3 and in plant 4 of generation T_4, an extra fragment of ± 6000 bp is visible. This fragment is the result of a partial digestion of the genomic DNA of these plants with BglII (1542 bp + 4500 bp = 6042 bp).] **CBI**

By means of Southern blot analysis, it was demonstrated that the hybridization pattern obtained by digestion of genomic DNA with [*Bgl*II] and probed with the complete T-DNA of three different generations of LLRICE601 are identical. These results show the stability of the event LLRICE601 at the genomic level over multiple generations. **CBI**

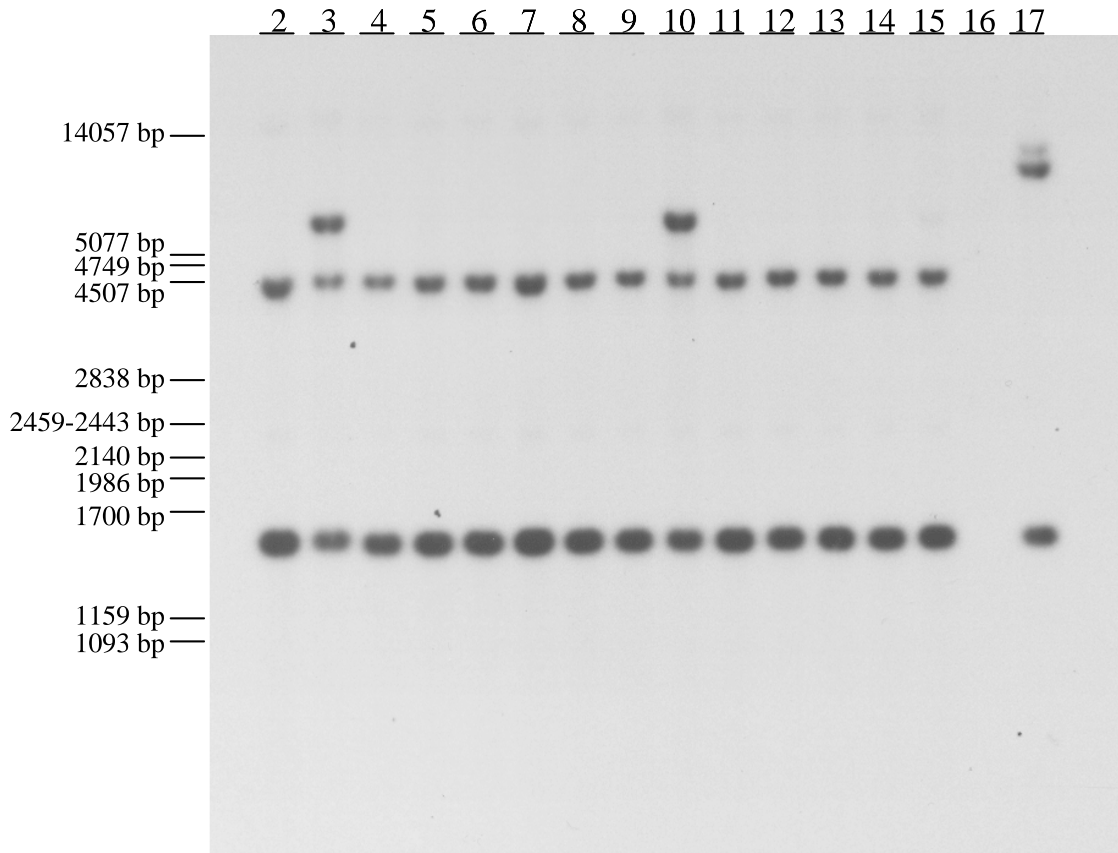


Figure A4-2. Demonstration of the stability of *Oryza sativa* transformation event LLRICE601- Southern blot analysis.

DNA was isolated from *Oryza sativa* event LLRICE601 (generations T_3, T_4 and T_5) and the wild type rice variety Cocodrie. Five µg genomic DNA was digested with *Bgl*II and probed with the 2250 bp *EcoR*I-*Hind*II pGSV71 fragment.

Lane 1. MW marker. Phage Lambda DNA - *Pst*I digested
Lane 2 - Lane 6. LLRICE601 (T_3 – plants 1 to 5) - *Bgl*II digested
Lane 7 - Lane 11. LLRICE601 (T_4 – plants 1 to 5) - *Bgl*II digested
Lane 12 - Lane 15. LLRICE601 (T_5 – plants 1 to 5) - *Bgl*II digested
Lane 16. Wild type rice var. Cocodrie - *Bgl*II digested
Lane 17. Wild type rice var. Cocodrie - *Bgl*II digested + 1 copy pGSV71 - *Bgl*II digested

The amount of restricted pGSV71 in lane 17 is equivalent to 1 copy of the plasmid integrated in 5 µg of *Oryza sativa* DNA. MW marker (Lambda DNA digested with *Pst*I) size given in base pairs

XIII. Appendix 5. Bioinformatics analysis

BIOINFORMATICS ANALYSIS

The flanking regions of the inserted sequence

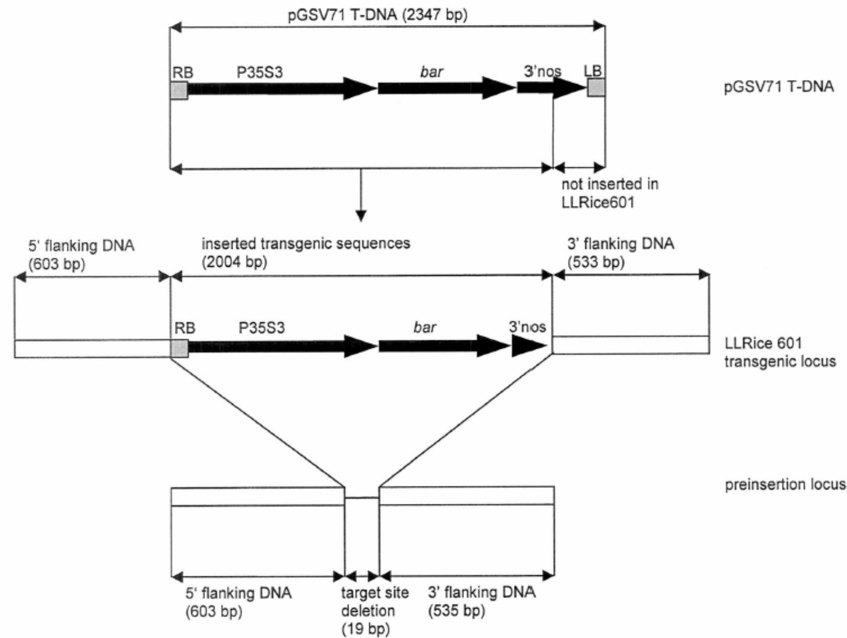
Rice plants transformed using *Agrobacterium tumefaciens*-mediated transformation inserting the T-DNA from vector pGSV71 into the rice genome generated the event LLRICE601. Due to the insertion of the P35S3-bar-3'nos gene cassette in rice, a 5-prime and 3-prime junction, where rice genomic DNA and inserted 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 *Stu*I 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

Database	***Posted date of database***	***Date of analysis***	***Number of sequences in database***
Non Redundant; Non Plant	July 30, 2006	August 3, 2006	1,35_3_1223
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	Ju1y 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.

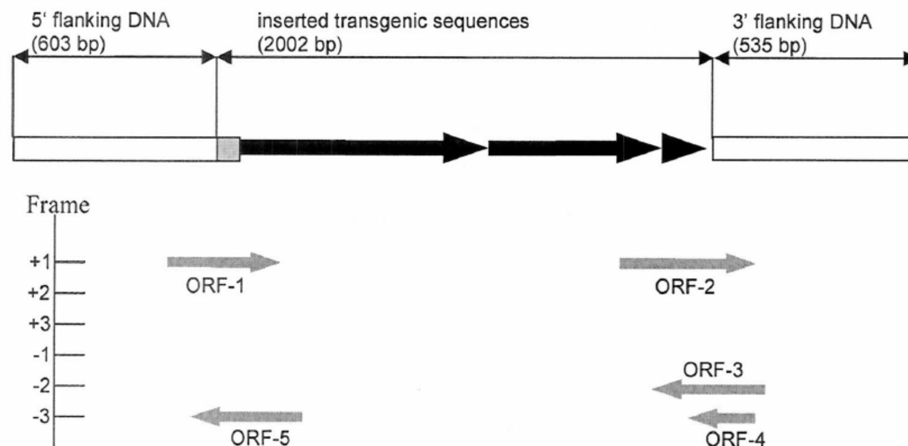
Gene prediction and open reading frame search

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 manufacture'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

UNITED STATES DEPARTMENT OF AGRICULTURE
AGRICULTURAL MAKRETING SERVICE
SCIENCE AND TECHNOLOGY
PLANT VARIETY PROTECTION OFFICE
BELTSVILLE, MD 20705

OBJECTIVE VARIETY DESCRIPTION
RICE (*Oryza sativa*)

Name of Applicant(s)	Temporary Designation	Variety name
Aventis CropScience	5201	to be determinedd

Address	FOR OFFICIAL USE ONLY
3926 Yana Place, Davis CA 95616	PVPO Number

Place the appropriate number that describes the character of this variety in the spaces provided below.
These numbers are also code numbers corresponding to descriptors developed by IBGR-IRRI Rice
Advisory committee and the US Rice Crop Advisory Committee. If no information is availble, leave blank.
Breeders will demonstrate distinctness more readily by describing as many characters as is possible.

1. MATURITY - Days to Heading (Seeding to 50% Heading) :

A. South (Location: _____ Leland, Mississippi 168

83 Number of days
3 No. of days earlier than Cypress
Heading days same as Cocodrie Known Varieties
______ No. of days later than ______

1 Maturity Class (50% heading) - South:
1= Very early (less than 86 days) 2= Early 86-100)
3= Intermediate (101-115) 4 = Late (more than 115)

B. California (Location: __ Not Grown

______ Number of days
______ No. of days earlier than ______
Heading days same as ______ Known Varieties
______ No. of days later than ______

______ Maturity Class (50% heading) - California
1= Very early (less than 90 days) 2= Early (91-97)
3= Intermediate (98-104) 4 = Late (more than 104)

2. CULM:

3 ANGLE (Degrees from Perpendicular after Flowering): (*See values on next page*)
2. CULM: (continued)
1= Erect (less than 30) 3 = Intermediate (about 45) 5 = Open (about 60)
7 = Spreading (more than 60 but the cu
9 = Procumbent (the culm or its lower part rests on the ground surface)

LENGTH 86.0 cm (Soil Level to topof extneded panicle on main stem)
9.0 cm shorter than Check variety: ______Cocodrie___________
Length same as Check variety: _________________
4.0 cm longer than Check variety: ________Cypress_________

1 HEIGHT CLASS: 1 = Semidwarf 2 = Short 3 = Medium 4 = Tall

1 INTERNODE COLOR (After flowering) : 1 = Green 2 = Light Gold 3 = Purple lines 4 = Purple

1 STRENGTH (Lodging resistance) :
1 = Strong (no lodging) 3 = Moderately strong (most plants leaning)
5 = Intermediate (most plants moderately lodged) 7 = Weak (most plants nearly flat)
9 = Very weak (all plants flat)

3. FLAG LEAF (After Heading) :
25.0 cm LENGTH 14.0 mm WIDTH

1 PUBESCENCE: 1 = Glabrous 2 = Intermediate 3 = Pubescent

3 LEAF ANGLE (after heading) 1 = Erect 3 = Intermediate 5 = Horizontal 7 = Descending

2 BLADE COLOR: 1 = Pale Green 2 = Green 3 = Dark Green 4 = Purple Tips
5 = Purple margins 6 = Purple blotch 7 = Purple

1 BASAL LEAF SHEATH COLOR: 1 = Green 2 = Purple lines 3 = Light Purple 4 = Purple

4. LIGULE:

1.5 cm LENGTH (from base of collar to the tip, at late vegetative stage)

1 COLOR (late vegetative stage): 1 = White 2 = Purple lines 3 = Purple

2 SHAPE: 1 = Acute to acuminate 2 = 2-Cleft 3 = Truncate

1 COLLAR COLOR (late vegetative stage): 1 = Pale green 2 = Green 3 = Purple

1 AURICLE COLOR (late vegetative stage): 1 = Plale green 2 = Purple

5. PANICLE:

21.0 cm LENGTH

5 TYPE: 1 = Compact 5 = Intermediate 9 = Open

2 SECONDARY BRANCHING: 1 = Absent 2 + Light 3 = Heavy 4 = Clustering

1.5 EXSERTION (near Maturity): 1 = Less than 90% 2 = 90-99% 3 = 100% exserted

2 AXIS: 1 = Straight 2 = Droopy

5 SHATTERING: 1 = Very low (less than 1%) 3 = Low (1-5%) 5 = Moderate (6-25%)
7 = Moderately high (26-50% 9 = High (more than 50%)

3 THRESHABILITY: 1 = Difficult 2 = Intermediate 3 = Easy

6. GRAIN (Spikelet):

0 AWNS (after full heading): 0 = Absent 1 = Short and partly awned 5 = Short and fully awned
7 = Long and partly awned 9 = Long and fully awned

2 APICULUS COLOR (at maturity): 1 = White 2 = Straw 3 = Brown (tanwy) 4 = Red
5 = Red apex 6 = Purple 7 = Purple apex

1 STIGMA COLOR: 1 = White 2 = Light green 3 = Yellow 4 = Light purple 5 = Purple

1 LEMMA AND PALEA COLOR (at maturity):

0 = Straw 1 = Gold and/or gold furrows on straw background 2 = Brown spots on straw (piebald)

3 = Brown furrows on straw 4 = Brown (tawny) 5 = Reddish to light purple

6 = Purple spots on strqw 7 = Purple furrows on straw 8 = Purple

9 = Black 10 = White

1 LEMMA AND PALEA PUBESCENCE: 1 = Glabrous 2 = Hairs on lemma keel 3 = Hairs on upper portion
4 = Short hairs 5 = Long hairs (velvety)

1 SPIKELET STERILITY (at maturity):1 = Highly fertile (>90%) 3 = Fertile (75-90%) 5 = Partly sterile (50-74%)
7 = Highly sterile (<50% to trace) 9 + Completely sterile (0%)

7. GRAIN (Seed):

1 SEED COAT (bran) COLOR: 1 = White 2 = Light brown 3 = Speckled brown 4 = Brown
5 = Red 6 = Variable purple 7 = Purple

1 ENDOSPERN TYPE: 1 = Nonglutinous (nonwaxy) 2 = Glutinous (waxy) 3 = Intermediate

3 ENDOSPERN TRANSLUCENCY: 1 = Clear 5 = Intermediate 9 = Opaque

1 ENDOSPERN CHALKINESS 0 = None 1 = Small (less than10% of sample)

0 SCENT (Aroma):) = Nonscented 1 = Lightly scented 2 = Scented

SHAPE CLASS (length/width ratio):

3.8 PADDY 1 = Short (2.2:1 and less) 2 = Medium (2.3:1 to 3.3:1) 3 = Long (3.4:1 and more)

3.4 BROWN 1 = Short (2.2:1 and less) 2 = Medium (2.3:1 to 3.3:1) 3 = Long (3.4:1 and more)

3.3 MILLED 1 = Short (2.2:1 and less) 2 = Medium (2.3:1 to 3.3:1) 3 = Long (3.4:1 and more)

MEASUREMENTS:

Grain Form	Length (mm)	Width (mm)	Thickness (mm)	L/W Ratio	1000 Grains (grams)	
Paddy	9.1	2.4	1.9	3.8	22.2	
Brown	7	2.1	1.6	3.4		18.9
Milled	6.9	2.1	1.6	3.4		18.7

7. GRAIN (Seed) (continued)

29% Milling quality (% hulls) __62/71__ Milling yield (% whole kernel (head) rice to total rice)

_____ % Protein (NIR) _______ % Amylose (NITR)

Alkali Spreading value: _______ 1.5% KOH Solution 3.6 1.7% KOH Solultion

5 GELATINIZATION TEMPERATURE TYPE: 1= High 5 = Intermediate 7 = Low

Amylographic Paste Viscosity (Brabender Units) - RVA

Peak Hot Paste Cooled Paste Breakdown' 'Setback'

_______ _______ _______ _______

8. RESISTANCE TO LOW TEMPERATURE:

2 GERMINATION AND SEEDLING VIGOR: 1 = Low 2 = Medium 3 = High

2 FLOWERING (Spikelet fertility): 1 = Low 2 = Medium 3 = High

9. SEEDLING VIGOR NOT RELATED TO LOW TEMPERATURE:

3 VIGOR: 1 = Low 2 = Medium 3 = High

10. BLAST RESISTANCE (*Pyricularia oryzae*). (International races found under references)

0 = Immune 1 = Resistant 3 = Moderately resistant 5 = Intermediate 7 = Moderately susceptible 9 = Susceptible

Group			IB			IC		ID		IE	IG	IH
Number	1	5	45	49	54	1	17	1	13	1	1	1
Resistance	__	___	___	___	_9_	___	___	1_	___	___	_1__	___

11. RESISTANCE TO OTHER DISEASES:

0 = Immune 1 = Resistant 3 = Moderately resistant 5 = Intermediate 7 = Moderately susceptible 9 = Susceptible

_____ Narrow Brown Leaf Spot *Cercospora oryzae*

_____ Leaf Smut *Entyloma oryzae*

_____ Brown Leaf Spot *Helminthosporium oryzae* (= *Bipolaris oryzae* and *Drechslera oryzae)*

_____ Leaf Scald *Gerlachia oryzae*

_____ Hoja Blanca Virus

_____ Sheath Spot *Rhizoctonia oryzae*

_____ Other: ______________________

_____ Aggregate Sheath Spot *Rhizoconia oryzae-sativae*

_____ Straight Head

_____ Kernel Smut *Neovossia horrida* (=*Tilletia barclayana*)

_____ White Tip Nematode *Aphelenchoides besseyi*

_____ Stem Rot *Sclerotium oryzae*

_____ Bacterial Blight *Xanthomonas campestris* pv.*Oryzae*

7 Sheath Blight *Rhizoctonia solani*

12.INSECT RESISTANCE: unknown

0 = Immune 1 = Resistant 3 = Moderately resistant 5 = Intermediate 7 = Moderately susceptible 9 = Susceptible

_____ Grasshopper

_____ Rice Leafhopper

_____ Rice Hispa

_____ Rice Midge

_____ Least Skipper

_____ Rice Stink Bug *Oebalus pugnax*

_____ Swarm Caterpillar

_____ Rice Water Weevil *:Lissorhoptrus oryzophilus*

_____ Rice Stalk Borer *Chilo plejadellus*

_____ Sugarcane Borer *Diatraea saccharalis*

13. OTHER DESCRIPTORS: If there are other characters that describe this variety, please indicate below:

Contains transformation event LLRICE601 and thus is tolerant to the herbicide, glufosinate ammounium.

REFERENCES

C.R. Adair *et al.* 1972. Rice in the United states: Varieties and Production. USDA Handbook No. 289 (Rev.), 124pp.

J.G. Atkins *et al* . 1967. An International Set of Rice Varieties for Differentiating Race of *Pyricularia Oryzae* . Phytopaht. 57:297-301.

IBPGR-IRRI Rice Advisory Committee. 1980. Descriptors for Rice *Oryza sativa* (L.). International Rice Research Institute. 21 pp.

K.C. Ling and S.H. Ou. 1969. Standardization of the International Race Numbers of *Pyricularia oryzae* . Phytopaht. 59:339-342.

B.D. Webb et al. 1985. Utilization Characteristics and Qualities of United states Rice. In Proceedings on Rice Grain Quality and Marketing. International Rice Reseaarch Institute (IRRI), Los Banos, Philippines. P. 25-35.

Pv Number	9900148
Name of variety	Cocodrie
Synonym	<LA9502008>
Similar variety	Cypress
Experimental no.	LA9502008
PI Number	606331
Year released	1998
Originator	Louisiana State University Agricultural Center
Kind of crop	RICE
Rice type	LONG-GRAIN
Maturity South	SOUTH
Days Mature Sout	78 |r Crowley, LA 165 Kg/Ha
Days earlr South	4 |r Cypress
Days same South	Jackson
Days later South	5 |r Maybelle
Maturity clas SO	VYEA
Plant habit	ERE
Plant height cm	089.0
Cm. shorter than	10.0 |r Maybelle
Length same as	|r Cypress
Cm. taller than	3 |r Lemont
Height class	SMDF
Internode color	LTGD
Stem strength	ST
Leaf color	MDGN
Leaf pubescence	GLA
Flag leaf angle	ERC
Flaglf length cm	41.0
Flaglf width mm	10.0
Ligule length mm	01.0
Ligule color	CO
Ligule shape	CLFT
sheath co inside	PU
Sheath co seedlg	GN
Collar color	PG
Auricle color	PG
Panicle type	IN
Pan Sec Branchng	LGHT
Panicle habit	ER
Panicle shattrng	LOWW
Threshability	SH
Pan length cm	24.0
Pan exsertion	<100
Stigma color	CO
Lemma color	SW
Apiculus col mat	PU
Lemma pubescence	GL
Spiklet Sterilty	HI
Awns af ful head	SHPA
Seed coat co pig	LBN
Seed scent	NON
Endosperm waxine	NWX
Endo transparncy	TLU
Endosperm Chalki	SMA
Paddy shape	LG
Paddy length mm	9.33
Paddy width mm	2.52

Paddy thicknes mm	1.94
Paddy L/W ratio	[illegible].70
Paddy weight grs	25.[illegible]
Brown length mm	[illegible]
Brown width mm	2.20
Brown thicknes mm	1.[illegible]
Brown L/W ratio	3.24
Brown weight grs	20.[illegible]
Milled length mm	7.09
Milled width mm	2.17
Miled thicknes mm	1.74
Milled L/W ratio	3.28
Milled weight g	20.2
% Hulls	020
% Totl mill rice	61.0
Germination vigr	ME
Spikelet fertity	ME
Seedling vigor	HI
Pyric race IB001	B0011 — Rating 1
Pyric race IB049	B0497 — Rating 7
Pyric race IC001	C0011
Pyric race IC017	C0177
Pyric race IE001	E0019
Pyric race IG001	G0011
Pyric race IH001	H0011
Cercospora oryza	CERC1
Entyloma oryzae	ENTY1
Bipolaris oryzae	BIPO1
Sclerotium oryza	SCLR5
Pythium seed blt	PYSB7
Rhizoctonia oryz	RHIZ5
Rhizoctonia sola	RZSL7
Agregte ShthSpot	RZOS5
Straight head	STRH7
Rice midge	RCMI9
Stem borer	STBR9
Sugarcane Borer	SCBR9
Stink bug	STBG9
Water weevil	WAWV9
Protein %P	7.7
Amylose %A	22.2
Alk Spr 1.7% KOH	3.98
Gelatin temp	ME
Peak Amy Past Vi	273
Hot Paste AmPaVi	201
Cooled Paste Vis	336
Breakdown AP Vis	081
Breeding history	'Cypress'//'L-202'/'Tebonnet' Variants observed include taller, pubescent, earlier, later, shorter, intermediate, medium or short grain type and gold hull. The total number of variants numbered fewer than 1 per 5,000 plants.
Coments	Appl: Basal Sheath Color = Green
Coments	Appl: Apiculus color at grain maturity = Very Light Purple

CONFIDENTIAL BUSINESS INFORMATION JUSTIFICATION

The information claimed as confidential within this application may fall into two categories, namely (1) the genotype/phenotype description and (2) commercial development information. The genotype/phenotype description category includes, among other items, names and information about the recipient plant, the phenotype of the regulated article, vectors, mode of transformation, gene coding regions, associated regulatory sequences and expressed traits. Commercial development information includes, among other items, the names and locations of cooperators, collaborators, investigators, and contacts.

This confidential business information justification is submitted by Bayer CropScience LP ("Bayer"). Bayer is the successor in interest of Aventis CropScience USA LP. Bayer is part of the worldwide Bayer CropScience group of companies which also includes Bayer BioScience N.V. (the former Aventis CropScience N.V., which was the former Plant Genetics Systems N.V.) and Bayer CropScience GmbH (the former Aventis CropScience GmbH, which was the former Hoechst Schering AgrEvo GmbH). All of these entities are referred to as Bayer CropScience in the statements given below.

GENOTYPE/PHENOTYPE DESCRIPTION

Central to the commercial value of Bayer CropScience's biotechnology products is the genetic information that confers the desired traits on the plant product, as well as the technical means by which the desired products have been achieved. Bayer CropScience has spent many person years in developing its expertise in the field of plant biotechnology, concurrent with the expenditure of millions of dollars on biotechnology research. In the rapidly growing and highly competitive industry of biotechnology products, Bayer CropScience has a leading edge.

Bayer CropScience has been working on the development of genetically enhanced plants, particularly those with herbicide tolerance, since the early 1980's and can document the large sums of money spent in research and testing costs. The uniqueness of Bayer CropScience's products lies in the transformation and regeneration methods and/or the combination of genetic components in the vectors transferred into the genomes of the recipient plants. The transformation and regeneration methods may be Bayer CropScience proprietary methods or available through licensing of others' proprietary methods. The genetic components in these vectors include the coding sequence for the expression of the trait(s), and regulatory sequences such as promoters, enhancers, introns, termination and polyadenylation sequences. In certain cases, the recipient plant strain used is tantamount for regeneration and other desired features. Although the information on the transformation methods, recipient plant strains, or on each of these vector components may be in the public domain, the particular combination of the components put together by Bayer CropScience is unique and represents a great expenditure of time and money.

Competitors (which include by way of example Monsanto/DeKalb, Syngenta, DuPont/Pioneer, Dow Mycogen, Stine Seeds) of Bayer CropScience cannot presently duplicate Bayer CropScience's commercially valuable products without going through the

painstaking process of trial and error development and testing of many different combinations of genetic information and plant strains. Access to genotype and/or phenotype description information, including the donor organisms and the recipient plant, for Bayer CropScience's products would allow competitors to create similar products that would result in a market share loss for Bayer CropScience of millions of dollars. By performing simple copy work, these competitors would avoid the significant expenditure of dollars, research time and effort used by Bayer CropScience to develop its commercial products. Furthermore, the release of genotype and phenotype information would provide competitors with commercially valuable knowledge about particular products that Bayer CropScience is planning to commercialize and the likely timeframe for commercialization. Such information would be extremely useful to these companies in developing their own marketing and development strategies.

COMMERCIAL DEVELOPMENT INFORMATION

The disclosure of information about the names of cooperators, collaborators, investigators, research farm on-site personnel or contacts and the location and characteristics of the field experiments will provide Bayer CropScience's competitors with invaluable information about Bayer CropScience's marketing strategy, and could cause severe harm to Bayer CropScience's competitive standing in the industry.

In particular, release of the choice of cooperators and collaborators provides the competition with knowledge about the individuals and organizations that Bayer CropScience has found, through experience and investigation, to be most expert. Information on the location and characteristics of the field experiments will directly, or with little effort, provide the identity of the cooperators and collaborators. There is no doubt that competitors would seek to use the services of the entities found most expert by Bayer CropScience, and limit or block access of these sources. This could be accomplished by prices for services being increased, or by competitors acquiring exclusive licenses with these individuals and organizations, or by entering into contracts that would essentially tie up the time and facilities of such entities.

Maintaining the good will of the cooperators and collaborators is also a very important consideration for Bayer CropScience's success. The release of information that would directly or indirectly identify these entities could cost Bayer CropScience considerable good will and the breach of an agreement with the entity concerned. This could lead to the loss of the entity as an expert source. If Bayer CropScience is forced to use alternative cooperators and collaborators, it would take time to identify high technical performance, and it would represent a loss of the valuable expertise and understanding built-up with former entities. This, in turn, could result in a delay in bringing products to market, which would cost Bayer CropScience sums into the millions of dollars.

Additionally, the disclosure of information about cooperators and collaborators would provide strong insights into Bayer CropScience's marketing strategy by revealing where Bayer CropScience is planning to introduce the products, and the schedule for such introduction.

Finally, all information deemed confidential is not known to others unless made available by appropriate secrecy agreements. Bayer CropScience takes the necessary precautions to prevent the intentional or unintentional disclosure to others of this information, supplemented with general site security system of gate guards, 24-hr security personnel, employee identification, limited access areas, escorts for visitors and restrictions for visitors, employee secrecy agreements, locked cabinets, files and data rooms, inside mail marked confidential and sealed, as well as other security measures.