AgrEvo

98-329-01p

Ms. Rebecca Bech
Director, Scientific Services
USDA, APHIS, PPQ, SS
4700 River Road, Unit 147
Riverdale, MD 20737-1237

November 20, 1998

Re: **Petition for Determination of Nonregulated Status for LibertyLink® Rice Transformation Events LLRICE06 and LLRICE62**

Dear Ms. Bech:

AgrEvo USA Company is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) for LibertyLink® Rice Transformation Events **LLRICE06 and LLRICE62.**

This petition requests a determination from APHIS that LibertyLink® Rice transformation events LLRICE06 and LLRICE62, and any progeny derived from crosses of events LLRICE06 and LLRICE62 with traditional rice varieties, and any progeny derived from crosses of events LLRICE06 and LLRICE62 with transgenic rice varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340. The petition contains a full statement explaining the factual grounds why LibertyLink® Rice transformation events should not be regulated under 7 CFR Part 340, including data and information required as set forth in paragraph (c) of 7 CFR 340 6. This petition does not contain any trade secrets or confidential business information (CBI) and is so marked.

Accompanying two copies of the petition and appendices 1-3 is one copy of certain literature reprints (appendix 4). All literature cited in the petition is not included in the reprints since the excluded literature is either a book, a government document, or a manuscript that is readily available to the public at many libraries in the United States. If these reprints are required for you to consider this petition complete, please contact me by December 11, 1998.

Please contact me at (302) 892-3155 if you have any questions concerning our petition.

Best Regards,

Sally Van Wert

Sally Van Wert, Ph.D.
Manager, Regulatory Affairs – Biotechnology

Enclosures (2)

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98-329-01p

Petition for Determination of Nonregulated Status:

LibertyLink® Rice Transformation Events LLRICE06 and LLRICE62

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

Submitted by:

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November 20, 1998

Contains No Confidential Business Information

11/25/98

Summary

AgrEvo USA Company is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) for LibertyLink® Rice Transformation Events **LLRICE06 and LLRICE62**. AgrEvo requests a determination from APHIS that LibertyLink® Rice transformation events LLRICE06 and LLRICE62, and any progeny derived from crosses of events LLRICE06 and LLRICE62 with traditional rice varieties, and any progeny derived from crosses of events LLRICE06 and LLRICE62 with transgenic rice varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340. Events LLRICE06 and LLRICE62 are considered regulated articles because they contain sequences from the plant pest, cauliflower mosaic virus (CaMV).

Glufosinate-ammonium (GA) is in the phosphinothricin class of herbicides. It is a non-systemic, non-selective herbicide that provides effective post-emergence control of many broadleaf and grassy weeds. GA controls weeds through the inhibition of glutamine-synthetase (GS), which leads to the accumulation of phytotoxic levels of ammonia in the plant. GS is responsible for the synthesis of the amino acid glutamine from glutamic acid and ammonia. It is the only enzyme in plants that can detoxify ammonia released by photorespiration, nitrate reduction, and amino acid degradation.

Transformation events LLRICE06 and LLRICE62 are rice genetic material that contain a stably integrated gene, *bar*, which encodes phosphinothricin-N-acetyltransferase (PAT). The PAT enzyme catalyzes the conversion of L-phosphinothricin, the active ingredient in GA, to an inactive form, thereby conferring resistance to the herbicide. The *bar* gene in events LLRICE06 and LLRICE62 is isolated from *Streptomyces hygroscopicus*, strain HP632. (Thompson,et al., 1987). The N-terminal codon of the wild type bar coding region have been substituted for the codons ATG and GAC respectively. The gene was introduced through direct gene transfer of a fragment of plasmid DNA. Southern blot analyses show events LLRICE06 and LLRICE62 contain several complete and partial copies and 1 complete copy of the *bar* gene, respectively.

Genetically engineered LibertyLink® Rice will provide a new weed management tool to rice growers. GA is currently registered in the United States as a nonselective herbicide for both non-crop and crop uses. It is highly biodegradable, has no residual activity, and has very low toxicity for humans and wild fauna. LibertyLink® Rice may positively impact current agronomic practices in rice by 1) offering a broad spectrum, post-emergence weed control system; 2) providing the opportunity to continue to move away from pre-emergent and residually active compounds; 3) providing a new herbicidal mode of action that allows for improved weed resistance management in rice acreage; 4) encouraging herbicide use on an as-needed basis; 5) decreasing cultivation needs; and 6) allowing the application of less total pounds of active ingredient than used presently.

Events LLRICE06 and LLRICE62 have been field tested by AgrEvo USA Company beginning in 1997 in winter nursery and in the rice growing regions of the United States. These tests have occurred at approximately 16 sites under field release authorizations granted by APHIS (USDA authorizations: 97-206-02n, 98-071-67n, 98-083-03n, 98-083-04n, 98-083-05n, 98-089-03n, 98-112-08n, 98-156-01n, 98-225-04n). Data collected from these trials, laboratory analyses, expert letters and reports, and literature references presented herein demonstrate that LibertyLink® Rice events LLRICE06 and LLRICE62: 1) exhibit no plant pathogenic properties; 2) are no more likely to become a weed than non-modified rice; 3) are unlikely to increase the weediness potential of any other cultivated plant or native wild species; 4) do not cause damage to processed agricultural commodities; and 5) are unlikely to harm other organisms that are beneficial to agriculture.

Primary transformation events LLRICE06 and LLRICE62 were selected for commercial development. They have been crossed with both commercially available public and proprietary germplasm of the rice. The primary transformation events and their progeny are collectively referred to as LibertyLink® Rice transformation events LLRICE06 and LLRICE62 in this petition.

AgrEvo USA Company requests a determination from APHIS that LibertyLink® Rice transformation events LLRICE06 and LLRICE62, and any progeny derived from crosses of events LLRICE06 and LLRICE62 with traditional rice varieties, and any progeny derived from crosses of events LLRICE06 and LLRICE62 with transgenic rice varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340.

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 are unfavorable to the petition.

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ACRONYMS AND SCIENTIFIC TERMS

APHIS - Animal and Plant Health Inspection Service
bar - phosphinothricin acetyltransferase gene (origin *Streptomyces hygroscopicus*)
CaMV - Cauliflower Mosaic Virus
ELISA - enzyme linked immunosorbent assay
GA - glufosinate-ammonium
GS - glutamine synthetase
PAT - phosphinothricin acetyltransferase (enzyme)
USDA - United States Department of Agriculture

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Statement of Grounds for Nonregulated Status

I. Rationale for Development of LibertyLink® Rice

Rice, *Oryza sativa* L., is grown world-wide and serves as a staple food for half of the world's population. Annually about 530 million tons of rice are harvested from the fields of 146 million hectares world-wide. More than 90% of the world rice production comes from Asia, 5% from the Americas, 3% from Africa, and another 1% from Europe and Oceania. Rice is adapted to diverse regions, and is cultivated between 53°N and 40°S (Kaneda, 1993).

In the United States for the 1997 production year, rice was grown on 1.3 million acres in Arkansas, 0.5 million acres each in California and Louisiana, 0.2 million acres each in Mississippi and Texas and 0.1 million acres in Missouri. The projected area in production for 1998 is 3.09 million acres, up 1% from 1997 (USDA-ERS, 1997).

Rice is grown in the United States using mechanized practices for planting and harvesting. Cultural practices, including irrigation and crop rotation, and herbicides are employed to control weeds. Weed management is critical to maximum rice yield and herbicides are used on most rice acreage grown in the United States. The grower is typically interested in applying a herbicide for weed control that has a broad weed spectrum, does not injure the crop, is cost effective, and has positive environmental attributes. Several classes of herbicides have effective broad spectrum weed control, however they may injure or kill the rice crop when used at the application rates suggested for weed control. On the other hand, some weeds such as red rice cannot be properly controlled with existing products.

The phosphinothricin herbicide glufosinate-ammonium (GA, chemical name: ammonium-DL-homoalanin-4-yl(methyl)phosphinate) is registered for nonselective weed control on both non-food use (Finale ®) and food use plants (Rely ®, Liberty®) in the United States. Outside of North America the herbicide is generally sold as Basta ®. Glufosinate is a contact, non-selective herbicide that provides effective post-emergence control of many broadleaf and grassy weeds. It is highly biodegradable, has no residual activity, and has very low toxicity for humans and wild fauna (Anonymous, 1991). Resistance to the herbicide has now been achieved, through the insertion of a resistance gene, in over 20 commercially important plant species including rice. Genetically engineered LibertyLink® Rice will provide a selective use for glufosinate and a valuable new weed management tool to rice producers.

Resistance of barnyardgrass or sedges has evolved to residual herbicides used in rice in recent years. Molecules with new modes of action are required to develop resistance management strategies. LibertyLink® Rice will offer the grower the choice and advantages of using a modern herbicide which features broad-spectrum weed control and favorable environmental features to manage weeds in production fields.

I. The Rice Family

A. History and Uses of Rice

Archaeological evidence from India, Indochina, China, Indonesia and in the southern Sahara show the cultivation of rice in these regions to be prehistoric (Oka, 1988). In the United States, rice was grown by the early colonists in the Carolinas and expanded to small plots along the Mississippi River following the Civil War. Rice culture began on a commercial, mechanized scale in southwest Louisiana in 1884 (Louisiana State University, 1987). Cultivation of rice in Brazil was initiated by the Portuguese in the 16th century (Morishima, 1984).

The domestication of rice is considered to have likely occurred in several locations and then widely distributed as a crop. Cultivated rice is recognized to have two varietal groups based upon adaptation, Indica (long grain) and Japonica (short grain). In very general terms, the Indica form is more adapted to tropical, rain-fed agriculture. The Japonica form is found more in temperate, irrigated agriculture, however, Japonica rice is often grown in the upland fields of tropical regions.

In the present, rice is considered to be the most important food crop for more than one-half of the world population. Rice provides more than 20% of the caloric intake for the world's people (USDA-ERS, 1997).

World rice production for 1998/99 is projected to be a record 387.9 million tons (milled basis), 1% larger than 1997/98 crop. World consumption is projected to be 387.8 million tons (USDA-ERS, 1997). For the current year, worldwide consumption and production are almost equal. Exports from rice growing countries with surplus compensate for local production shortages. The top six rice exporting nations are, in order of export volume, Thailand, Vietnam, the United States, India, Pakistan and China. Thailand is the consistent leader in exports. For 1998, the estimated export volumes are 5.8 million metric tons from Thailand and 2.8 million from the United States (USDA, 1998). Historical export destinations for rice produced in the United States are provided in Table II.1. Rice is consistently shipped to our NATO trade partners and the European Union. Exports of rice to South America and Japan have varied according to annual crop supply. However, a new trade agreement with Japan now provides quotas for the import of United States-grown rice.

Table II.1. Historical United States exports and current year commitments in metric tons of rough rice equivalents

United States Export Destination	1993/1994	1994/1995	1995/1996	1996/1997	1997/1999*
European Union	362,000	474,000	417,000	342,000	370,000
Turkey	67,000	259,000	187,000	202,000	130,000
Saudi Arabia	157,000	153,000	130,000	168,000	136,000
Japan	568,000	2,000	192,000	212,000	250,000
Mexico	177,000	327,000	318,000	309,000	386,000
Canada	91,000	127,000	107,000	109,000	123,000
Brazil	Not listed	368,000	1,000	0	Not listed
Columbia	0	0	28,000	34,000	297,000
Ecuador	5,000	0	0	0	144,000
Total for the Western Hemisphere	513,000	1,585,000	1,010,000	934,000	1,708,000
Total US exports	2,521,000	3,723,000	2,564,000	2,500,000	2,740,000

Volume in metric tons, milled rice is ca. 70% of rough rice.
Source: USDA-ERS, 1997. Rice Yearbook. (Table 29, 31)
* Combined exports plus sales commitments as of 7/2/98.

In the United States, rice cultivation is concentrated in two regions, the southern Mississippi River Valley, beginning in the Missouri Bootheel and moving south through Arkansas and Louisiana to the Gulf Coastal Plain into Texas, and in north central California. White, long grain rice is the predominant type in production; however, other varieties of food grade rice are grown under identity preserved conditions. In California, medium grain varieties comprise 90% of the state's production with a mixture of short grain, long grain and specialty rice varieties making up the balance. Basmatti rice, a special aromatic rice, is planted in some regions of Louisiana, Arkansas and Texas. The area devoted to rice production is the largest in the state of Arkansas, but the greatest productivity per area is achieved in California. There are small areas of land in rice agriculture and winter breeding nurseries in the state of Hawaii and the commonwealth of Puerto Rico. Table II.2 provides production estimates for the six rice producing states.

Following milling and polishing, rice grains are sorted. The whole grains are used for premium direct food products and the broken grains are moved into the product stream for beer, starch and processed products. The bran is used for human food and animal feed. Rice bran is composed of dietary fiber, protein, and oil.

In addition to human food uses, the whole grain and hulls may be ground for animal feed. Rice straw has uses as biomass for co-generation operations and can be pressed to manufacture fiberboard products. Due to the high silicate content of rice plants, rice straw has been used to produce ceramics. Traditional Asian uses of rice straw include fiber for coarse weaving (Kaneda, 1993).

Table II.2. Projections for United States Domestic Production for 1997

State	Area 1,000 acres[a]	Yield lbs. per acre[b]	Production million cwt[b]
Arkansas	1,180	5,600	74.9
Louisiana	535	4,800	27.1
California	502	8,400	43.6
Texas	300	5,600	14.5
Mississippi	210	5,500	14.7
Missouri	92	5,400	5.1
TOTAL	2,819	Ave. 5,883	179.9

Source: [a]USDA-NASS and [b]USDA-ERS (June 1998 web site posting, usda.mannlab.cornell.edu/reports/)
cwt. = 100 weight

B. Taxonomy of the Genus *Oryza*

Rice belongs to the grass family, Gramineae, in a subfamily that includes rice and bamboo (*Bambusoideae* or *Oryzoideae*). The general adaptation of this subfamily is for marshy, tropical regions. The basic chromosome number of this group is 6 or 12. The genus *Oryza* is known for its diversity and as a result, the classification of species within this genus is often in debate.

There are many species in the genus *Oryza* (Table II.3), but only two species are cultivated – *O. sativa* and *O. glaberrima*. *Oryza sativa* is distributed throughout the tropics and parts of the temperate regions of the world, while *O. glaberrima* is limited to West Africa. There are few morphological differences between the two cultivated species, most notably *O. glaberrima* has a red pericarp. Hybrids between *O. sativa* and *O. glaberrima* are sterile (Oka, 1988).

Rice is an annual crop, however, in regions with sufficiently long season, a second crop can be obtained from tillers bud initials. There are three stages of rice plant development - vegetative, reproductive and grain maturation. Leaves emerge one at a time from the main stem or culm of the plant. Tillers are evident when the fifth leaf emerges. Panicle primordia differentiate at the uppermost internode of the culm. Internode elongation begins with panicle initiation. At

heading time, panicles come out of flag-leaf sheaths. Flowering takes place in spikelets on a panicle, followed by pollination on stigmata and fertilization in ovules, which is complete within six hours. Flowering proceeds from the upper branches of the panicle and continues to the lower branches as they emerge from the leaf sheath. Flowering time for an entire panicle is four to seven days. Embryo and endosperm mature in the ovule and become a seed for the next generation. Rice plants are very easily propagated by seeds or tiller buds (Kaneda, 1993).

Wild and cultivated rice do not rely on insects for pollination, although some aromatic varieties may attract bees. The floral development of cultivated rice insures a high degree of self-pollination. For some species of wild rice, cross-pollination is more likely; the flowers are more receptive to foreign pollen. Most cross-pollination occurs within two meters (Grist, 1975).

The rice flower is capable of receiving pollen from a source other than itself for only a short time. In a survey of wild rice strains, Oka found the time interval from flower opening to pollen emission to be quite short, from 0.5 to 9 minutes. Pollen grains have a short life. Pollen from cultivated varieties loses viability within 3 to 5 minutes of shedding. The longest lasting pollen from a wild *Oryza* species is only 9 minutes (Oka and Morishima, 1967).

C. Genetics of Rice

Members of the *Oryza* species in the AA genome group (Table II.3) can cross and hybrids with wild species have contributed adaptative traits to the cultivated varieties. There are various levels of cross-incompatibility and successful introgression of a resistance gene is a lengthy process. Germplasms have been developed with both acceptable grain quality and pest resistance. Three broad breeding objectives include agronomic performance, pest resistance and milling quality. Common techniques for variety development are crossbreeding and bulk selection to identify lines with local adaptation. Selected lines are then increased as breeder seed, under careful evaluation and removal of off-types. Hybrid rice varieties account for half the cultivated area in China, but are considered basic research in Japan and the United States (Kaneda, 1993).

D. Weediness Potential of Rice, *Oryza sativa*

The primary adaptation of rice is the humid tropics as a semi-aquatic plant, but modern rice varieties are produced in a variety of climates (Morishima, 1984). In the United States, rice is produced in temporate climates and solely in irrigated agricultural systems (Hill, Smith and Bayer, 1994). Rice is cultivated annually, however, under favorable water and temperature conditions, rice plants can grow vegetatively and continuously. In the irrigated rice production regions of the

United States, rice cannot compete outside of cultivation (personal communication, Steve Linscombe, Louisiana State University).

Table II.3. Generally recognized *Oryza* species, chromosome number, genome symbol and geographic distribution (after Oka, 1988)

Oryza species	2n	Genome	Geographic distribution
O. sativa	24	AA	Worldwide, cultivated
O. rufipogon (syn. *O. perennis*)	24	AA	Asia, America
O. glumaepatula (syn. *O. perennis*)	24	AA	Central and South America
O. longistaminata	24	AA	Africa
O. glaberrima	24	AA	Africa, cultivated
O. breviligulata (syn. *O. barthii*)	24	AA	Africa
O. australiensis	24	EE	Australia
O. eichingeri	24	CC	Africa
O. punctata	24, 48	BB, BBCC	Africa
O. officinalis	24	CC	Asia
O. minuta	48	BBCC	Asia
O. latifolia	48	CCDD	Central and South America
O. alta	48	CCDD	Central and South America
O. grandiglumis	48	CCDD	Central and South America

Wild rice and older rice cultivars are noted for ease of seed shattering, however, modern cultivars developed for machine harvesting have been selected for lack of shattering. Most cultivated rice has been selected for seed expressing dormancy that can be broken with a short period of dry afterripening. Such seed dormancy can be important to allow ripening and harvest of the grain without precocious germination (Kaneda, 1993).

Red rice (*O. sativa*) is considered a weed in the United States rice production fields because it is highly competitive with crop rice (Pantone and Baker, 1991) and the dark seeds blemish the cultivated rice yield and quality, and bring down its economic value (Craigmiles, 1979). It was established in the United States, most likely introduced with the planting seed, long before the large-scale cultivation of rice. Red rice biotypes easily shatter and have strong dormancy, becoming a weed problem in rice fields. Red rice seed, when buried in the soil can remain viable for many years and thus create a recurring weed problem (Noldine, 1998; Appendix 2.a and 2.b, also see Section VI.A.1).

Red rice in not a native species, but rather an introduced weed which inhabits only agricultural-eco systems. In his 35-year career as an agronomist, Roy Smith has never seen a red rice population growing in non-agricultural land nor one growing in non-irrigated crops, like wheat or oats (personal communication, Roy Smith, University of Arkansas, retired).

E. Potential for Outcrossing

In the genus *Oryza*, viable hybrids between distantly related species or varieties are difficult to achieve. Any of the *Oryza* species containing the AA genome (Table II.3) can intercross and produce hybrid seed. The opportunity for outcrossing depends upon the proximity of the growing range and overlapping flowering period. However, the formation of hybrid seed is not sufficient to establish a new trait in a new population, certain biological factors act to limit gene flow.

Reproductive barriers exist for F_1 progeny derived from parents having the AA genome in common. These include non-viability of young F_1 zygotes, F_1 weakness, F_1 sterility, F_2 sterility, and F_2 weakness. Two or more barriers are often found (Morishima, 1984). Thus, hybrid seed may be produced, but sustained populations of hybrids and the subsequent introgression of genes from one population into another may not be realized.

Introgression is the incorporation of genes from one population of close genetic relatives into another with a different adaptive norm. In the case of the United States, only one potential for outcrossing exists and that is between red and crop rice. Weedy, red rice is the same species as crop, white rice. Introgression between rice and red rice has been documented (Langevin, et al., 1990). Rice-red rice crop-companion weed hybridization is common, and given overlapping flowering, outcrossing is to be expected. Populations of red rice are diverse genetically. Homogenous populations of red rice likely exist only in weed science programs where the well characterized biotypes are important for research (Dr. Steve Linscombe, Louisiana State University, personal communication).

III. The Transformation System and Plasmid Used

The LibertyLink® Rice transformation events LLRICE06 and LLRICE62 contain the *bar* gene derived from *Streptomyces hygroscopicus*, strain HP632 (Thompson, 1987). The *bar* gene encodes the enzyme phosphinothricin acetyltransferase (PAT), which confers resistance to the herbicide GA. The *bar* gene is fused to a 35S promoter and terminator from Cauliflower Mosaic Virus (CaMV) forming a *bar* gene cassette. The transforming DNA fragment was derived from the plasmid, pB5/35Sbar, which contains no other plant expressible gene besides *bar*. Direct gene transfer of the *bar* cassette (1501 bp PvuI-HindIII fragment) from pB5/35Sbar was the method of transformation. Stable insertion of the *bar* gene into the rice genome results in the expression of the PAT enzyme.

A. Transformation System

Rice tissue for transformation was prepared by surface-sterilizing rice seeds, then placing them on callus induction medium. Expanding callus tissue was used for transformation. The DNA delivery was performed by direct gene transfer. Cells that received the transforming DNA and expressed *bar* gene were selected on media containing phosphinothricin. These cells were allowed to develop into transgenic callus. This callus was transferred to regeneration medium, which induces shoot and root development. The seedlings that developed were transferred to the greenhouse, and allowed to flower and set seed.

B. Parent Lines

Transformation event LLRICE06 has its origin in the variety M202. The variety M202 is a medium-grain rice developed by the California Co-Operative Rice Research Foundation. It is a pure line selection from a cross made in 1977. Foundation seed was made available to seed growers in 1985. Medium grain rice are grown on the vast majority of California's 500,000 acres and M202 is the principal variety. Johnson, et al. (1986) describes the pedigree and variety registration information.

Transformation event LLRICE62 was derived from the variety Bengal. The variety Bengal is a medium-grain rice developed by the Rice Research Station of the Louisiana Agricultural Experiment Station. The variety was officially released in 1992. Linscombe, et al, proves pedigree and variety description (1993). Medium grain rice is grown on ~250,000 acres in Arkansas and Louisiana, and Bengal is the principal variety.

C. Construction of the Plasmid Used for Transformation

Plasmid pB5/35Sbar is a derivative of the vector pUC19 (Yanisch-Perron, et al., 1985). The pUC19 vector was modified by replacing the β-lactamase gene with the 3'5"-aminoglycoside phosphotransferase III (*nptIII*) gene from *Streptococcus faecalis* (Trieu-Cuot, et al.,1983). The *nptIII* gene was subcloned from the binary transformation vector pBIN19 (Bevan, 1984) and inserted into the β-lactamase gene-deleted pUC vector. The resulting plasmid was further modified by insertion of a DNA element containing the right border of the *Agrobacterium tumefaciens* octopine plasmid pTiAch5 (Gielen, et al., 1984) into the single NdeI site of the vector. It was into the EcoRI site of this vector, pB5, that the *bar* cassette was inserted.

The chimeric *bar* cassette consists of the promoter and transcription-termination and polyadenylation sequences from the 35S-transcript of CaMV and the complete coding sequence of the *bar* gene from *S. hygroscopicus*, strain HP632 (Thompson, et al., 1987). The N-terminal codon of the wild type bar coding region has been substituted for the codon ATG . The *bar* gene was inserted between the 35S promoter and terminator of pDH51 (described in Pietrzak, et al. 1986). The chimeric *bar* cassette was isolated as an EcoRI fragment and inserted into pB5 to create pB5/35Sbar.

To obtain the transforming DNA the plasmid pB5/35Sbar was digested with restriction enzymes PvuI and HindIII, and the resulting restriction fragments were separated on an agarose gel. The cleavage position of the restriction enzymes in pB5/35Sbar for HindIII is at nucleotide 2140, and for PvuI at nucleotide 3641. The 1501 bp HindIII/PvuI fragment contains the *bar* cassette (P35S-*bar*-T35S), and this was purified from the gel, and used in transformation of parental lines M202 and Bengal to generate transformation events LLRICE06 and LLRICE62, respectively. The *nptIII* gene was not included in the transforming DNA.

A map of the pB5/35Sbar vector is shown in Figure III.1. A description of the DNA elements in pB5/35Sbar is shown in Table III.1.

D. Open Reading Frames and Associated Regulatory Regions in pB5/35Sbar

Transformation was performed by direct gene transfer of the 1501 bp PvuI-HindIII fragment of pB5/35Sbar. This fragment contains one open reading frame, *bar*, which is intact and functional in transformation events LLRICE06 and LLRICE62, as will be shown in Section IV. The LibertyLink® Rice transformation events LLRICE06 and LLRICE62 have been considered regulated articles because they contain DNA sequences from CaMV, an organism, which is a plant pest. This section contains a more thorough description of the inserted genetic

material responsible for expression of the glufosinate resistance trait. Refer to Table III.1 for a description of all other introduced genetic sequences.

Table III.1. Genetic Elements of the Vector pB5/35Sbar

Position in Vector	Genetic element and Function
0001 – 1025	Sequence from pBIN19 (Bevan, 1984) containing *npt III* gene. The ntpIII coding sequence is from nt 172 – 966.
1026 – 2195	Sequence derived from pUC19, (Yanisch-Perron, et al., 1985)
2196 – 2204	Synthetic polylinker sequence
2205 – 2398	Complement of 35S terminator (T35S) from Cauliflower Mosaic Virus (Franck, et al., 1980; Pietrzak, et al., 1986)
2399 – 2417	Synthetic polylinker sequence
2418 – 2969	Complement of bar gene from *Streptomyces hygroscopicus* (Thompson, et al., 1987).
2970 – 2985	Synthetic polylinker sequence
2986 – 3517	Complement of 35S promoter (P35S) from Cauliflower Mosaic Virus (Franck, et al., 1980; Pietrzak, et al., 1986)
3518 – 3730	Sequence derived from pUC19 (Yanisch-Perron, et al., 1985)
3731 – 3791	Synthetic right border fragment (RB) of the *Agrobacterium tumefaciens* octopine plasmid pTiAch5 (Gielen, et al., 1984)
3792 – 4161	Sequence derived from pUC19, (Yanisch-Perron, et al., 1985)

1. CaMV *35S* promoter and terminator

The *35S* promoter and terminator sequences are derived from CaMV and control expression of the *bar* gene. CaMV is a doublestranded DNA caulimovirus with a host range restricted primarily to cruciferous plants. The region of the CaMV genome used corresponds to nucleotides 6909 to 7437 for the promoter and nucleotides 7439 to 7632 for the terminator (described in Pietrzak, et al., 1986). The *35S* promoter directs high level constitutive expression and is widely used as a promoter for high expression of genes (Harpster, et al., 1988). The CaMV sequences, as used in the LibertyLink® Rice, do not cause the rice to become a plant pest.

2. *bar*

The *bar* gene was isolated from *Streptomyces hygroscopicus*, strain HP632 (Thompson, et al, 1987). It encodes the enzyme phosphinothricin acetyltransferase (PAT), which imparts resistance to the phytotoxic activity of GA. Genes encoding PAT enzymes have been isolated from

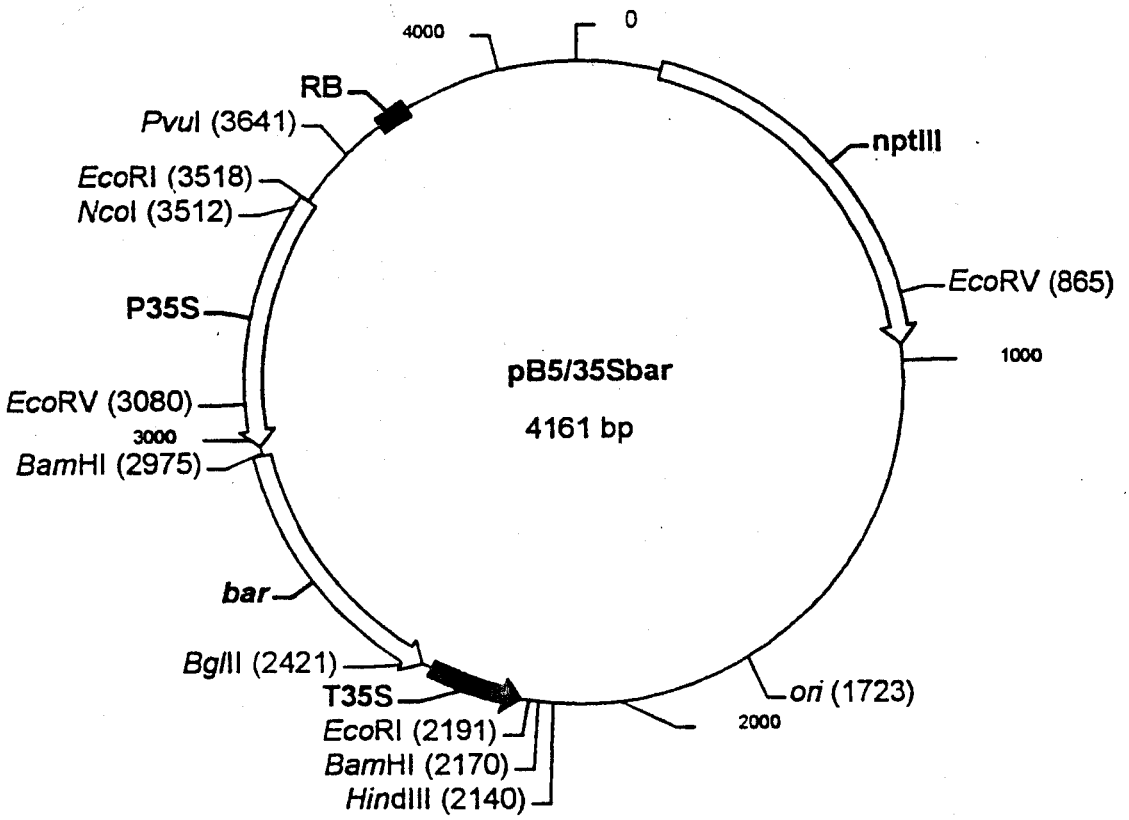
S. viridochromogenes (Hara, et al., 1991) and *S. hygroscopicus* (Thompson, et al., 1987).

Members of the genus *Streptomyces* are gram-positive sporulating soil bacteria. These organisms synthesize numerous unique compounds, secondary metabolites, that often possess antibacterial, antitumor, or antiparasitic activity (Demain, et al., 1983). Both *S. viridochromogenes* and *S. hygroscopicus* produce one such compound, the antibiotic bialaphos. Bialaphos (syn. L-phosphinothricyl-L-alany-L-alanine) is an herbicidally active tripeptide consisting of two L-alanine molecules and an analog of L-glutamic acid called phosphinothricin. When it is released by peptidases, the L-phosphinothricin moiety, is a potent inhibitor of glutamine synthetase (GS) (Bayer, et al., 1972). L-phosphinothricin is the active component of the commercial herbicides, Herbiace® (Meiji Seika Ltd.) and Liberty®, Basta®, Ignite®, Rely®, Remove® and Finale® (AgrEvo GmbH). Herbiace® is bialaphos that is commercially produced using *S. hygroscopicus*. The other herbicides are the ammonium salts of phosphinothricin, common name GA, and are chemically synthesized.

L-phosphinothricin is a potent inhibitor of the enzyme GS in both bacteria and plants, where it apparently binds competitively to the enzyme by displacing L-glutamate from the active site. Evidently GS binds L-phosphinothricin better than the substrate. GS plays a central role in nitrogen metabolism of higher plants where it is the only enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid degradation and photorespiration (Miflin and Lea, 1976). Ammonia, although a plant nutrient and metabolite, is toxic in excess and leads to death of plant cells (Tachibana, et al., 1986).

Although the GS from both *S. viridochromogenes* and *S. hygroscopicus* are sensitive to L-phosphinothricin, the bacteria produce an inactivating enzyme, PAT. PAT catalyzes the conversion of L-phosphinothricin to N-acetyl-L-phosphinothricin in the presence of acetyl CoA as a co-substrate. N-acetyl-L-phosphinothricin does not inactivate GS, and, thus, has no herbicidal activity. Therefore, plants expressing the PAT enzyme are resistant to the phosphinothricin class of herbicides. The PAT enzyme is encoded by the *bar* (bialaphos-resistance) gene in *S. hygroscopicus*, and by the *pat* gene in *S. viridochromogenes*. These genes function both as an integral part of the biosynthetic pathway of bialaphos and as an enzyme which confers resistance (Kumada, 1988).

Figure III.1. Vector Map of pB5/35Sbar



IV. Molecular Characterization of Transformation Events LLRICE06 and LLRICE62

A. Mendelian Inheritance of Events LLRICE06 and LLRICE62

Primary transformation events LLRICE06 and LLRICE62 were derived from the transformation of rice cells as described in Section III. These have been crossed with both commercially available public lines and proprietary lines. Through traditional breeding with these fertile transformation events individuals homozygous at the *bar* locus have been produced. Plant breeding will be used to continue to transfer the glufosinate resistance locus events in LLRICE06 and LLRICE62 to a wide range of rice varieties with a wide range of commercially important grain types.

1. Mendelian Inheritance of LLRICE06

T_1 seed harvested from self-pollinated T_0 plants surviving a Liberty® Herbicide greenhouse screen were planted in September 1997 in Puerto Rico (USDA authorization 97-206-02N). T_1 plants were selected for survival following Liberty® application. Mendelian inheritance for a single gene locus would predict three resistant plants in every 4 or 75% of the plants surviving the herbicide application. A survival rate of 77% was observed, which fits the expected segregation ratio of 3:1 for a single locus (Table IV.1).

Table IV.1. Segregation analysis of Event LLRICE06

Individual T_1 plants

Resistant plants	Sensitive plants	Total plants	Expected ratio	Chi ^{2} value[a]
71	21	92	3:1	0.23

T_2 panicle rows

Fully Resistant Rows	Partially Resistant Rows	Total Rows	Expected ratio	Chi ^{2} value[a]
20	47	67	1:2	0.37

[a] No significant difference for the Chi Square goodness-of-fit test for the hypothesis of either 3:1 or 1:2 segregation. Significance test level at p=0.05 for Chi ^{2} values greater than 3.84, with one degree of freedom.

Panicles were harvested from individual plants and T_2 panicle rows were planted in January 1998 in Puerto Rico for evaluation. Application of Liberty® Herbicide was used to score the rows for segregation of the PAT phenotype. Rows

containing no sensitive plants were considered to be homozygous for the *bar* gene. Mendelian inheritance for a single gene locus would predict one fully resistant row for every two partially resistant rows. The segregation observed fit the expected ratio of 1:2 for a single locus (Table IV.1). In a total of 67 T_2 panicle rows, 20 rows contained no sensitive plants and were harvested as independent populations for advanced variety evaluation.

2. Mendelian Inheritance of LLRICE62

T_1 seed harvested from self-pollinated T_0 plants surviving a Liberty® Herbicide greenhouse screen were planted in December 1997 in Puerto Rico (USDA Authorization 97-206-02N). T_1 plants were selected for survival following Liberty® herbicide application. Panicles were harvested from individual plants and T_2 panicle rows were planted in May 1998 at Louisiana State University for evaluation. Application of Liberty® Herbicide was used to score the rows for segregation of the PAT phenotype. 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. For each population of LLRICE62 the expected ratio of 1:2 was observed (Table IV.2). In a total of 211 T_2 panicle rows, 69 rows contained no sensitive plants. The fully resistant rows were harvested as independent populations for advanced variety evaluation.

Table IV.2. Segregation analysis of populations of LLRICE62 T_2 panicle rows[a]

Populations	Fully Resistant Rows	Partially Resistant Rows	Total Rows	Expected Ratio	Chi ^{2} value[b]
1	19	30	49	1:2	0.65
2	11	30	41	1:2	0.78
3	24	53	77	1:2	0.16
4	15	29	44	1:2	0.01

[a] Segregation of entire versus partially resistant T_2 panicle rows derived from resistant T_1 plants.

[b] There was no significant difference (p=0.05) for the Chi Square goodness-of-fit test for the hypothesis of 1:2 segregation. To prove the null hypothesis, the Chi^2 value must be greater than 3.84, with one degree of freedom.

The T_2 individual plants of the partially resistant panicle rows represent a population of homozygous, hemizygous and null individuals. Mendelian inheritance would predict a segregation three resistant plants to one sensitive plant, that is to say 1/4 or 25% of the plants will not survive Liberty® herbicide

applications. Census of living and dead plants within the panicle rows found that 23% of the plants did not survive Liberty® Herbicide treatment (Table IV.3). The living / dead plant census of some lines did not match the predicted Mendelian ratios at the 0.05 level of probability (data not shown). The lack of fit is attributed to the difficulty in counting closely spaced plants in the panicle rows and not to instability in the gene insertion. The percentage of dead plants for these lines is less than the predicted 25%. If gene instability were observed, the number of non-surviving plants would be greater than expected, not less. No further testing of the segregating rows was performed. None of the progeny from the segregating rows were advanced to generate commercial lines.

Table IV.3. Segregation analysis of LLRICE62 T_2 progeny from panicle rows partially resistant to Liberty® Herbicide

Partially resistant panicle rows	Resistant plants	Sensitive plants	Total plants	Expected ratio	Chi ^{2} value[a]
1	64	21	85	3:1	0.003
2	38	13	51	3:1	0.005
3	109	37	146	3:1	0.007
4	64	21	85	3:1	0.091
5	38	13	51	3:1	0.138
6	109	37	146	3:1	0.300
7	165	58	223	3:1	0.372
8	65	24	89	3:1	0.662
9	109	32	141	3:1	1.561
10	168	50	218	3:1	2.273
11	215	81	296	3:1	2.273
12	131	33	164	3:1	2.409
13	156	38	194	3:1	2.814
14	156	38	194	3:1	2.898
15	93	20	113	3:1	2.909
16	140	32	172	3:1	2.909
17	119	26	145	3:1	2.944
18	74	14	88	3:1	3.524
19	74	14	88	3:1	3.675
20	107	50	157	3:1	2.944
21	70	12	82	3:1	3.524
22	180	41	221	3:1	3.675

[b] There was no significant difference (p=0.05) for the Chi Square goodness-of-fit test for the hypothesis of 3:1 segregation. To prove the null hypothesis, the Chi^2 value must be greater than 3.84, with one degree of freedom.

B. DNA Analysis of Events LLRICE06 and LLRICE62

To determine the nature, number, integrity and stability of insertions, which occurred in transformation events LLRICE06 and LLRICE62, Southern hybridization was used. In the experiments restriction digested genomic DNA from transgenic plants homozygous for the integrated DNA were run in parallel with digested genomic DNA from a nontransgenic plant, and with digested genomic DNA from a nontransgenic plant supplemented with approximately 1 copy of digested transforming plasmid. The determination of the number of integrated copies was deduced from analyzing all obtained Southern blot data.

Several aliquots of LLRICE06 and LLRICE62 DNA were digested with restriction enzymes. See Figure III.1 and Figure IV.1 to locate restriction sites in pB5/35Sbar. After separation of the DNA by electrophoresis, the DNA was transferred to a nylon membrane and hybridized with gel purified ^{32}P-labeled P35S-*bar*-T35S cassette (1501 bp HindIII/PvuI fragment) or with ^{32}P-labeled vector backbone (2660 bp HindIII/PvuI fragment) probes (Figure IV.1 and Table IV.4). Lanes contain approximately 10 µg of restricted DNA. The amount of restricted pB5/35Sbar in positive control lanes is equivalent to 1.0 copy of the plasmid integrated in 10 µg of rice DNA. The probed membranes were visualized by autoradiography. Electronic scans of the autoradiographs are presented in this document. Standard molecular biology methods were used (Sambrook, et al., 1989).

The hybridizing fragments expected and observed when using the probes are listed in Tables IV.5, 6, 7 and 8. The sizes of some hybridizing fragments can be predicted by the location of restriction enzyme cleavage sites internal to the inserted DNA. Those hybridizing fragments whose sizes cannot be predicted result from cleavage in the integrated DNA and in the adjacent plant DNA, or only in the adjacent plant DNA.

Table IV.4. Probes used

Probe	Features	Position in pB5/35Sbar	Length
2660 bp HindIII/PvuI	Vector backbone *(NptIII)*	3642 bp → 2139 bp	2660 bp
1501 bp HindIII/PvuI	P35S-*bar*-T35S (*bar* cassette)	2140 bp → 3641 bp	1501 bp

1. DNA Analysis Event LLRICE06

For the molecular verification of the absence of pB5/35Sbar vector sequences in event LLRICE06 Southern blot analysis was performed using the 2660bp HindIII-PvuI fragment as probe (Table IV.4). When hybridizing this probe with HindIII, EcoRV, EcoRI and NcoI digested *O. sativa* LLRICE06 DNA, no hybridization signals were observed in the transgenic LLRICE06 samples nor in the wild-type M202 DNA samples (negative controls). In the DNA positive controls the expected fragments were observed (Table IV.5 and Figure IV.2). The results obtained with the DNA positive controls showed that the hybridization was performed under conditions allowing hybridization of the probe with target sequences. From the obtained data we can conclude that the pB5/35Sbar vector backbone sequences are absent in *O. sativa* transformation event LLRICE06.

Table IV.5. Demonstration of the absence of vector sequences in LLRICE06 - Summary of obtained hybridization results

Digest	LLRICE06	Negative Control (M202)	Positive Control (M202 +pB5/35Sbar)
HindIII	No hybridization signal	No hybridization signal	4161 bp
EcoRV	No hybridization signal	No hybridization signal	2215 bp 1946 bp
EcoRI	No hybridization signal	No hybridization signal	2834 bp
NcoI	No hybridization signal	No hybridization signal	4161 bp

To ascertain the integrity of the transforming *bar* cassette in *O. sativa* transformation event LLRICE06 Southern blot analysis was performed using the *bar* cassette as probe (1501bp HindIII-PvuI fragment). A summary of the obtained hybridization results is presented in Table IV.6 and the hybridization results are shown in Figure IV.3.

When hybridizing HindIII digested genomic LLRICE06 DNA (Figure IV.3, lane 2) with the 1501bp HindIII-PvuI fragment two hybridizing fragments were observed (14kb and 13kb respectively). The two fragments are not completely resolved on the blot presented in Figure IV.3. The weakly hybridizing 16kb fragment is the result of partial digestion of the genomic DNA.

With EcoRI digested DNA (Figure IV.3, lane 8) a number of hybridizing fragments are observed. The most prominent fragment of 1327bp represents an intact copy of the transgenic gene cassette minus vector sequences upstream of the EcoRI site (see Figure III.1 and IV.1). The smaller fragments of 700bp and 500bp

represent incomplete copies of the *bar* cassette. The larger EcoRI fragments might represent incomplete and/or rearranged and/or partially digested copies.

Table IV.6. Transforming DNA in LLRICE06 - Summary of obtained hybridization results

Digest	LLRICE06	Negative Control (M202)	Positive Control (M202 + pB5/35Sbar)
HindIII	16 kb 14 kb 13 kb	No hybridization signal	4161 bp
EcoRV	13 kb 10 kb 4900 bp 2700 bp 1700 bp 1600 bp 1050 bp 1000 bp 550 bp	No hybridization signal	2215 bp 1946 bp
EcoRI	15 kb 10 kb 8 kb 2900 bp 2100 bp 1980 bp 1700 bp **1327 bp** 700 bp 500 bp	No hybridization signal	2834 bp **1327 bp**
NcoI	10 kb 7000 bp 2900 bp 2300 bp 2000 bp 1800 bp 1000 bp	No hybridization signal	4161 bp

A complex integration pattern is also observed in the fingerprint obtained with NcoI digested genomic DNA (Figure IV.3, lane 11). When an intact copy of the transforming DNA is inserted in the genome a fragment with a minimal size of 1373bp is expected (*bar* cassette digested with NcoI). A fragment of 1000bp, which represents an incomplete copy, was observed. The other observed fragments, which are listed in Table IV.6, represent integration and/or incomplete

and/or rearranged and/or partially digested copies. Similarly, with EcoRV digested genomic DNA (Figure IV.3, lane 5), many fragments were observed.

The Southern blot hybridization data obtained with *O. sativa* transformation event LLRICE06 demonstrates that at least one intact copy of the transgenic gene cassette is integrated into the plant genome. Event LLRICE06 contains no vector backbone sequences (including *nptIII*). The insert is complex and certainly carries incomplete transgenic gene cassettes.

To demonstrate the stability of *O. sativa* transformation event LLRICE06 over multiple generations (T2, T3 and T4) Southern blot analysis was performed using the 1501bp HindIII-PvuI fragment as probe. When hybridizing EcoRV digested DNA (generations T2, T3 and T4) the transgene structure is identical for all tested progeny plants, thus showing the stability of the LLRICE06 event at the genomic level over multiple generations (Figure IV.3, lane 5, for T2, Figure IV.4 for T3 and T4)(see Table VI.6 for hybridizing fragment sizes). Segregation data (Section IV.A) further confirm the stability of the insert, and show that it segregates as one dominant Mendelian locus.

2. DNA Analysis Event LLRICE62

For the molecular verification of the absence of pB5/35Sbar vector sequences in event LLRICE62 Southern blot analysis was performed using the 2660bp HindIII-PvuI fragment as probe (Table IV.4). When hybridizing this probe with HindIII, EcoRV, EcoRI and NcoI digested *O. sativa* LLRICE62 DNA, no hybridization signals were observed in the transgenic LLRICE62 samples nor in the wild-type Bengal DNA samples (negative controls). In the DNA positive controls the expected fragments were observed (Table IV.7 and Figure IV.5). The results obtained with the DNA positive controls showed that the hybridization was performed under conditions allowing hybridization of the probe with target sequences. From the obtained data we can conclude that the pB5/35Sbar vector backbone sequences are absent in *O. sativa* transformation event LLRICE62.

Table IV.7. Demonstration of the absence of vector sequences in LLRICE62 - Summary of obtained hybridization results

Digest	LLRICE62	Negative control (Bengal)	Positive Control (Bengal + pB5/35Sbar)
HindIII	No hybridization signal	No hybridization signal	4161 bp
EcoRV	No hybridization signal	No hybridization signal	2215 bp 1946 bp
EcoRI	No hybridization signal	No hybridization signal	2834 bp
NcoI	No hybridization signal	No hybridization signal	4161 bp

To ascertain the integrity of the transforming *bar* cassette in *O. sativa* transformation event LLRICE62 Southern blot analysis was performed using the *bar* cassette as probe (1501bp HindIII-PvuI fragment). A summary of the obtained hybridization results is presented in Table IV.8 and the hybridization result is shown in Figure IV.6, and a schematic drawing of the insert is presented in Figure IV.7.

Table IV.8. Transforming DNA in LLRICE62 - Summary of obtained hybridization results

Digest	Rice62	Negative control (Bengal)	Positive Control (Bengal + pB5/35Sbar)
HindIII	5300 bp	No hybridization signal	4161 bp.
EcoRV	14 kb 3500 bp	No hybridization signal	2215 bp 1946 bp
EcoRI	3000 bp **1327 bp**	No hybridization signal	2834 bp **1327 bp**
NcoI	3800 bp 2800 bp 1000 bp	No hybridization signal	4161 bp

When hybridizing HindIII digested DNA (Figure IV.6, lane 2) with the 1501 bp HindIII-PvuI fragment, a 5300bp fragment was observed. Since the HindIII-PvuI fragment that was used for transformation has no internal HindIII restriction site, one can conclude that the insert resides on this 5300bp HindIII fragment.

With EcoRV digested transgenic DNA (Figure IV.6, lane 5) two fragments (14kb and 3500bp respectively) were observed. Since EcoRV has one restriction site in the transforming purified HindIII-PvuI fragment it can be concluded that both fragments represent integration fragments (plant DNA-transgenic DNA junctions).

With EcoRI digested DNA two fragments (Figure IV.6, lane 8) are observed: a 3000 bp and a 1327 bp fragment. The 1327 bp fragment is the expected internal fragment, carrying the *bar* gene cassette (compare with Figure IV.6, lane 10). The 3000 bp fragment represents the upstream integration fragment (one transgenic DNA-transgenic DNA junction, and one plant DNA-transgenic DNA junction).

Three fragments were observed (3800bp, 2800bp and 1000 bp respectively) when hybridizing NcoI digested LLRICE62 (Figure IV.6, lane 11). The 3800 bp fragment is most likely the result of partial digestion of the genomic DNA. The

2800bp fragment represents the downstream integration fragment. The weakly hybridizing 1000bp fragment represents the upstream integration fragment.

The Southern blot hybridization data obtained with *O. sativa* transformation event LLRICE62 demonstrates that the transferred DNA in the plant genome corresponds to the DNA configuration as designed in the pB5/35Sbar plasmid vector. The obtained data show that there's one intact copy of the complete gene cassette present in *O. sativa* transformation event LLRICE62 and no vector backbone sequences. A schematic drawing of the insert of *O. sativa* transformation event LLRICE62 is presented in Figure IV.7.

To demonstrate the stability of *O. sativa* transformation event LLRICE62 over two generations (T2 and T3) Southern blot analysis was performed using the 1501bp HindIII-PvuI fragment as probe. When hybridizing EcoRV digested DNA of *O. sativa* transformation event LLRICE62 two fragments were observed: a 14 kb and a 3500 bp fragment (Figure IV.8.)(see Table VI.8 for hybridizing fragment sizes). Both fragments represent transforming DNA – plant DNA junctions. Both integration fragments were observed in the two generations of *O. sativa* LLRICE62 event, thus showing the stability of the event at the genomic level over generations. Segregation data (Section IV.A) further confirm the stability of the insert, and show that they segregate as one dominant Mendelian locus.

C. Gene Expression in Events LLRICE06 and LLRICE62

The content of phosphinothricin acetyltransferase (PAT) protein, a *bar* gene product, in the transformation events LLRICE06 and LLRICE62 were determined in grain by an Enzyme Linked Immunosorbent Assay (ELISA). Polyclonal antibodies recognizing PAT protein were used in the ELISA. PAT ELISA detects both inactive and intact PAT enzyme. Therefore, the enzyme detected may not be functional.

The PAT ELISA is a sandwich immunoassay in which PAT specific antibodies are used to coat the wells and serve as capture antibodies for PAT protein. Samples consisting of transformant extracts, non-transformant extracts as controls, and pure PAT protein as a standard are added to the wells. Following incubation, during which time the PAT in the sample is captured by the coated antibodies, the unbound material is removed by rinsing with a wash solution. The plate was subsequently incubated with the second antibody, which recognizes another epitope of PAT protein. The binding of the second PAT antibody to the PAT protein was detected by the incubation of a third antibody labeled with horseradish peroxidase (HRP). A peroxidase substrate, Tetramethylbenzidine (TMB), is then added and converted by the peroxidase to a blue product in proportion to the amount of PAT protein present in the sample. The reaction is stopped with 0.5 M H_2SO_4 which changes the color to a yellow product. The resultant color development is proportional to the concentration of PAT protein in each microwell. Three dilutions of each extract are tested and the

value nearest to the midpoint of the standard curve is used to determine the PAT content. ELISA assays were performed on grain harvested from the transformation events. Results from the ELISA are shown in Table VI.9. These data indicate that a small amount of PAT protein is present in the grain, which can constitute a significant part of the diet for poultry (EPA, 1995) and humans.

Table IV.9. Quantities of PAT in Events LLRICE06 and LLRICE62 as Detected by ELISA[a]

Plant [b]	mg TEP [c] / g sample	µg PAT/ g sample	% PAT/TEP	% PAT/fresh weight (g/g)
LLRICE06	1.89 ±0.49	0.419 ±0.04	0.02	0.00005%
M202 –NT	1.99 ±0.17	ND [d]	0.00	0.00
LLRICE62	2.54 ±0.09	12.4 ±2.4	0.63	0.00124%
Bengal - NT	1.98 ±0.30	ND [d]	0.00	0.00

[a] Values reported are the average from two replicate extractions from two samples of grain ± standard deviation of the mean.

[b] NT = nontransformed.

[c] TEP = total extractable protein

[d] ND = not detectable. Limit of detection is 0.0047 µg PAT/g matrix.

Figure IV.1. Schematic drawing of the probes used

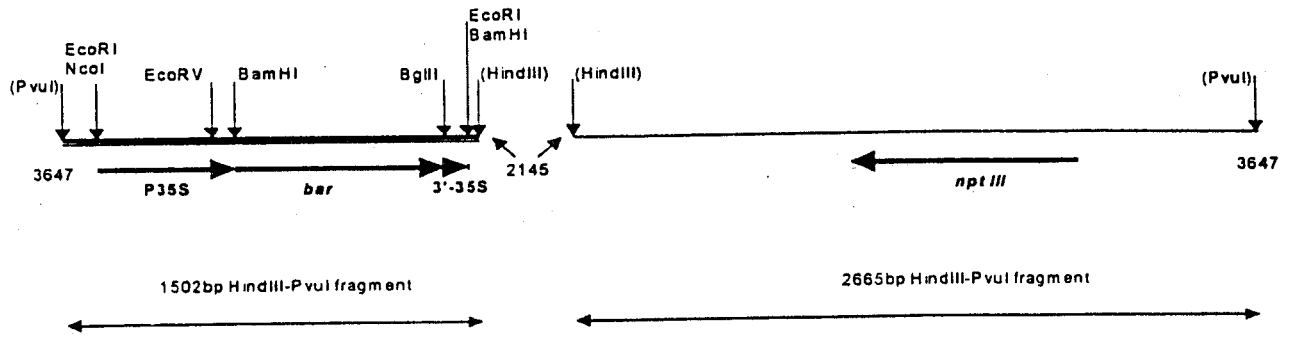


Figure IV.2. Southern blot analysis of event LLRICE06 – vector backbone probe

DNA was isolated from event LLRICE06 (T2 generation) and the nontransgenic parent line M202. DNAs (10 µg) were digested with the indicated restriction enzymes. The vector backbone (2665 bp)(see Figure IV.1) was used as probe. Lane 1. MW-marker. Lane 2. LLRICE06: HindIII digest. Lane 3. M202: HindIII digest. Lane 4. M202 supplemented with pB5/35Sbar plasmid: HindIII digest. Lane 5. LLRICE06: EcoRV digest. Lane 6. M202: EcoRV digest. Lane 7. M202 supplemented with pB5/35Sbar plasmid: EcoRV digest. Lane 8. LLRICE06: EcoRI digest. Lane 9. M202: EcoRI digest. Lane 10. M202 supplemented with pB5/35Sbar plasmid: EcoRI digest. Lane 11. LLRICE06: NcoI digest. Lane 12. M202: NcoI digest. Lane13. M202 supplemented with pB5/35Sbar plasmid: NcoI digest. Lane 14. MW-marker. The amount of restricted pB5/35Sbar in lanes 4, 7, 10 and 13 is equivalent to 1.0 copy of the plasmid integrated in 10 µg of rice DNA. MW marker (λ DNA digested with PstI) sizes given in base pairs.

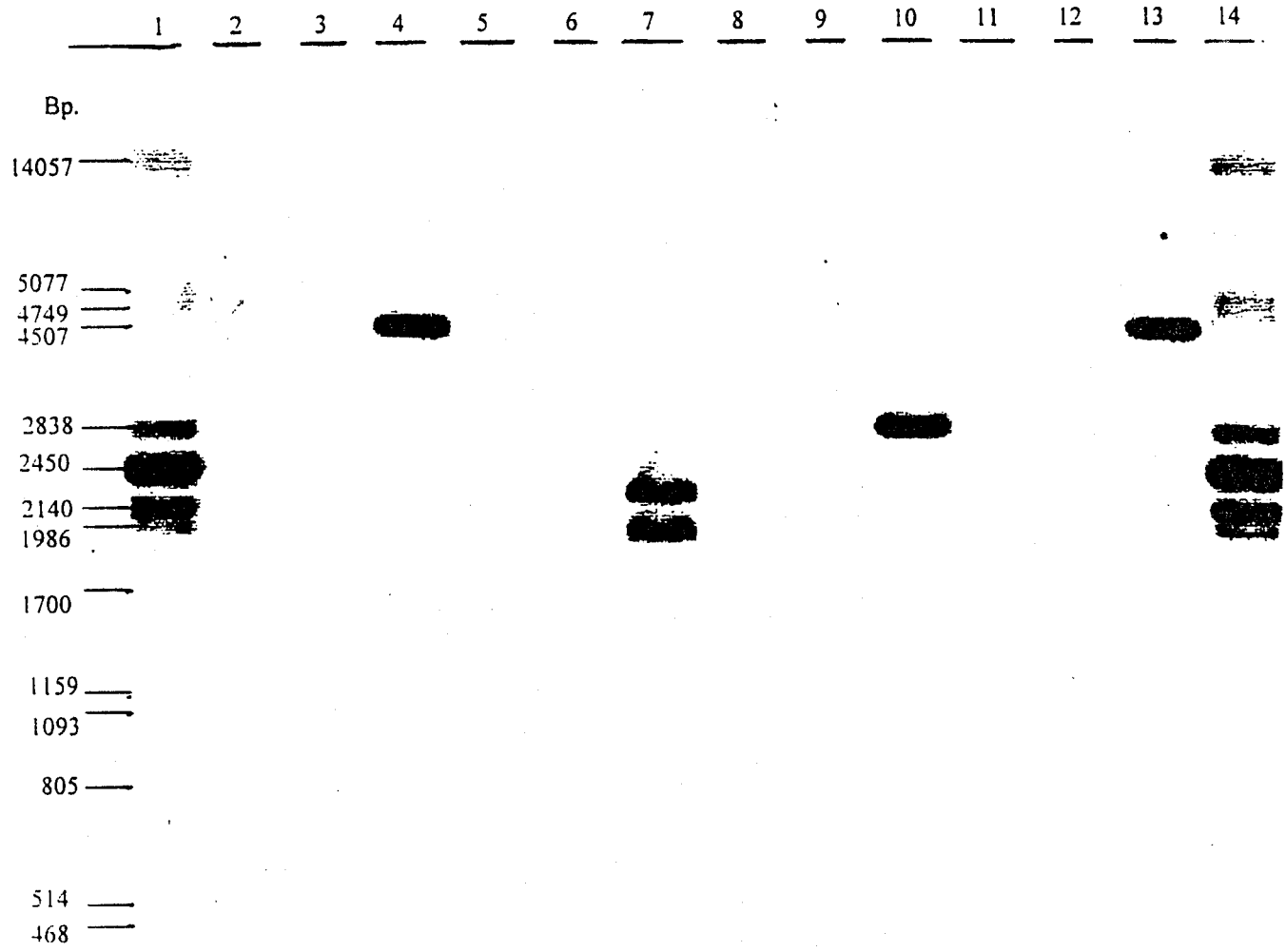


Figure IV.3. Southern blot analysis of event LLRICE06 – *bar* cassette probe

DNA was isolated from event LLRICE06 (T2 generation) and the nontransgenic parent line M202. DNAs (10 µg) were digested with the indicated restriction enzymes. The *bar* cassette (1502 bp)(see Figure IV.1) was used as probe. Lane 1. MW-marker. Lane 2. LLRICE06: HindIII digest. Lane 3. M202: HindIII digest. Lane 4. M202 supplemented with pB5/35Sbar plasmid: HindIII digest. Lane 5. LLRICE06: EcoRV digest. Lane 6. M202: EcoRV digest. Lane 7. M202 supplemented with pB5/35Sbar plasmid: EcoRV digest. Lane 8. LLRICE06: EcoRI digest. Lane 9. M202: EcoRI digest. Lane 10. M202 supplemented with pB5/35Sbar plasmid: EcoRI digest. Lane 11. LLRICE06: NcoI digest. Lane 12. M202: NcoI digest. Lane13. M202 supplemented with pB5/35Sbar plasmid: NcoI digest. Lane 14. MW-marker. The amount of restricted pB5/35Sbar in lanes 4, 7, 10 and 13 is equivalent to 1.0 copy of the plasmid integrated in 10 µg of rice DNA. MW marker (λ DNA digested with PstI) sizes given in base pairs.

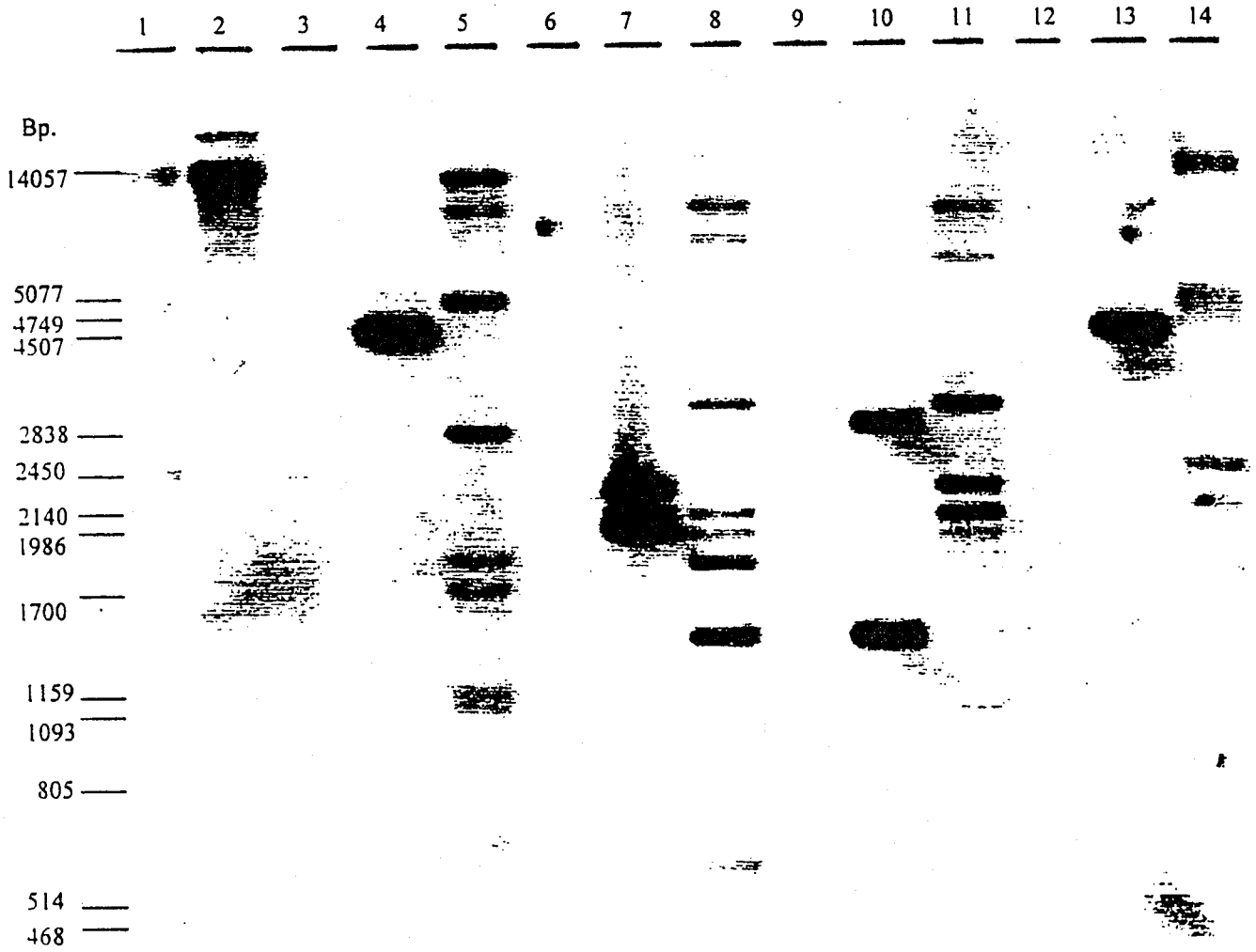


Figure IV.4. Southern showing stability of insertions in event LLRICE06

DNA was isolated from event LLRICE06 and the nontransgenic parent line M202. DNAs (10 µg) were digested with the indicated restriction enzymes. The *bar* cassette (1502 bp)(see Figure IV.1) was used as probe. Lane 1. LLRICE06 (T3 generation): EcoRV digest. Lane 2. LLRICE06 (T4 generation): EcoRV digest. Lane 3. M202: EcoRV digest. Lane 4. M202 supplemented with pB5/35Sbar plasmid: EcoRV digest. The amount of restricted pB5/35Sbar in lane 4 is equivalent to 1.0 copy of the plasmid integrated in 10 µg of rice DNA. MW marker (λ DNA digested with PstI) sizes given in base pairs.

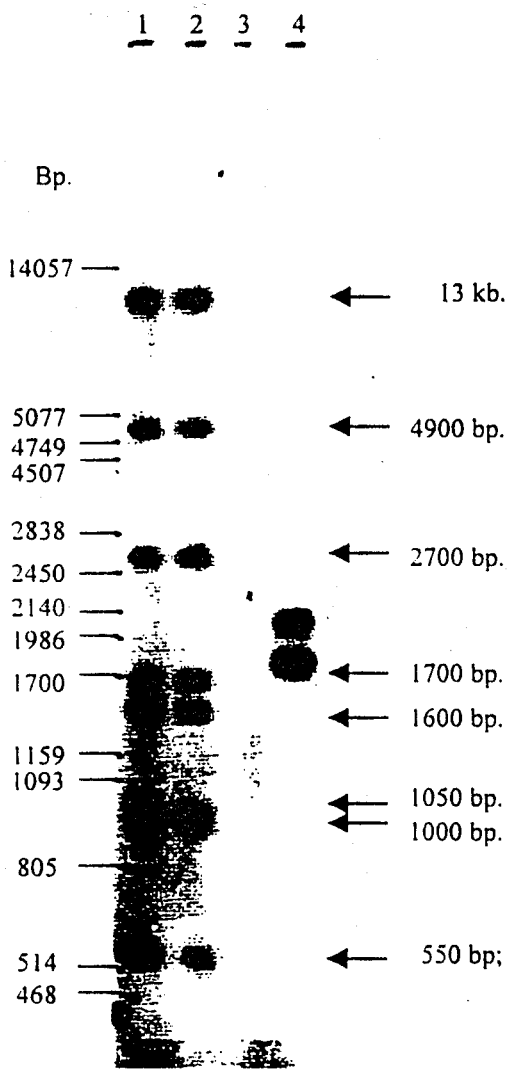


Figure IV.5. Southern blot analysis of event LLRICE62 – vector backbone probe

DNA was isolated from event LLRICE62 (T2 generation) and the nontransgenic parent line Bengal. DNAs (10 µg) were digested with the indicated restriction enzymes. The vector backbone (2665 bp)(see Figure IV.1) was used as probe. Lane 1. MW-marker. Lane 2. LLRICE62: HindIII digest. Lane 3. Bengal: HindIII digest. Lane 4. Bengal supplemented with pB5/35Sbar plasmid: HindIII digest. Lane 5. LLRICE62: EcoRV digest. Lane 6. Bengal: EcoRV digest. Lane 7. Bengal supplemented with pB5/35Sbar plasmid: EcoRV digest. Lane 8. LLRICE62: EcoRI digest. Lane 9. Bengal: EcoRI digest. Lane 10. Bengal supplemented with pB5/35Sbar plasmid: EcoRI digest. Lane 11. LLRICE62: NcoI digest. Lane 12. Bengal: NcoI digest. Lane13. Bengal supplemented with pB5/35Sbar plasmid: NcoI digest. Lane 14. MW-marker. The amount of restricted pB5/35Sbar in lanes 4, 7, 10 and 13 is equivalent to 1.0 copy of the plasmid integrated in 10 µg of rice DNA. MW marker (λ DNA digested with PstI) sizes given in base pairs.

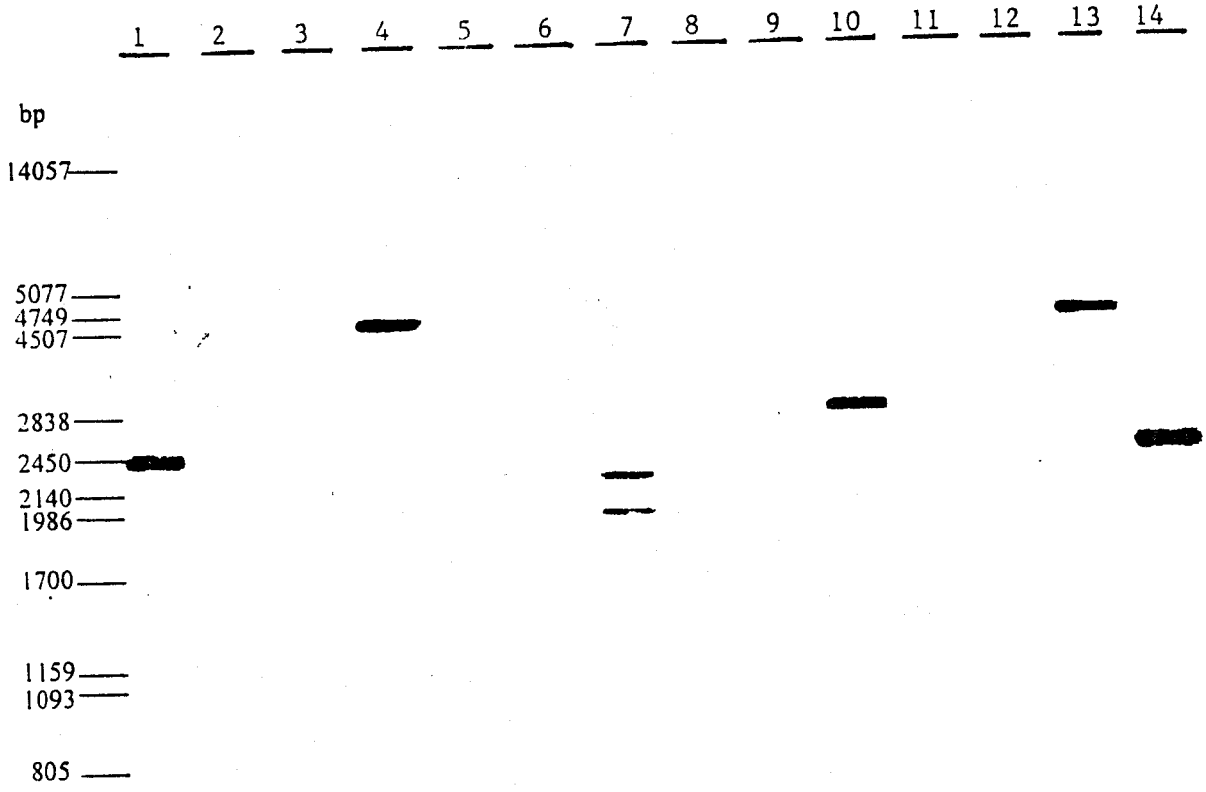


Figure IV.6. Southern blot analysis of event LLRICE62 – *bar* cassette probe

DNA was isolated from event LLRICE62 (T2 generation) and the nontransgenic parent line Bengal. DNAs (10 µg) were digested with the indicated restriction enzymes. The *bar* cassette (1502 bp)(see Figure IV.1) was used as probe. Lane 1. MW-marker. Lane 2. LLRICE62: HindIII digest. Lane 3. Bengal: HindIII digest. Lane 4. Bengal supplemented with pB5/35Sbar plasmid: HindIII digest. Lane 5. LLRICE62: EcoRV digest. Lane 6. Bengal: EcoRV digest. Lane 7. Bengal supplemented with pB5/35Sbar plasmid: EcoRV digest. Lane 8. LLRICE62: EcoRI digest. Lane 9. Bengal: EcoRI digest. Lane 10. Bengal supplemented with pB5/35Sbar plasmid: EcoRI digest. Lane 11. LLRICE62: NcoI digest. Lane 12. Bengal: NcoI digest. Lane13. Bengal supplemented with pB5/35Sbar plasmid: NcoI digest. Lane 14. MW-marker. The amount of restricted pB5/35Sbar in lanes 4, 7, 10 and 13 is equivalent to 1.0 copy of the plasmid integrated in 10 µg of rice DNA. MW marker (λ DNA digested with PstI) sizes given in base pairs.

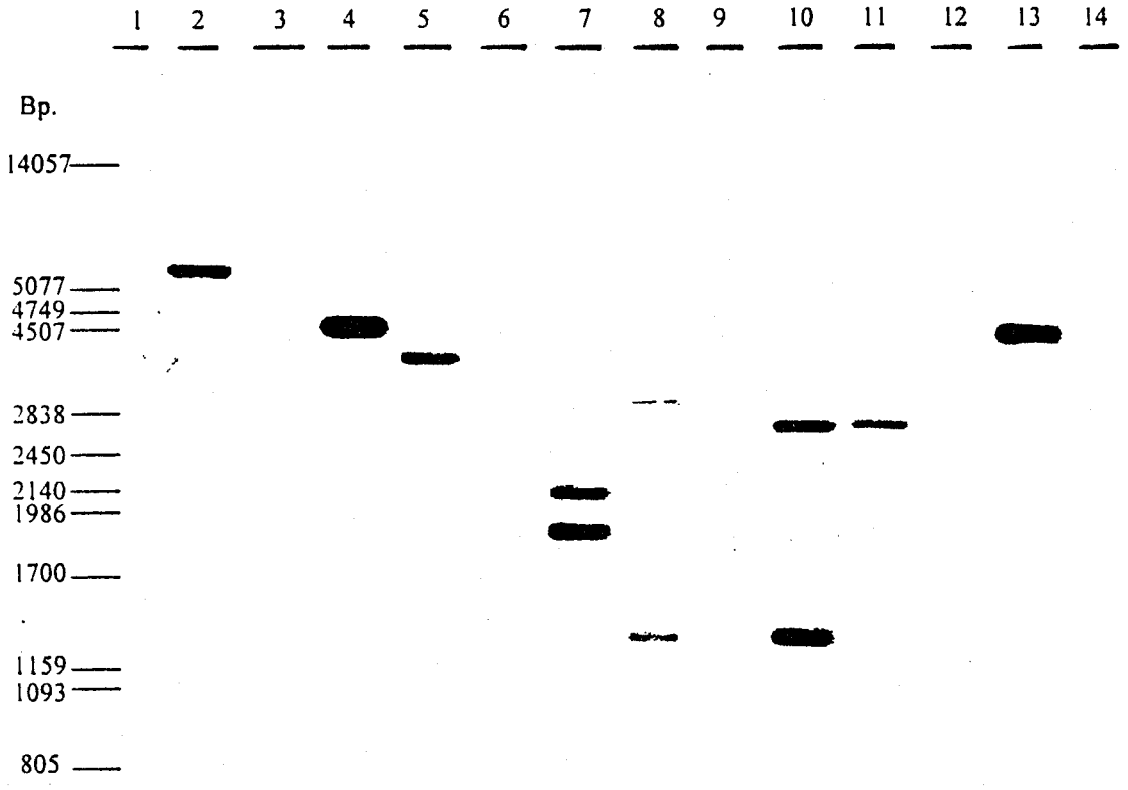


Figure IV.7. Schematic drawing of the insert in event LLRICE62

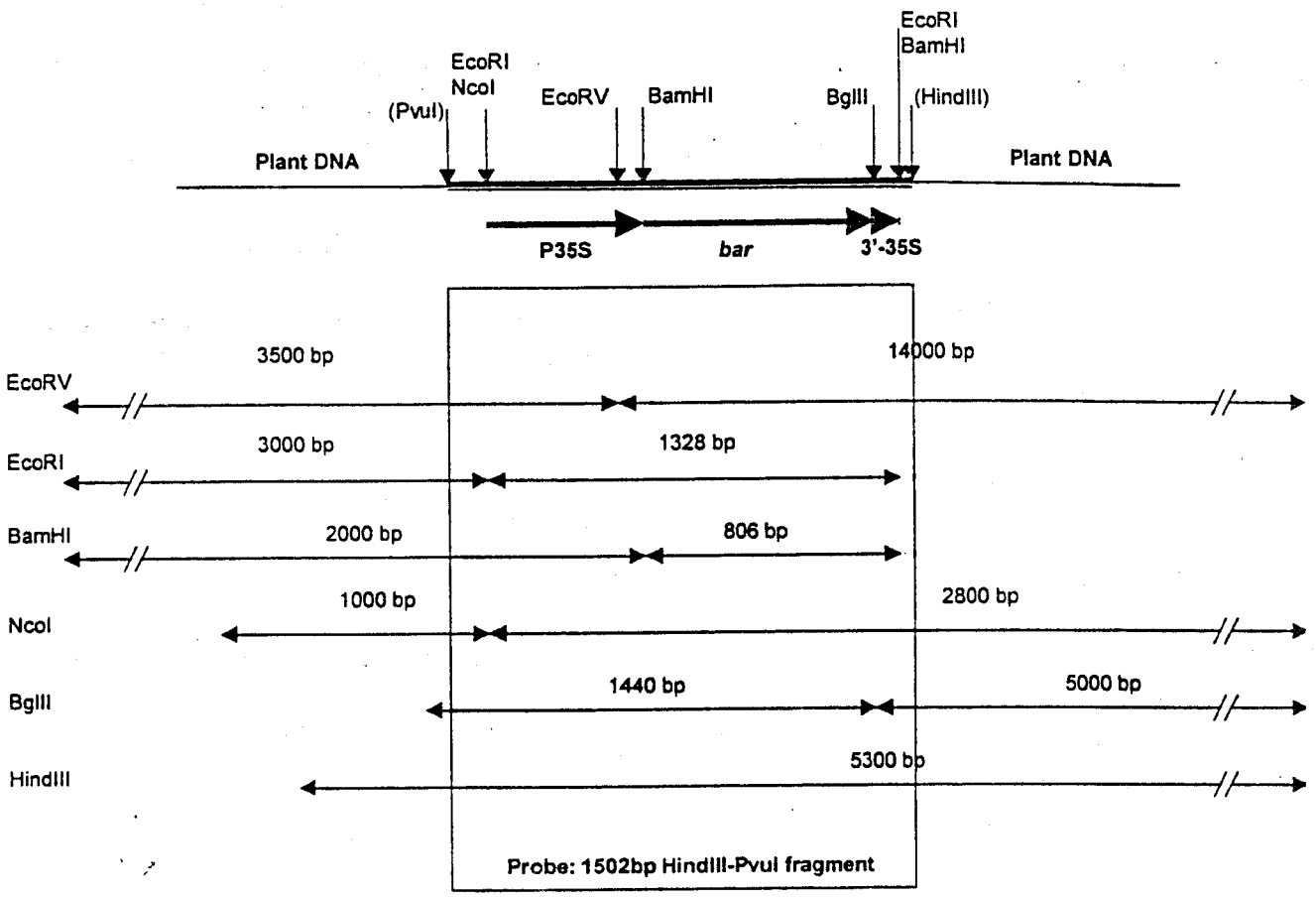
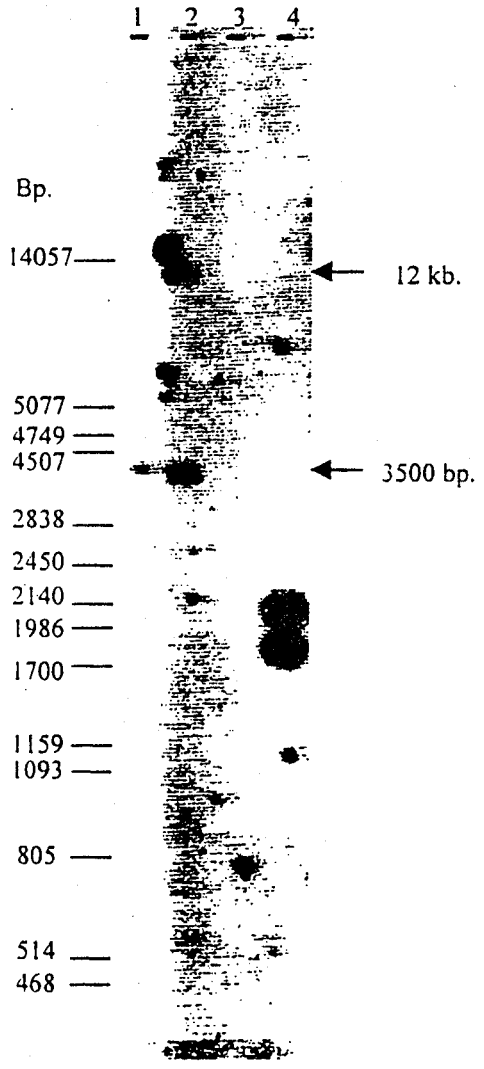


Figure IV.8. Southern showing stability of insertions in event LLRICE62

DNA was isolated from event LLRICE62 and the nontransgenic parent line Bengal. DNAs (10 µg) were digested with the indicated restriction enzymes. The *bar* cassette (1502 bp)(see Figure IV.1) was used as probe. Lane 1. LLRICE62 (T2 generation): EcoRV digest. Lane 2. LLRICE62 (T3 generation): EcoRV digest. Lane 3. Bengal: EcoRV digest. Lane 4. Bengal supplemented with pB5/35Sbar plasmid: EcoRV digest. The amount of restricted pB5/35Sbar in lane 4 is equivalent to 1.0 copy of the plasmid integrated in 10 µg of rice DNA. MW marker (λ DNA digested with PstI) sizes given in base pairs.



V. Agronomic Performance and Compositional Analysis of LibertyLink® Rice Events LLRICE06 and LLRICE62

A. Field Tests of Events LLRICE06 and LLRICE62

Transformation events LLRICE06 and LLRICE62 have been field tested by AgrEvo USA Company in winter nursery and adapted growing regions of the United States. LLRICE06 is adapted to California and LLRICE62 is adapted to the Delta and Gulf Coast rice production regions. These tests have occurred at approximately 16 sites under field release authorizations granted by APHIS (USDA authorizations: 97-206-02n, 98-071-67n, 98-083-03n, 98-083-04n, 98-083-05n, 98-089-03n, 98-112-08n, 98-156-01n). A field release is currently in progress under notification 98-225-04n. (At the writing of this petition LibertyLink® Rice events have not been field tested in any other country).

Data was collected for plant breeding, herbicide efficacy and registration from field trials in the United States. In the breeding trials, extensive notes were taken to facilitate the selection of the best lines. In the efficacy trials, different rates of herbicide were applied to evaluate weed control, and observations were made for agronomic characteristics and disease / pest characteristics. Additionally, material was harvested for compositional analyses. Rice plants were observed from emergence through maturity. Tables V.1 and V.2 summarize the USDA field trial authorizations for LLRICE62 and LLRICE06, respectively. Appendix 1 contains termination reports submitted to the USDA for the environmental releases that have been completed in the United States.

B. Agronomic Characteristics

The best lines derived from transformation events LLRICE06 and LLRICE62 have been evaluated for agronomic characteristics since their first greenhouse screens of the T_0 generation. T_0 plantlets were transitioned from tissue culture, transferred to greenhouse soil, and allowed to flower and set seed. Plantlets were evaluated for fertility, fecundity and tolerance to GA. Seed (T_1 generation) collected from plants that passed the greenhouse screen was planted in winter nursery for the primary field evaluations. Panicles were selected fromT_1 plants that survived an increased herbicide pressure and continued to express acceptable fertility and fecundity. Selected panicles were harvested and advanced to a secondary field evaluation of the T_2 generation. Each row was planted with the seed of a single panicle. The panicle rows were evaluated for Mendelian inheritance (Section IV.A) and seed from homozygous rows was harvested for further evaluation.

Of the over 200 T_2 generation panicle rows planted in the summer of 1998 of the Delta medium grain lines (LLRICE62), 69 were homozygous. Of that number, 25 were selected for advancement based upon uniformity, maturity, heading, height, disease resistance, lodging resistance, fertility, plant type and general vigor.

Replicated yield trials are underway in counter season locations (winter 1998-99).

Table V.1. Summary of field release authorizations granted for LLRICE06

USDA Authorization	Planting dates	Number of locations	Type of Trial	Location (county/state)
97-206-02N	9/97 1/98	1 1	Breeding < 1 acre	Lajas, PR
98-083-03N	5/30/98 6/4/98	2	Residue	Sutter, Glenn CA
98-083-04N	6/13, 6/14 6/17/98	3	Nutritional Composition Studies < 1 acre per site	Sutter, Glenn, Butte CA
98-089-03N	5/27/98	1	Breeding < 1 acre	Butte County, CA
98-112-08N	5/27/98 6/1, 6/3	3	Yield and Efficacy trials < 1 acre per site	Sacramento, Yolo, Yuba CA
98-156-01N	6/26/98	1	Nutritional Composition Study < 1 acre	Merced County, CA

Table V.2. Summary of field release authorizations granted for LLRICE62

USDA Authorization	Planting dates	Number of locations	Type of Trial	Location (county/state)
97-206-02n	12/97	1	Breeding < 1 acre	Lajas, PR
98-071-67n	5/5/98	1	Breeding < 1 acre	Acadia Parish, LA
98-083-05n	5/8/98	1	Breeding and Nutritional Composition Studies < 1 acre	Acadia Parish, LA
98-225-04n	9/18/98	1	Breeding < 1 acre	Lajas, PR

In the summer of 1998, the lines adapted for the California medium grain market (LLRICE06) were advanced to replicated yield evaluations. T_3 generation seed were planted as panicle rows in isolated blocks for seed production and for yield evaluation at two locations, in randomized, complete block replicated trials. All the parameters necessary for plant variety protection application were collected at all three locations (panicle rows in isolated blocks and replicated yield trials). Statistical analysis of the agronomic parameters and ranking statistics of the plant conformation and other non-parametric data were completed to identify the best commercial candidate to compare with the non-transgenic counterpart variety, M202 (Table V.3). In addition, lines with desirable characteristics for entry into breeding programs to create varieties targeted for other uses and regions of adaptation were identified.

Table V.3. Summary of characteristics of LLRICE06 advanced lines compared to the non-transgenic counterpart, M202*

Characteristics	Different	Not different
Agronomic Traits		
Germination		+
Seedling Vigor		+
Herbicide Tolerance	+	
Yield		+[1]
Maturity		+[2]
Low temperature		+
Height		Variable[3]
Panicle Conformation		Variable[4]
Stem rot		+
Plant Morphology[5]		
Culm angle		+
Internode color		+
Flag leaf		+
Panicle		+
Spikelet		+

* Comparisons were made in California in the summer of 1998.

1 The majority of lines were not significantly different compared to non-transformed M202 for yield (28 of 34 in the untreated setting and 30 of 34 in the treated test). Significance based upon analysis of variance, p=0.05.

2 Some of the advanced lines were 1-2 days later in maturity than M202. Small shifts in maturity are common for somaclones of rice.

3 Variation for plant height was noted to be within the range expected for somaclones, for example anther culture derived lines.

4 Six lines had panicles and kernels which conformed to the M202 type.

5 Mean of 20 measurements.

Comparisons were also made to determine the possibility of reduced yield for transformation events (Table V.4). Only one factor, line, was a significant source

of variation for each of the parameters measured. Neither the seeding date, replications, date by variety interactions or error term was a significant source of variation. Of the 36 lines included in this test, 20 were derived from transformation event LLRICE06 and 4 of these lines were significantly different from all the others (lower yield). None of these four were continued in the breeding program. The three lines listed in Table V.4 were not different from the parent variety and were advanced in the breeding program.

Table V.4. Summary of replicated yield trial results for selected lines derived from transformation event LLRICE06

Line	Yield (lbs/acre)	Seedling vigor (1-5, 5 best)	Days to 50% heading	Plant Height (cm)
M202	8532	5	78	94
Line 22	8331	5	79	97
Line 32	7572	5	80	97
Line 34	8378	4.9	80	100
Grand Mean	8105	5.0	79	96
LSD, 1%	906	0.1	1	5
C.V.	6.9	1.1	1.2	3.4

For LLRICE06, yield of all lines treated with GA (1600 gm ai/ha) was 8,124 lbs/acre and non-treated lines was 8,085 lbs/acre (Table V.5). The majority of lines tested were not significantly different ($p < 0.05$) compared to non-transformed M202 for yield (28 of 34 in the untreated setting and 30 of 34 in the treated test displayed no yield penalty). To select for lines with high levels of herbicide tolerance, the rate of GA applied to the breeding trials is in excess of the anticipated labeled rate.

Table V.5. Overall means compare LLRICE06 lines with and without glufosinate-ammonium applications

Herbicide treatment*	Yield	Seedling vigor	Heading date	Height
None	8,085	4.97	80	97
Two applications	8,124	4.96	79	96

* a single application consists of 1600 gm ai/ha

Rice plants were observed from emergence through maturity in all the field release sites (Table V.1 and V.2). No differences were observed between transgenic and nontransgenic rice in emergence, seedling vigor, stand establishment, lodging and seed shattering. Observations were conducted at

least 4 times in the growing season; 1) emergence, 2) early tiller, 3) panicle initiation to boot stage and 4) at harvest. Seedling emergence observations are provided for LLRICE06 planted at seven sites in California (which included studies for chemical registration, herbicide efficacy and nutritional composition) where transgenic and parental lines could be directly compared (Table V.6). Mean germination observed at these various sites was ~80%, indicating no tendency for the weedy characteristic, seed dormancy in the lines. Germination at the Glenn County sites was the lowest, however, no yield differences were observed at harvest.

Table V.6. Field emergence, 12-21 days post-planting for LLRICE06

Location (California county)	Planting date (1998)	Evaluation date	LLRICE06	Non-transgenic parent
Butte	30-May	12-Jun	90%	90%
Glenn	13-Jun	26-Jun	50%	50%
Glenn	30-May	24-Jun	90%	50%
Merced	26-Jun	14-Jul	90%	90%
Sutter	10-Jun	30-Jun	75%	90%
Sutter	14-Jun	30-Jun	75%	90%
Yolo	29-May	12-Jun	95%	95%
	Mean		81%	79%

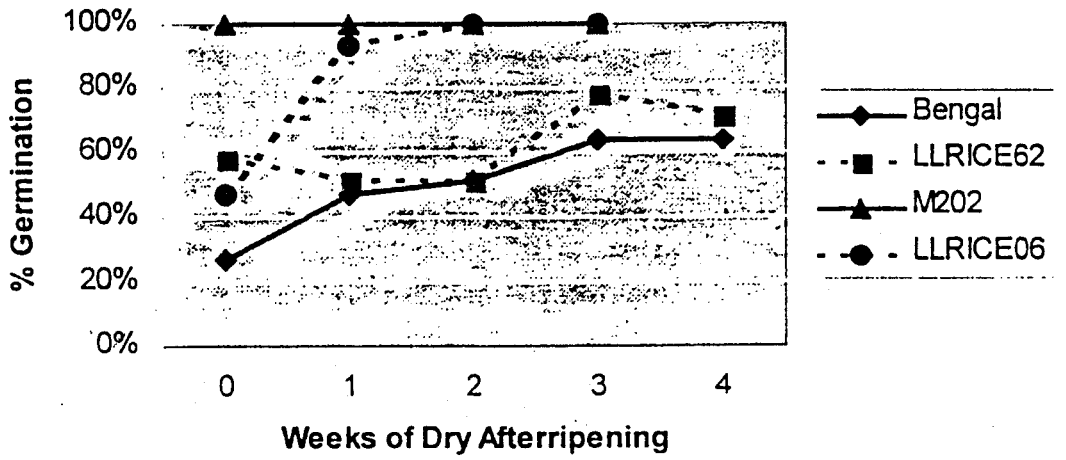
C. Seed Characteristics

Seed characteristics of long dormancy and shattering are important adaptive traits for weedy rice that are determined by both genetic and environmental factors. Thus, to access the potential for weediness of LibertyLink® Rice, evaluations specifically designed to measure seed germination, dry afterripening requirements, dormancy and shattering were conducted using panicles grown in the regions of adaptation.

Panicle samples of LLRICE62 and its parent, Bengal, were collected from the breeding plots in Louisiana and LLRICE06 and its parent, M202, from breeding plots in California. In both locations, panicle samples were collected at physiological maturity and the seed germination and dormancy protocol developed by Dr. Marc Cohn at Louisiana State University was followed (Appendix 2.c, seed dormancy evaluation protocols). Panicles were evaluated for shattering and none of the panicles exhibited the typical easy shattering of weedy, red rice.

Following the shatter test, seed were removed by hand from the panicles and samples were transferred to containers for dry afterripening. Germination was tested until the dry afterripening requirement was met (Figure V.1). A difference of one week was observed for the California grown material (M202 and LLRICE06). The M202 seed samples exhibited no requirement for dry afterripening. Within one week of dry afterripening, LLRICE06 had reached complete germination. The Louisiana grown material (Bengal and LLRICE62) required a longer period of dry afterripening. No difference in germination, length of dry afterripening requirement or seed dormancy was observed between the transgenic and non-transgenic pairs for each location after one week of dry afterripening (Figure V.1). At the end of the germination test, there were no un-germinated and firm seeds remaining indicating no dormancy beyond the dry afterripening. The seed lots may be characterized as expressing Class 1 dormancy, as defined in Appendix 2 (c).

Figure V.1. Germination and Dormancy Evaluation for Transgenic Events and Non-transgenic parents*



* Mean of three panicles, 15 seed per panicle.

In direct comparison with their non-transgenic counterparts and grown in their respective regions of adpatation, LibertyLink® Rice did not exhibit any seed character that is associated with weediness. LibertyLink® Rice was not different from the non-transgenic counterparts (Table V.7).

Table V.7. Summary of seed characteristics of transformation events LLRICE06 and LLRICE62 compared to non-transgenic counterparts

Characteristics	Different	Not different
Seed Traits		
Shattering		+
Dry afterripening requirement		+
Dormancy		+

D. Disease and Pest Characteristics

There are many viral, bacterial, fungal, and insect pests that can damage rice and cause disease. In any given year one such pest infestation could result in severe damage and yield reduction to the rice crop. However, high disease pressure is rare in rice. Company researchers and cooperators made visual observations for plant pathogenic organisms in trials containing LibertyLink® Rice events LLRICE06 and LLRICE62 during the 1997 and 1998 growing seasons. Such observations revealed some minor pathogen infections but no infestations (see Appendix 1). Diseases observed included panicle blight (causal agent unknown), sheath blight (*Rhizoctonia solani*), blast (*Pyricularia oryzae*) and stem rot (*Sclerotium oryzae*). Infestations with rice water weevil (*Lissorhoptrus oryzophilus*, Kurshel) were noted in California. Whenever pests were observed there were no differences in damage or populations found between LibertyLink® Rice events LLRICE06 and LLRICE62 and nontransgenic counterparts. In addition, no differences were observed between plots of LibertyLink® Rice treated with no, 400, and 1600 gms GA per hectare (Appendix 1). Events LLRICE06 and LLRICE62 did not influence susceptibility to disease or pest organisms in diverse genetic backgrounds.

In conclusion, transformation events LLRICE06 and LLRICE62 are no more susceptible to disease or insect infestation or severity than their nontransgenic counterparts. The genetic background in which the *bar* locus was placed does not appear to influence susceptibility to disease and insect pests.

E. Compositional Analysis

Whole grain (rough rice) was harvested for field tests in Puerto Rico for LLRICE06 and from Louisiana for LLRICE62. To provide an indication of potential pleiotrophic effects resulting from the gene insertion, proximate analyses and a check of key minerals were conducted using rough rice samples. Proximates include: total protein, total fat, moisture, carbohydrate and ash. Minerals tested included: calcium, phosphorus and iron. The values reported are corrected for moisture. In addition, the three known antinutrients found in rice;

trypsin inhibitor, lectin, and phytic acid, were also analyzed in the rough rice samples. Levels were found to be within the expected range normal for rice (Table V.8 and V.9).

Further analysis is underway of processed fractions and from samples produced at additional locations. The remainder of the data will be provided to the FDA in support of AgrEvo's food and feed safety assessment of transformation events LLRICE06 and LLRICE62. However, there are no apparent differences between the transgenic and nontransgenic counterparts. All the results clearly demonstrate that LibertyLink® Rice is substantially equivalent to non-transgenic counterparts.

Table V.8. Compositional data of whole seed of transformation events LLRICE06 and LLRICE62 and their non-transgenic counterparts

Component[1]	Non-Transgenic M202	LLRICE06	Non-Transgenic Bengal	LLRICE62	Literature ranges
% Fat[2]	2.6	2.2	3.5	2.4	1.9-2.7
% Protein[3]	6.4	7.1	10.0	8.7	6.7-8.4
% Ash[4]	5.4	5.6	6.54	4.1	3.4-6.0
% Carbohydrates[5]	86	85	80	85	85
% TDF[6]	19	15	23	19	19
% Crude Fiber	11	9	18	14	8-12
% Calcium	0.05	0.03	0.03	0.03	0.03-0.07
% Phosphorus	0.2	0.3	0.3	0.3	0.27-0.36
% Iron	0.005	0.007	0.008	0.007	0.0016-0.0045

[1] All data adjusted to dry weight basis and reported as %w/w.

[2] Fat (Crude) or Ether Extract in Animal Feed, AOAC Official Methods of Analysis (1990), 920.39

[3] Modified Kjeldahl Method, AOCS Official Method (1991), Ba 4d-90

[4] Ash of Animal Feed, AOAC Official Methods of Analysis (1990), 942.05

[5] By calculation: % carbohydrate = 100% - (% protein + % moisture + % fat + % ash)

[6] TDF = Total Detergent Fiber and Lignin in Animal Feed, AOAC Official Methods of Analysis (1990), 973.18

Table V.9. Analysis for antinutrients in whole seed of transformation events LLRICE06 and LLRICE62 and their non-transgenic counterparts

Component	Non-Transgenic M202	LLRICE06	Non-Transgenic Bengal	LLRICE62
Lectins [1] (H.U./mg protein)	152.5	191.7	87.1	20.3
Phytic Acid [2]	0.62%	0.83%	0.44%	0.62%
Trypsin Inhibitor [3] (TIU/mg)	Not detected	Not detected	Not detected	Not detected

[1] Lectins have been found in rice seed, confined to the germ, and values reported in the literature are variable. Lectins are measured according to the method of Klurfeld and Kritchevsky (Lipids 22 (1987), 667-668) and are reported as hemagglutinating units per mg protein extracted (H.U/mg protein).

[2] Phytic acid is typically found in rough rice in the range of 0.64-0.74% and was measured by AOCS Official Methods of Analysis (1995) locator 32.5.18.

[3] Trypsin inhibitor can be found in rice bran at less than 0.02 units/mg. Trypsin inhibitor was measured according to the Methods of the American Association of Cereal Chemists (1995) AACC method 71-10 and is report as trypsin inhibitor units per mg of sample (TIU/mg).

VI. Potential for Environmental Impact from Noncontained Use of LibertyLink® Rice Events LLRICE06 and LLRICE62

The introduction of a new weed management system using LibertyLink® Rice varieties and Liberty® Herbicide will provide the rice grower greater flexibility in his/her weed control program. Defining the options appropriate for each of the rice growing regions is a task shared by rice researchers in both the public and private sectors. Liberty® Herbicide will allow farmers to control problem weeds like rice mimic (*Echinochloa* species) and red rice (*Oryza sativa*) and will change rice agriculture by reducing the amount of herbicides currently applied.

In the United States red rice is an introduced weed existing only in the agro-eco system of rice agriculture and its rotational crops. There are no genetic barriers to gene flow of the *bar* gene into red rice (Section II.C and II.E). There are, however, botanical and agricultural management barriers, which will be discussed in this section. It must be noted that red rice is present in only a portion of the rice growing area and that the Liberty® Herbicide weed control program is targeted to other important rice weeds. Management programs for the use of Liberty® Herbicide and the growing of LibertyLink® Rice varieties will be part of the stewardship initiative communicated by AgrEvo to growers. As an integral part of the product development program, management strategies to contain the potential for the development of Liberty® Herbicide resistance in red rice have been examined.

A. Potential for Gene Transfer from LibertyLink® Rice to Other Organisms

1. Outcrossing with wild and weedy relatives

Crop-companion weed complexes often have a common progenitor and a parallel evolution with the crop (Harlan, 1992). There are two rice relatives, "wild rice" and "red rice" that can mimic the cultivated crop and are considered to be weed problems in various parts of the world. In the United States, *O. rufipogon*, commonly called wild rice, is considered to be a separate species from domestic rice. *Oryza rufipogon* is included on the USDA Federal Noxious Weed List. It has only been identified in the United States as a single patch in the Everglades, Florida, and does not exist in any of the rice production regions (Vandiver, 1992).

The second weed, red rice, is a variant or ecotype of domestic rice, *O. sativa*. It does not share the perennial nature of *O. rufipogon* and persists in cultivated rice fields primarily by the characteristic of highly dormant seed. Seed banks of red rice can be long lived and management of the weed is often based upon depletion of the seed bank. Red rice can compete with the commercial rice and if not controlled, is considered to be a weed problem (Craigmiles, 1979; Noldine, 1998). Red rice mimics the crop and often causes yield reduction and quality loss by the admixture of red grains with the harvest. Red rice has been

described as a dominant competitor. In competition studies, as many as three rice plants were required to impact yield as much as one red rice plant (Pantone and Baker, 1991). The red pericarp, the result of anthocyanin pigmentation and inherited as a dominant gene, Rc, is undesirable in commercial rice production and considered a milling defect for the white rice.

In some of the older literature, red rice in the United States was misidentified as *O. rufipogon*, however the taxonomy was later confirmed to be *O. sativa* (Vandiver, 1992). Key differentiation between the two species is the annual nature *of O. sativa* and the perennial nature of *O. rufipogon.*

Historically, the origin of red rice is believed to be in the cultivated fields of India where both red and white rice were grown. Red rice is considered to have been introduced in the United States as a seed mixture, which source has been attributed to the East Indian Seed Company. Red rice was established in the rice fields of the American colonists and published USDA reports in 1850 list four red rice types (Craigmiles, 1979). Strict quality standards for rice planting seed combined with agricultural practices designed to deplete the red rice seed bank have eliminated red rice populations from California and sections of the southeastern production area (Hill, Smith and Bayer, 1994). Red rice populations have been and continue to be hybridized naturally with cultivated rice. The gene flow is predominately from the cultivated crop into the weed, red rice population. The cultivation of early maturing commercial varieties have, in effect, provided a partial hybridization barrier (based on phrenology) to the later maturity red rice populations (Langevin, et al., 1990). Although the pollination periods for red rice may be later than most of our currently cultivated rice varieties, red rice exhibits an uneven maturity in the panicle and can produce some seed capable of germination in a rapid time frame. Thus, red rice allowed to produce seed in a commercial rice field can shatter mature grains in advance of even the early harvested varieties.

Although biotypes of red rice are described in the literature (Kwon, Smith and Talbert, 1992; Diarra, Smith and Talbert, 1985) red rice populations are very diverse. Distinctive homogenous populations are rare. In a single field, red rice plants can be found that vary for such traits as culm coloration (blackhull or strawhull), plant height, flag leaf angle, and may range in awn length from long to awnless (personal communication, Steve Linscombe, Louisiana State University). However, for red rice collected from the rice growing regions of Arkansas, Louisiana and Texas, the diagnostic characters remain constant. Characteristics that distinguish red rice from crop rice are hispid, light green leaves; tall, slender culms; pubescent plants and seeds; profuse tillering; shattering of grains; and seed dormancy in the soil (Kwon, Smith and Talbert, 1992; Diarra, Smith and Talbert, 1985). In addition, red rice can produce more seed per plant than rice (Pantone and Baker, 1991).

Red rice seed can express a long seed dormancy when submerged and buried in the soil. In field studies of five red rice populations buried at three locations, red rice survived more than 6.5 years, however the length of seed survival varied with location and population (Goss and Brown, 1939). In lab tests of submerged seeds or seeds continuously buried in flooded soil at 25 to 30°C, red rice will remain viable and dormant for 4 to 6 years. Anecdotal evidence from farmers indicates survival for 20 years in soil (e.g., old levees knocked down in reworking previously abandoned fields (personal communication, Marc Cohn, Louisiana State University). A summary of the Goss and Brown (1939) buried seed study is provided in Appendix 2.a. For a discussion of red rice seed bank dynamics and dormancy characterization, see Appendix 2.b.

2. Outcrossing to cultivated rice

Cultivated rice is primarily self-pollinating, however when LibertyLink® Rice events LLRICE06 and LLRICE62 are grown for commercial grain production they may participate in unconfined outcrossing with other rice. The opportunity for outcrossing depends upon the proximity of the growing range and overlapping flowering period. However certain biological factors act to limit gene flow. Rice pollen is only viable for a few minutes and, thus, can only be transferred a short distance by wind to outcross. The self-pollinating nature of rice results in stigma with a preponderance of self-pollen, thus it is not likely that pollen from another source can compete and result in outcrossed seed (see Section II.E - Potential for Outcrossing). To illustrate the strong tendency for rice to self-pollinate, the separation distance between varieties for certified rice seed production in the United States range between 10 to 20 ft (3-6 m) depending upon the class of seed and region of production. Should seed result from outcrossing, volunteer rice can be controlled in rotational crops (see Section VI.E - Effects on Agricultural Practices of Rice).

3. Transfer of genetic information to organisms with which it cannot interbreed

Movement of transgenes from genetically engineered plants to microorganisms has been suggested as a risk if such plants are released into the environment. As initially stated in the USDA's Interpretive Ruling on Calgene, Inc. Petition for Determination of Regulatory Status of FLAVR SAVR™ Tomato (USDA-APHIS, 1992), and subsequently repeated in other USDA Determination documents, "There is no published evidence for the existence of any mechanism, other than sexual crossing" by which genes can be transferred from a plant to other organisms. As summarized in these Determination documents, evidence suggests that, based on limited DNA homologies, transfer from plants to microorganisms may have occurred in evolutionary time over many millennia. Even if such transfer were to take place, transfer of the *bar* gene to a microbe would not pose a plant pest risk. Genes encoding both PAT enzymes and acetyl transferases are found in microbes in nature. Indeed, as described earlier in this

document, the *bar* gene present in LibertyLink® Rice events LLRICE06 and LLRICE62 was isolated from a naturally occurring soil microbe.

4. Likelihood of Appearance of Glufosinate-Resistant Weeds

Herbicide resistance may be achieved by either a) gene flow to sexually compatible species and subsequent introgression of the trait into weed populations or b) through intensive use of the herbicide, which can select for naturally occurring resistant mutants in weed populations. For example, propanil resistant barnyardgrass and bensulfuron resistant sedges were found where continuous cropping to rice and no rotation to alternative herbicides was practiced (Hill, Smith and Bayer, 1994; Heap, 1998). Both these herbicides have residual activity and, thus, offer a prolonged selection pressure for weed populations. GA has no residual activity (see Section VI.E).

a. Gene Flow

In North America, two *Oryza* species exist that are potential recipients of gene flow from LibertyLink® Rice. They are red rice (*O. sativa*) and wild rice (*O. rufipogon*). Only red rice is present in rice production areas in the United States. See Section II.E - Potential for Outcrossing and Section VI.A.1 - Outcrossing with Wild and Weedy Relatives, for further discussion of potential recipients.

When red rice is present and flowering periods overlap, LibertyLink® Rice varieties may exchange pollen with red rice. If seed is allowed to mature from these crosses, a dormant seed bank containing the *bar* gene will likely be established. If no backcrossing or introgression into the red rice population occurs, one can expect the F_1 hybrid seed to persist no longer than red rice seed (Section VI.B). If introgression does occur, a herbicide resistant red rice population will have been created. In the case of such an occurrence, current red rice weed control practices still can be used to combat the weed.

Studies conducted by rice researchers at Louisiana State University, USDA-ARS, and the University of Arkansas concerning the conditions and consequences of gene flow into red rice are summarized and appended to this petition to illustrate that; 1) gene flow from rice can occur into red rice, however, the rate is likely to be very low (Appendix 2.d., Langevin, Clay and Grace, 1990) or not detected (Appendix 2.e. and Appendix 3, Sanders, et al., 1998), 2) the *bar* gene has no effect on the predicted inheritance of herbicide resistance in either red or crop rice backgrounds (Appendix 2.f., Sankula, Braverman and Oard, 1998, and Appendix 3, Oard, et al., 1998), and 3) the presence of glufosinate tolerance does not change the fitness characteristics of white/red rice hybrid populations (Appendix 3 for Linscombe reports to USDA; Hybrid Fitness Report; Rush disease evaluation; Oard, et al., 1998; Gealy and Gravois, 1998). See also discussion in Section VI.B - Weediness Potential of LibertyLink® Rice.

1) Detection of gene flow

A Louisiana State University study (Langevin, Clay and Grace, 1990) directed to the question of the fate of rice / red rice hybrids and the likelihood of introgression of domestic traits into red rice is summarized in Appendix 2.d. Using seed collected from red rice plants in the second year of an intensive weed control study including six varieties as the experimental set, the researchers established common garden plots and measured the red rice plants for morphological and isozyme characters. In brief, the group found evidence in all six of the varieties included in the test for the formation of F_1 hybrids. The frequency of hybrids was higher as the coincidence of flowering increased. For the only late season variety tested, the group reports introgression of cultivated traits into red rice within 2 seasons of conspecific habitation (growing side-by-side in a cultivated field situation). A gap in phenology was observed between the hybrids of the early varieties and red rice, but not for the hybrids made with the one late season cultivar, Nortai. The lack of opportunity for backcrossing in the F_1 populations may explain the differences between varieties.

A field study completed by researchers at Louisiana State University in 1997, interplanted a single red rice biotype with a Cypress transgenic rice line containing the *bar* gene. Although the flowering period of the Cypress and red rice were overlapping, gene flow was not detected (Appendix 2.e. and Appendix 3, Sanders, et al., 1998).

2) Inheritance of bar gene in red rice

Two studies have made reciprocal crosses between red rice and four different rice varieties containing the *bar* gene (Appendix 2.f., Sankula, Braverman and Oard, 1998, and Appendix 3, Oard, et al., 1998). The F_1 hybrids were resistant to glufosinate-ammonium and the F_2 families segregated according to Mendalian inheritance.

3) Fitness characteristics of white/red rice hybrid populations

Segregation and expression of the phenotype, glufosinate resistance, did not change the fitness levels of various traits measured in the transgenic rice parents or the F_2 families. For a more detailed discussion see Section VI.B - Weediness Potential of LibertyLink® Rice.

In summary, the first line of defense against potential development of Liberty® Herbicide resistant red rice populations is the control of the movement of the herbicide resistance gene. There is very little cross pollination in cultivated rice (generally less than 1%). However, pollen from domestic rice can outcross into conspecific red rice populations, if both are flowering at the same time. Studies to identify best agronomic practices have been conducted in the various growing regions and recommendations have been made to prevent outcrossing of

LibertyLink® Rice into red rice. See Section VI. E. 5 for the results of agronomic field studies and recommended management practices.

b. Mutation and Selection

Today there are large numbers of herbicide resistant weed biotypes, with over half of them resistant to triazines (Heap, 1998). GA is unrelated to triazines and has a different mode of action, i.e., it inhibits GS. It is unlikely that weeds or any plant species will spontaneously develop resistance to GA under selective pressure, because a plant must either develop mutant forms of GS that do not bind L-phosphinothricin, but still recognize glutamic acid, and/or evolve a L-phosphinothricin detoxification system. Experimental work to create GA resistant crop plants by selection has been ongoing for several years with no success. Below is an accounting of attempts to create GA resistant crop plants in the laboratory by selection for mutants that can tolerate L-phosphinothricin or overproduce GS.

Over the last 10 years AgrEvo has not succeeded in selecting a glufosinate resistant corn mutant from protoplast cultures. There have been no survivors when wild-type corn protoplasts are placed on medium containing L-phosphinothricin. On the other hand, using sulfonylureas as selective agents we have been able to select 44 independent sulfonylurea-resistant mutants within 3 months. Using fenoxaprop-ethyl as a selective agent we have been able to select 2 independent fenoxaprop-resistant mutants during one year. In all cases, there is a correlation with observations in weed populations where glufosinate-resistant weeds have never been observed, but weeds resistant to the other chemicals have been found.

Glutamine synthetase exists in multiple isozymic forms in different plant organs (McNally, et al., 1983). These forms can be cytosol or plastid localized, and encoded by a multigene family. Overproduction of the GS isozymes could provide a degree of tolerance to L-phosphinothricin. Donn, et al. (1984) selected alfalfa suspension cell lines that were more tolerant to L-phosphinothricin than wild-type cells. These cell lines have a 3- to 7- fold increase in their GS activity, due to an increase in GS mRNA resulting from amplification of a GS gene. When the amplified GS gene, under the regulation of the CaMV 35S promoter, was integrated into the tobacco plant genome, a 5-fold increase in GS specific activity and a 20-fold increase in resistance to L-phosphinothricin was measured *in vitro* (Eckes, et al., 1989). Neither the amino acid composition of the plant tissue was altered significantly by GS overproduction; nor were the fertility and growth of the overproducing GS plants affected. Although overproduction of GS in plants has been demonstrated following intensive laboratory manipulation, it is doubtful that weeds will be selected in nature which overproduce GS, thereby conferring commercial levels of resistance to GA.

The likelihood that GS mutants will occur that do not bind L-phosphinothricin, but still recognize glutamic acid seems to be extremely low. *In vitro* mutagenesis studies in Dr. Howard Goodman's lab, Massachusetts General Hospital, several years ago showed that GS mutants that could no longer bind L-phosphinothricin could be obtained for the alfalfa GS gene (personal communication, Günter Donn, AgrEvo GmbH). However, these mutants were very ineffective in using glutamic acid as a substrate. A plant bearing such a mutation would have difficulties surviving because its ability to detoxify ammonia would be seriously decreased. This theoretical consideration is in accordance with the observations *in vitro* and in the field.

Differential sensitivity to GA has been observed in rice varieties (Sankula, Braverman and Oard, 1998; personal communication, Steve Linscombe, Louisiana State University) and in red rice biotypes (Noldine, 1998). In screening a collection of red rice biotypes, Gealy and co-workers found that none survived the recommended application rates (personal communication, David Gealy, USDA-ARS, NRGEEC). Most biotypes did not survive the ¼ rate and only the most tolerant biotype survived the ½ rate. None survived the full application rate (1.12 kg ai/ha) used in this study.

There is little potential for the development of GA resistant red rice due to mutations in the weed and strong selection for resistant weeds due to the overuse of the chemical, as was the case for bensulfuron-methyl resistant weed biotypes that developed in California rice production and Propanil resistant weed biotypes in the Delta. The only foreseeable way by which a weed could develop true resistance to GA is through sexual transmission of the *bar* gene. In the temperate rice production regions, the potential for gene transfer is limited to red rice. This can and will occur where crop rice and companion weed rice are growing together and flowering periods overlap.

In conclusion, the likelihood of appearance of glufosinate-resistant weeds in the United States is extremely low. The only chance is for the case of gene flow into red rice, *O. sativa*.

B. Weediness Potential of LibertyLink® Rice

The relevant literature and studies that were designed to address the weediness potential of LibertyLink® Rice are reviewed in Appendices 2 & 3. In Appendix 2, there is a summary of the study findings and in Appendix 3, expert letters and unpublished reports describing the work. Manuscripts for peer-review are in preparation by the respective researchers. Study outcomes have been reported at scientific meetings and the related abstracts have been provided to AgrEvo via personal communication (see Appendix 3). In some cases, raw data and expert opinion were provided by the researchers to use in the safety evaluation via personal communication.

1. Crop-weed hybrid study findings

Studies to obtain baseline information for the agronomic consequences of red rice hybrids containing the *bar* gene were initiated by researchers in the southern rice production region (Louisiana and Arkansas). Hybrids between several lines of transgenic rice containing the *bar* gene and red rice were made and tested in greenhouse studies (Sankula, Braverman and Oard, 1998). The glufosinate resistance trait was found to be inherited in red rice following the rules of Mendel and the trait provided no cross resistance to other herbicides.

Field studies were planted in 1997 to expand upon this work and evaluate fitness under field conditions (see Appendix 3 for Louisiana State University reports submitted to USDA regarding field release 97-029-04R). In 1996, hybrids were constructed using red rice isolated from production fields and two regionally adapted rice varieties, Cypress and Bengal including transgenic and non-transgenic lines (see Oard, et al.,1996, for a description of the origin of the transgenic lines). Seed of F_2 families was produced and in the spring of 1997, these populations were planted in randomized complete block design at two locations (Arkansas and Louisiana). The field sites were selected for their isolation from current rice production, but were in areas with climate similar to the rice regions. The field sites were not naturally infested with red rice and thus the introduction of red rice biotypes and hybrid populations were carefully contained. Life stage and reproductive characteristics were measured as the season progressed (see Appendix 3, Hybrid Fitness Report for materials and methods) and mature seed were harvested for laboratory dormancy evaluation (see Appendix 2.c. for description of the dormancy testing methodology). Analysis of life history and seed traits found the hybrid families to be intermediate to their weed and crop parents. The presence of glufosinate resistance does not change the fitness characteristics of white/red rice hybrid populations. The differences identified by statistical analysis can be attributed to the contribution of red rice vs. commercial crop rice genetic background (see Appendix 3 for Hybrid Fitness Report).

The suite of characters that make red rice a successful weed include; 1) competitiveness from seedling to tillering stage, 2) height to shade out the crop, 3) panicles which shatter seed as the grain matures, and 4) seed dormancy. Characteristics held in common with crop rice that limit red rice to agro-ecosystems include; 1) susceptibility to disease and pests, 2) requirements for irrigation (continuous flood) and other agronomic inputs, 3) seed bed preparation for germination and 4) red rice is an annual plant, its mode of reproduction is by seed.

Thus, the hybrid fitness study was designed to measure parameters associated with the fitness of the weed, red rice. The following section provides a summary comparison of the crop, the crop-weed hybrids and the weed.

Life Stage. No statistically significant differences were observed for emergence, vigor or final height when comparing the hybrid populations and their parents. As expected, the commercial rice cultivars, Cypress and Bengal were separated based upon maturity (tillering date and heading date, both first and 50%). When contrasts between classes are tested, red rice is the dominant effect. The transformation had no effect on life history traits in these contrast analyses (Appendix 3, Hybrid Fitness Report, Tables 4 and 5). Hybrid populations have a wide maturity range which could provide more opportunities for overlapping flowering and outcrossing. Red rice and the hybrid populations had difficulty setting viable seed at the Ben Hur, Louisiana site.

Disease Resistance For naturally occurring infestations of sheath rot and leaf scald, no effect of the transgene was observed. There are significant differences in the disease ratings. The white rice tends to be above average in both traits; some of the hybrids and one of the reds tend to be below average for both. Most of the reds tend to be low for sheath rot, high for leaf scald (Appendix 3, Rush report for disease score).

Fecundity Hybrid populations had reduced seed number and size when compared to commercial varieties.

Shattering Populations with red parents expressed shattering. Analysis of mean shatter scores group red parents as highest, F_2 hybrid populations as intermediate and commercial rice in lowest group with little or no shattering (Appendix 3, Hybrid Fitness report, Figure 1).

Dormancy Dry afterripening requirements were shorter for the commercial than the red and white-red hybrid populations. Commercial lines lacked dormancy, imbibed seed either germinated or rotted as the testing progressed. Contrast analyses for the dry afterripening requirement for seed produced at the Louisiana and the Arkansas site show that although length of dry afterripening varies, there is no consistent pattern associated with parentage or transformation. However, when red rice is included in the genetic background, the dry afterripening requirement and length of dormancy is always greater (Appendix 3, Hybrid Fitness report, Tables 7 and 8).

Seed of the red and white-red hybrid populations either germinated or remained firm under prolonged, imbibed conditions (Appendix 3, Hybrid Fitness report, Table 6, Figure 2). Testing of the firm seed found many of the hybrids did not germinate when cut. Thus indicating that the hybrid seed was not able to respond to a common germination cue (removal of the husk and cutting the seed in half) (personal communication, Marc Cohn, Louisiana State University).

Transgenic vs. Non–transgenic For no fitness traits were the populations distinguished based on the presence of the *bar* gene, with the exception of resistance to Liberty® Herbicide.

Persistence In a fallow setting, techniques commonly used to induce germination of red rice and/or volunteer white rice, such as irrigation and cultivation, were successful to germinate hybrid volunteers. Following a single germination flush early in the season, no subsequent volunteers were observed for the remainder of the season (Appendix 3, Hybrid Fitness Report).

2. Rice is not a weed

Rice is generally not regarded as a weed. Rice is not listed as a noxious weed in the United States (USDA, 1995), nor is it listed as a weed anywhere else in the world (Holm, et al., 1979). However, red rice is a troublesome weed in the drilled-seed rice growing regions of the southern United States (Craigmiles, 1979).

3. Agronomic observations

For the transformation events LLRICE06 and LLRICE62, we cite the agronomic observations provided in Section V to add support to the assertion that the introduction of resistance to the herbicide GA has not caused LibertyLink® Rice to become a weed. LibertyLink® Rice retains the same growth rate and growth habit as nontransgenic rice (see Appendix 3, and Section V.B). It continues to be an annual which produces panicles that do not shatter and disperse their seed. As shown in Section V.B, LibertyLink® Rice events LLRICE06 and LLRICE62 germinate uniformly without extended seed dormancy. In addition, LibertyLink® Rice is equally susceptible to disease and insect pests as its nontransgenic counterparts (Section V.C and Appendix 1). Although LibertyLink® Rice events LLRICE06 and LLRICE62 may volunteer, the range in numbers of volunteers is no different from the number expected in commercial rice production (see Appendix 1, and Section V.B). If one chooses to eliminate LibertyLink® Rice events LLRICE06 and LLRICE62 and their progeny by chemical management, they can be removed by treatment with herbicides other than GA (see Termination Reports, Appendix 1).

C. Effects of LibertyLink® Rice on Non-target Organisms

LibertyLink® Rice transformation events LLRICE06 and LLRICE62 have been field tested at numerous sites across the United States and no toxicity or alteration of population levels have been observed for beneficial insects, birds or other species that frequent rice fields (see Termination Reports, Appendix 1). There were no qualitative differences between beneficial species and populations present on transgenic and nontransgenic rice plants. This observation was expected since LibertyLink® Rice contains a gene which encodes a protein that shares no homology with proteins that are known to be toxic (see Section VI.E). The three known antinutrients found in rice, trypsin inhibitor, lectin, and phytic acid were measured in seed of the two transformation events and their

nontransgenic counterparts. The levels were found in the expected range for rice seed (Section V.E, and Table V.9)

D. Indirect Effects of LibertyLink® Rice on other Agricultural Products

As indicated in Section V.D most of the rice grown in the United States is used for the production of food and feed. Rice grain is generally not consumed raw by humans, but is subjected to a number of processing steps including drying, milling, polishing of the grain and cooking for human food, and drying and grinding of the grain for animal feed. The levels of naturally occurring antinutrients found in rice (trypsin inhibitor, lectin, and phytic acid) were not out of the range for rice in commerce (Section V.E, and Table V.9).

AgrEvo GmbH has conducted studies on purified, synthetic PAT enzyme which show that the enzyme is both heat and acid labile. The enzyme looses 100% of its activity upon incubation at 75°C (167°F) or greater for 30 minutes. At pH values of 4 or less it is inactive after exposure for 30 minutes. Cooking of the rice grain should eliminate most PAT activity.

Should there be any PAT enzyme remaining after cooking, the only route of exposure for humans and livestock to PAT in LibertyLink® Rice would be via oral ingestion. In addition, animals would be exposed orally to PAT present in unprocessed grain and straw. AgrEvo GmbH has confirmed experimentally that PAT protein and *pat* DNA (a gene sharing 65.6% homology with *bar*) in a plant matrix is rapidly degraded *in vitro* by the gastric juices from swine, chicken, and cattle. These animals represent the three primary types of gastric systems among livestock. It has also been experimentally confirmed that PAT is readily degraded in simulated human gastric fluids within minutes. The PAT protein from *bar* and *pat* are very similar in their physicochemical characteristics (Wehrmann, et al., 1996).

The PAT enzyme does not have the characteristics of an allergen or a toxin. It is acid and heat labile and contains no glycosylation motifs. The protein has no homology to proteins other than PAT genes from other organisms. The substrate specificity for the PAT enzyme is very strict in that the only substrate is L-phosphinothricin. Neither any protein amino acid nor D-phosphinothricin is acetylated by PAT. Acetyl transferases are abundant and ubiquitous in nature where they share the common function of transferring an acetyl group from acetyl CoA to a substrate. Acetyl transferases differ in substrates and the metabolic pathways in which they function (Webb, 1992).

Based on 1) the substrate specificity of PAT; 2) the physicochemical properties of PAT; 3) its rapid degradation upon ingestion; 4) the low levels of PAT in whole seed (Table IV.9); and 5) the ubiquitous presence of acetyl transferases in nature, no adverse effects are predicted if the PAT enzyme is a minor constituent of human and animal food.

E. Effects on Agricultural Practices of Rice

1. Current Practices

In the United States, rice cultivation is concentrated in two regions, the southern Mississippi River Valley, beginning in the Missouri Bootheel and moving south through Arkansas and Louisiana to the Gulf Coastal Plain into Texas, and in north central California. White, long grain rice is the predominant type in production, however, other varieties of food grade rice are grown under identity preserved conditions. In California, medium grain varieties comprise 90% of the state's production with a mixture of short grain, long grain and specialty rice varieties making up the balance. Basmati rice, a special aromatic rice, is planted in some regions of Louisiana, Arkansas and Texas.

a. Weed control overview

Rice in the United States is grown using mechanized practices. It is not transplanted. The practices in the United States are typical of temperate rice production, in that both cultural practices (e.g., crop rotation and irrigation management) and herbicides are needed to control weeds (Hill, et al., 1994). Weed management is critical to maximum rice yield and is used on most rice acreage grown in the United States. The grower is typically interested in applying a herbicide for weed control that has a broad weed spectrum, does not injure the crop, is cost effective, and has positive environmental attributes. Products are applied pre-plant, pre-emergence and post-emergence to the rice crop. Herbicide programs in rice can vary due to the geographic area, seeding method (water, drill or broadcast), weed spectrum, and first-year versus continuous rice. Farmers have traditionally relied upon propanil products to a great extent. Several weeds, however, have developed resistance to propanil, e.g. barnyardgrass. Pre-plant herbicides used are molinate, glyphosate, 2,4-D, and paraquat. Pre-emergence herbicides used are thiobencarb, quinclorac, pendimethalin or combinations of these herbicides. Typical post-emergence herbicides used are propanil + molinate, bentazon, acifluorfen, trichlopyr, bensulfuron, molinate, propanil, 2, 4-D, quinclorac and fenoxaprop. Also, many products are used in combination as premixes or tank mixes to widen the spectrum of control. Herbicide products are combined to prevent rice injury, reduce weed pressure on the crop, and avoid rotational restrictions for the following season.

Problem weeds in the Delta and Coastal rice production areas include barnyardgrass (*Echinochloa crus-galli*), red rice (*Oryza sativa*), bearded sprangletop (*Leptochloa fascicularis*), broadleaf signalgrass (*Brachiaria platyphylla*), Johnsongrass (*Sorghum halepense*), nutsedge (*Cyperus esculentus*) and flat sedges (*Cyperus spp*). Ducksalad (*Heteranthera limosa*),

dayflower (*Commelina* spp,), eclipta (*Eclipta alba*), hemp sesbania (*Sesbania exaltata*), morninglories (*Ipomoea spp*), smartweed (*Polygonum pennsylvanicum*) and jointvetch (*Aeschynomene* spp.) are broadleaf concerns. Perennials, such as redvine (*Brunnichia ovata*) and trumpetcreeper (*Campsis radicans*), are difficult to control because they propagate by seed and/or underground plant parts.

For California rice production the most important weed is watergrass (*Echinochloa oryzoides*) followed by ricefield bullrush (*Scirpus mucronatus*), smallflower umbrellaplant (*Cyperus difformis*) and redstem (*Ammania coccinea*). Others include arrowhead (*Sagittaria sp.*), sprangletop (*L. fasicularis*), and ducksalad (*H. limosa*).

Control of these diverse species requires the use of multiple herbicide families and multiple applications. Historically, the two most important herbicide introductions for rice farming have been 2,4-D in the early 1950's and propanil for grass control in 1961. Before propanil, the only tool for grass weed control was irrigation. It was common practice to plow under and replant entire fields if rice seedlings could not compete with grass weeds in stand establishment. Very tall rice varieties were favored to allow deep flooding. Semi-dwarf rice varieties and improvements in weed control have been the foundation for increases in rice productivity. Since the 1961 introduction of propanil, a number of herbicides have been registered for rice production. However, today, herbicides registered for control of weeds in rice are declining, especially in California. To summarize the historical record for California-grown rice, in 1983, monitoring of surface water for herbicide residues from rice fields was instituted. Holding periods were established for the herbicides molinate and thiobencarb to reduce potential residue in the water leaving the fields. In 1988, the herbicide bentazon was banned for use in California due to its detection in the ground water. In 1990, a new herbicide, bensulfuron-methyl, tradename Londax, was registered for use in California. By 1993, the first reports of weeds resistant to bensulfuron-methyl were reported and tank mixes with phenoxy herbicides were required to provide a complete weed control program. In 1994, the California rice growers established a 2 cents per cwt. (hundred weight) assessment to sponsor the reregistration of MCPA [4-(2 Methyl - 4 - chlorophenoxy) butyrate], as the original MCPA registrant did not reregister aquatic use for the herbicide. In 1995, cotton production was begun in the rice production regions. The use of phenoxy herbicides is detrimental to cotton production and serious damages were observed. To avoid damage to cotton, phenoxy herbicide registrations for use on rice have been restricted. All existing phenoxy rice registrations in CA have been pulled. Existing inventory can be used subject to local Agriculture Commissioner's approval.

No single weed management strategy is successful for the control of weeds in rice. Combinations of preventative, cultural and chemical practices are common. Practices include the planting of weed-free seed, crop rotation to break weed

cycles, precision land leveling for irrigation, seed bed preparation, conservation tillage programs, irrigation management to suppress weeds and the application of one or more herbicides (Hill, Smith and Bayer, 1994). There are two general types of rice culture that can best be described by the method of planting, either dry or water seeding. Both systems have elements focused to manage the weed species competing at differing stages of rice production (Table VI.1). Barnyardgrass and red rice (for regions outside California) are the most competitive weeds for the entire season. Aquatic and broadleaf weeds often become established when the flood is dropped and can reduce yield and reduce grade of the final grain, as many have dark-colored seed (Guy, Baldwin and Helms, 1992).

Table VI.1. Weed pressure in Arkansas rice production[a]

Early Season	Midseason
Barnyardgrass	Red rice
Bearded sprangletop	Ducksalad
Amazon sprangletop	Hemp sesbania
Broadleaf signalgrass	Northern jointvetch
	Eclipta

[a]After Guy, Baldwin and Helms, 1992.

b. Seeding Practices

In dry seeded rice production systems (often called, drilled-seeded rice) typical for most of the Delta region, rice seed is planted with a grain drill. In some regions, no-till systems have been developed for rice, using herbicides to burn down the seed beds prior to drilling. Water-seeding rice production is typical for sections of Texas, southern Louisiana, most of California and some regions of Arkansas. In the Delta regions, the primary reason for water-seeding is suppression of red rice, as red rice seeds do not germinate when held under water. Imbibed rice seed are spread over fields submerged under a shallow flood using airplanes. Rough preparation or grooving of the seed bed is essential to minimize seedlings drifting on the water surface before the roots can establish contact with the soil.

c. Irrigation Practices

In all rice production systems, water level is managed to suppress weed seed germination, while allowing the rice seedlings to establish. An extremely level field surface is key to even flooding. Two methods, discontinuous flood and continuous flood, are utilized to allow seedling establishment. If environmental conditions are cool or the seeds are covered by soil due to high winds the field is usually drained to allow seedling to peg their roots into the soil. The field is flooded with a shallow water depth and the flood is increased as the rice

seedlings develop. In the drain period of the discontinuous flood method, weed seed also germinate and the drain period is often a window for herbicide application.

d. Rice Weed Control Programs

A chronology of rice weed control programs provides agronomic details for three management systems; dry-seeded, no-till, and water-seeded. Chemical names of herbicides are provided in parenthesis and the registered product names in brackets.

1. Delta / Coastal – Standard Program
   - Spring plant by drilling or broadcasting the seed after land preparation.
   - Apply residual material pre-emergence (thiobencarb [Bolero], quinclorac [Facet], pendimethalin [Prowl]).
   - Flush as needed.
   - Apply post-emergent herbicides (propanil + molinate [Arrosolo], bentazon [Basagran], acifluorfen [Blazer], triclopyr [Grandstand R], bensulfuron [Londax], molinate [Ordram], propanil, fenoxaprop [Whip 360]).
   - Flood when rice is in early to mid-tillering stage.
   - Apply salvage weed control if needed (propanil + molinate [Arrosolo], bentazon [Basagran], acifluorfen [Blazer], triclopyr [Grandstand R], bensulfuron [Londax], molinate [Ordram], propanil, fenoxaprop [Whip 360]).

2. Delta / Coastal – No-Till System
   - Fall field preparations, apply burndown (paraquat [Gramoxone Extra] or in the Spring, apply glyphosate [Roundup Ultra]).
   - Spring plant by drilling or broadcasting the seed.
   - Apply residual material pre-emergence (thiobencarb [Bolero], quinclorac [Facet], pendimethalin [Prowl]).
   - Apply post-emergent herbicides; (propanil + molinate [Arrosolo], bentazon [Basagran], acifluorfen [Blazer], triclopyr [Grandstand R], bensulfuron [Londax], molinate [Ordram], propanil, fenoxaprop-p-ethyl [Whip 360]).
   - Flush as needed.
   - Flood when rice is in early to mid-tillering stage.
   - Apply salvage weed control if needed (propanil + molinate [Arrosolo], bentazon [Basagran], acifluorfen [Blazer], triclopyr [Grandstand R], bensulfuron [Londax], molinate [Ordram], propanil, fenoxaprop-p-ethyl [Whip 360]).

3. California – Standard Program
    - Spring plant, 95% is water seeding of pre-germinated seed via airplane.
    - Herbicide choices include; thiobencarb (Abolish) preflood, molinate (Ordram), thiobencarb (Bolero), bensulfuron (Londax)1.5 - 2. 5 rice leaf stage of growth (SOG), triclopyr (Grandstand CA) 3 leaf to 1 tiller rice SOG, carfentrazone (Shark)(Sec 18) 10-12 days after seeding in the water or 30 days after seeding (DAS) foliar, 2,4-D 2,4-Dor MCPA 30 - 35 DAS, propanil (primarily Super Wham) 20-35 DAS, and fenoxaprop(Whip 1 EC) (30-45 DAS).

e. Control of Rice Volunteers

In the Gulf Coast and Delta rice growing regions, rice is primarily grown in rotation with soybeans and grain sorghum. In some regions, rice is grown following rice from the previous year. The ideal program is rice grown once in three years. Once in two years is acceptable provided a good red rice and weed control program is used in the rotation crop. Volunteer rice in soybeans can present a potential problem to farmers. The severity of the problem largely depends on harvest conditions for the rice the previous fall. If rice falls down before or during harvest, there can be a significant amount of rice growing amongst the soybeans in the following year. 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.

In California, 70% of rice land is dedicated strictly to rice production. In rotational areas, off-year crops may include wheat or corn oilseed crops such as safflower and sunflower, tomatoes, dry beans, and melons or vegetables. Rice volunteers are not considered to be a weed problem.

2. Agricultural management practices for control of red rice

Key to red rice control are long term programs aimed at the reduction of the seed reservoir in the soil. For example, in southwest Louisiana, cropping rice and soybean in rotation provides the opportunity to employ a range of management practices and herbicides with differing specificities (Griffin, Baker, Dunand and Sonnier, 1986). Some principles of best management practices that are in use to control red rice include:

a. Plant as early as possible

The early establishment of the crop will allow flooding before red rice has begun germination. Standing irrigation water will suppress red rice germination.

Early plantings of the rice crop will help to insure that rice and red rice flowering periods do not overlap. [The same management practices that control red rice today can be used to control red rice containing the *bar* gene.]

b. Plant clean seed

Red rice populations can be established in production fields by planting contaminated seed or spread by equipment used previously in a red rice infested field. Seed certification regulations define standards for red rice contamination in planting seed. Rouging of red rice out of certified seed fields is an important point of control that requires skilled, hand labor to walk the field and remove the red rice plants all through the season. Gibberellic acid or maleic hydrazide can be used as seed head suppressants.

LibertyLink® Rice will allow the chemical clean-up of rice seed production fields. Glufosinate-ammonium can be applied over the top and late in the season providing more control options.

c. Irrigation management to suppress red rice seed germination.

Several irrigation methods have been adapted for specific growing condition and grower equipment. Rice will not germinate through soil and water. This same principle is true for red rice. In general, the principal is to establish the rice seedlings and keep the flood level high enough to suppress weed competition yet allow the rice to set roots and grow. For example, the application of molinate (Ordram) or thiobencarb (Bolero) herbicides to water-seeded rice with the use of continuous flood culture is recommended for control of red rice (University of Arkansas, 1992). Fenoxaprop (Whip 360) can be applied at both preflood and postflood to suppress red rice.

d. Depletion of the seed bank

Red rice seed shatters and is often cultivated into the soil in the course of normal field activities. It creates a resident seed bank in fields that survive crop rotations. Crop rotation patterns that promote spring germination of red rice followed by cultivation to destroy the seedlings before the next crop is planted will help to deplete the red rice seed bank. For example late planted soybeans (May plantings) can allow two flushes of red rice germination prior to planting (Eastin, 1979).

e. Use of grass-active herbicides in rotational crop

Crop rotations with corn, sorghum, soybeans and cotton for one or two years in combination with herbicides have been used successfully to reduce red rice infestations (Griffin, Baker, Dunand and Sonnier, 1986; Hill, Smith and Bayer,

1994). A number of over-the-top grass herbicides are labeled for use in cotton and soybean that can be used to control red rice in the rotational crop.

f. Seed head suppression of red rice

Maleic Hydrazide, trade name MH-30, has recently been registered for use in rice to delay red rice seed development.

g. Herbicide tolerant rice varieties

Herbicide tolerant rice varieties (IMI® and Roundup Ready® rice varieties, which are tolerant to sulfonlyureas and imidazilinones, both are ALS inhibitors; glyphosate; and LibertyLink® Rice)[1] representing three separate herbicidal modes of action (Schmidt, 1997) are currently in advanced breeding programs targeted for temperate rice production.

3. Possible Effect of LibertyLink® Rice on Current Practices

a. The Herbicide Glufosinate-ammonium and Current Uses

L-phosphinothricin, the active ingredient in GA herbicides, is a potent inhibitor of the enzyme GS in both bacteria and plants, where it apparently binds competitively to the enzyme by displacing L-glutamate from the active site. GA is a nonselective herbicide for both non-crop and crop uses. It is highly biodegradable, has no residual activity, and has very low toxicity for humans and wild fauna. There are presently no registered uses for GA in rice. However, GA is registered for use as a non-selective herbicide on turf (trade name Finale™) and apples, grapes, and tree nuts (trade name Rely®) in the United States. GA is registered as the herbicide Liberty® for use on LibertyLink® varieties or varieties warranted by AgrEvo USA as being tolerant to Liberty® Herbicide of corn and soybean in the USA and for canola in Canada. Outside the United States, GA is registered for use on plantation crops, tree nuts, and vines, and for industrial/non-agricultural weed control under a variety of trade names including Basta® and Ignite®.

b. Possible Effects as Indicated by Results from Agronomic Practice and Weed Control Efficacy Trials

Studies across the rice growing regions of the United States have tested Liberty® Herbicide against many of the common weeds found in rice. Study results have shown acceptable control for every important rice weed (Table IV.2). The use of Liberty® Herbicide fits well with the current agronomic practices in use for rice. It

[1] IMI rice, developed by the Louisiana State University Agricultural Center and American Cyanamid, is derived from somoclonal mutation. Round Ready rice and LibertyLink rice are derived from recombinant DNA techniques and are being developed by Monsanto and AgrEvo, respectively.

can be applied in either dry or water seeded rice production systems. The option to wait for crop establishment to assess the need for weed control, allows the grower flexibility and avoids blind application of pre-plant and pre-emergent herbicides. Liberty® Herbicide will give the farmer more flexibility in application timing and to use the herbicide on an "as-needed" basis. Liberty® Herbicide allows the grower the option to delay herbicide application until the level of weed infestation is known (Appendix 3, Schwarzlose, et al., 1998).

Table VI.2. Weeds in rice which can be controlled by Liberty® Herbicide Provided are the common and scientific names.

Grass weeds	
Barnyardgrass	*Echinochloa crus-galli*
Crabgrass, large	*Digitaria sangulinalis*
Red rice	*Oryza sativa*
Signalgrass, broadleaf	*Brachiaria platyphylla*
Sprangletop	*Leptochloa fascicularis*
Watergrass, early	*Echinochloa oryzoides*
Broadleaf weeds	
Redstem	*Ammannia coccinea*
California Arrowhead	*Sagittaria montevidensis*
Cocklebur, common	*Xanthium strumarium*
Dayflower	*Commelina communis*
Eclipta	*Eclipta prostrata*
Morningglory, ivyleaf	*Ipomoea hederacea*
Sesbania, hemp	*Sesbania exaltata*
Sedges.	
Bulrushes, ricefield	*Scirpus mucronatus*
Flatsedge	*Cyperus iria*
Yellow nutsedge	*Cyperus esculentus*
Smallflower umbrella plant	*Cyperus difformis*

Some of the more important herbicides used today require high dosage uses for weed control. For example, propanil recommends doses of up to 4480 gms ai/acre X 2 applications, or 8960 grams ai/acre. Liberty® Herbicide will be recommended with much lower dose rates (up to 800 gm ai/acre). Effective Liberty® Herbicide dose rates are variable depending upon the weed species and age. The herbicide can kill large and small weeds. This range in efficacy is in direct contrast to Facet, Propanil and Ordram, which are only effective against small weeds. Some herbicides can cause injury to crop; e.g. Propanil can cause injury when applied after 4-leaf stage. The use of Liberty® Herbicide on LibertyLink® Rice varieties will not injure the crop.

Rice extension specialists, Drs. Joe Street and Dearl Sanders have provided their opinion on the importance of LibertyLink® Rice to combat the increasing area of red rice infestation in Mississippi and Louisiana (Appendix 3). They both concur that red rice cannot be eliminated from their production regions without additional management tools. Both experts have active evaluation programs to define recommendations for use of LibertyLink® Rice by their growers. Both believe that outcrossing can be managed and the risk of potential gene flow is out weighted by the current economic loss to the industry due to red rice infestations of rice production lands. Long seed dormancy and tendency to shatter seed continue to renew the seed bank in the soil that sustains the weed population. Depletion of the seed bank is the only long-term solution for the elimination of this highly competitive rice weed. LibertyLink® Rice varieties will provide the farmer with a weed control system that allows elimination of red rice without damage to the rice crop. Liberty® Herbicide can be applied to prevent the red rice from surviving to maturity and shedding more seed.

LibertyLink® Rice and GA may positively impact current agronomic practices in rice by 1) offering a broad spectrum, post-emergence weed control system; 2) providing the opportunity to continue to move away from pre-emergent and residually active compounds; 3) providing a new herbicidal mode of action that allows for improved weed resistance management in rice acreage; 4) encouraging herbicide use on an "as-needed" basis; 5) decreasing cultivation needs; and 6) allowing the application of less total pounds of active ingredient than used presently.

4. Resistance management recommendations

A product stewardship program will be communicated and provided to growers in a grower's guide and through targeted educational programs. The program will include agronomic practices designed to minimize the occurrence of resistant weed populations. In regions of the United States where red rice is present, removal of the red rice by herbicidal application prior to seed set will help to prevent potential crop-weed hybrid seed from reaching maturity. Red rice resistance management practices need to be identified for rotational and continuous rice cropping systems as well as dry and water seeded planting practices. The lessons learned from the experiences with the development of propanil resistant barnyardgrass and bensulfuron resistant sedges, teach us that resistance developed where continuous cropping to rice and no rotation to alternative herbicides were practiced (Hill, Smith and Bayer, 1994).

a. Agronomic practice recommendations

- **Never allow red rice plants to set seed**

For optimum weed control in rice, apply Liberty® Herbicide twice. Red rice may germinate throughout the crop season. It is key to prevent it from flowering at the

same time as the cultivated rice crop. Application timing will be pre-flood and post-flood in the Delta. If red rice is present in a LibertyLink® Rice field, Liberty® Herbicide must be applied to the field in accordance with the Liberty® Herbicide label directions for red rice control.

- **Monitor for signs of herbicide resistance**

When following a LibertyLink® Rice crop with a LibertyLink® Soybean crop, the red rice plants will need to be monitored for signs of Liberty® Herbicide resistance. If survivors are noted, an alternative herbicide can be used to control red rice in a rotational crop other than white rice. A number of post-emergence grass herbicides are labeled for use in soybean that will control red rice (Nastasi and Smith, 1989).

Diligence in seed bank depletion strategies must be maintained for at least two cycles of a three-year rotation program.

- **Rotate crops and herbicides**

The most important time to control red rice is in the rotational crop where effective grass weed herbicide can be used without damage to the following white rice crop (Smith, 1989). If using a three year crop rotation of rice: rice: row crop or fallow, observe the field for signs of red rice that may be resistant to Liberty® Herbicide. In the third year of row crop or fallow, employ a red rice control measure other than Liberty® Herbicide.

- **Red rice and white rice are resistant to some herbicides**

Most herbicides have similar activity on white rice and red rice. Thus, red rice which is resistant to some herbicides already exists. Herbicide resistant red rice is not new to the rice growing region. Knowledge of red rice biology and currently developed weed management practices can be applied to minimize the potential occurrence of glufosinate resistant red rice populations and to control those populations that may develop.

b. Targeted educational program

Prior to commercial release, resistance management strategies will be in place for agronomic management recommendations, communication, monitoring and response. An example outline plan for herbicide tolerant rice follows:

- **Management recommendations**
  - Rotation of crops and herbicide mode of action
  - Techniques to prevent red rice from flowering
  - Techniques for depletion of red rice seed in the soil
  - Identification of the best practices for the region

- **Communication**
  - Develop grower education program combining best agronomic practices for local region and monitoring for volunteers
  - Training for field representatives, local pest control advisors, extension agents and consultants
  - Point of sale product education brochures
  - Alternation of herbicides with different modes of action

- **Monitoring**
  - Independent surveys by private and/or public sector weed specialists
  - Grower responsibility

- **Response**
  - Management plan for unexpected results
  - 800 phone access and follow up
  - Apply the "Weed Resistance Management Action Tree" recommendations
  - Sampling procedures to confirm presence of herbicide tolerant gene

F. Conclusion of Environmental Impact Assessment

In 1992, the World Bank, Rockefeller Foundation and USDA brought together members of the rice research community and regulatory agencies representing the rice producing countries of the world (41 experts representing 15 nations). The proceedings were published as a white paper to provide biosafety guidance for rice researchers worldwide (Clegg, et.al. 1993). Three scientific issues relevant for the environmental impact assessment of transgenic rice were identified:

Issue 1: What is the likelihood that pollen movement or other mechanisms could transfer genetic materials out of rice planting to adjacent crop or wild and weedy relatives?

Issue 2: What are the likely consequences of gene transfer to other crops or to wild and weedy relatives (including impact on biodiversity)?

Issue 3: What other biological risks and consequences might be posed by transgenic rice?

The experts acknowledged that outcrossing to weed and/or wild rice is possible in certain production regions and recommended two inquiries to access the likely consequences of a herbicide tolerance gene moving into red rice or a wild rice species; *i*) fitness evaluation of the potential hybrids, and *ii*) an evaluation of the likely effects on current agricultural systems. A key question identified by the symposium for herbicide tolerance genes was; 'Will the transfer of herbicide

tolerance to wild and weedy rice relatives exacerbate problems of weed control and thus lead to decreased rice production yields?'

In the environmental impact assessment for LibertyLink® Rice, each of the three issues and the special considerations for herbicide tolerance have been addressed.

Issue 1. Likelihood of Gene Flow

Assessment: Gene flow from rice can occur into red rice, however, the rate is likely to be very low because there are numerous barriers.

Genetic barriers to outcrossing

a) gene flow via pollen and no other mechanism	Section II.B, C, D, and E Section VI.A.3
b) no genetic barrier within the AA genome, however reproductive barriers exist for F_1 progeny	Section II.B Section II.E
c) only two *Orzya* species in AA genome in North America, *O. sativa* and *O. rufipogon*	Section II.B
d) red rice and crop rice, *O. sativa,* grown conspecific in rice production fields	Section VI.A.1, VI.A.2
e) wild rice, *O. rufipogon*, one patch, confined to Everglades, Florida and not near commercial rice production	Section VI.A.1

Botanical barriers to outcrossing

f) rice is self pollinating (less than 1% outcrossing)	Section II.B, C
g) crop rice pollen has a short life (5-9 minutes)	Section II.B.
h) no insect pollen vectors that could move the pollen long distances	Section II.E.
i) successful outcrossing depends upon proximity of plants and coincidence of flowering, although red rice is conspecific with rice cultivation, the phenology of the two often do not overlap	Section VI.
j) height differences, many red rice populations are taller than crop rice, thus pollen shed from the crop rice falls to the ground, not reaching the red rice flowers above	Section VI.A.1

Agricultural barriers to outcrossing

k)	production of planting seed free of red rice can be aided by the use of Liberty® Herbicide to remove red rice from seed production fields	Section VI.E.2
l)	management of red rice flowering hand rouging crews or by applications of Liberty® Herbicide can remove red rice before it can flower	Section VI.E.2
m)	sub-lethal dose of Liberty® Herbicide can delay flowering and suppress seed formation	Section VI.E.2
n)	depletion of seed bank via shallow cultivation and crop rotation	Section VI.E.2
o)	irrigation management to prevent red rice germination	Section VI.E.2

Issue 2. Consequences of Gene Flow

Assessment: Gene flow to red rice will not exacerbate problems of weed control or adversely impact agriculture. The fitness of crop-weed hybrids is not greater than that of red rice.

Impacts on crop and crop-weed hybrid fitness

p)	no impact of the *bar* gene on life history, reproductive or seed dormancy traits	Section VI.B Section V.B, C
q)	no observed changes for weediness characters in agronomic and breeding evaluations	Section V.B, C, D
r)	crop-weed hybrids were intermediate to parents in seed shattering and dormancy traits	Section VI.B

Impacts on agriculture and rice productivity

s)	new weed control options: - no long residual activity - new mode of action - minimum till to reduce cultivation - reduced environmental load	Section VI.E.3
t)	no yield reduction with LibertyLink® Rice	Section V.B
u)	no new resistance management techniques required	Section VI.B, E
v)	stewartship initiative to manage Liberty® Herbicide resistant red rice that may arise	Section VI.E.4

Impact on biodiversity

w) neither red rice or wild rice are important genetic resources for modern rice breeding. Red rice is an introduced weed that resides only in agro-ecosystem and wild rice, *O. rufipogon*, is listed as a noxious weed by the USDA	Section II.D Section VI.E.2
x) no impact by *bar* gene on other organisms that may consume rice grain for food or feed and no change in nutritional composition or levels of anti-nutrients	Section V.E and VI.D

Issue 3: Other biological risks

Assessment: No other biological risks are identified.

y) the PAT protein is not toxic and is found in nature	Section III. D
z) no unanticipated harmful effects have been observed in commercial transgenic crops containing the *bar* gene nor in any of the field trials with rice containing the *bar* gene	Section I Section V.A, B
aa) molecular characterization shows normal inheritance and only the intended DNA was inserted.	Section III.A, D and IV.B

In summary, a review of studies that address the questions surrounding gene flow to red rice and its consequences indicates that the large scale release of herbicide tolerant rice varieties will not exacerbate red rice weed control problems. Studies to identify best agronomic practices have been conducted in the various growing regions and recommendations have been made to 1) prevent outcrossing of LibertyLink® Rice into red rice, 2) prevent the selection of natural genetic variation for resistance to various herbicides by rotation of crops and use of herbicides with different modes of action, and 3) manage any resistant red rice populations that may arise.

VII. Statement of Grounds Unfavorable

No unfavorable information and data has been demonstrated for LibertyLink® Rice Transformation Events LLRICE06 and LLRICE62.

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IX. Appendices

Appendix 1. USDA Field Trial Termination Reports

Appendix 2. Background and Summaries of Related Studies

Appendix 3. Expert Letters and Unpublished Reports

Appendix 4. Cited Literature

Appendix 1. USDA Field Trial Termination Reports

AgrEvo®

Field Trial Termination Report for LibertyLink® Rice Transformation Events

Date of Report:	October 23, 1998
Notification Numbers:	98-083-03N, 98-083-04N, 98-156-01N
Dates of Release:	May to June 1998
Dates of Terminations:	September to November 1998
Number of States and Sites:	California (6)

Purpose:
Produce seed from LibertyLink® rice plants for nutritional and compositional analysis. All plots were treated with standard herbicide treatments to control California weeds, Ordram and Lonax.

Plot types -

1) Transgenic plots treated with two applications of glufosinate (Liberty Herbicide) at 0.45 lb.ai. /A (0.5%), once at the 2 to 4 leaf stage, and once at the 2 to 3 tiller stage of growth.
2) Non-transgenic counterpart plots with no Liberty application.
3) Transgenic plots with no Liberty application.

Results:
Seed was produced for analysis at six locations, spanning the rice growing regions of California. Rough rice samples were harvested from all study sites for analysis of the raw agricultural commodities; whole grain and straw. A larger sample of rough rice was harvested from one site to be processed into the common fractions of rice in commerce.

Observations:
The plots were visited on at least a weekly basis during the duration of the release. The area planted to transgenic rice was 0.5 acre at each site. Observations were recorded at emergence, tiller stage, mid-season, and at harvest.

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

Insect Susceptibility:
The primary insect pest of rice grown in California is rice water weevil. Slight numbers of water weevils were observed in both the transgenic and non-transgenic rice plots.

Disease Susceptibility:
Common diseases to California rice production are sheath blight, rice blast, stem rot, and aggregate sheath spot. As expected, some disease symptoms were observed, however, applications of fungicide held the disease in check and there were no differences between the transgenic and non-transgenic plots.

Weather Related Conditions
The cool, wet El Nino weather pattern delay planting by almost one month. The summer months were hot and dry and the rice grew normal for the region. Cooler weather and rains were experienced in late September. The late planting and cool fall delayed the harvest until early October.

Physical Characteristics
Rice plants were observed from emergence through maturity. No differences were observed between transgenic and nontransgenic rice in emergence, seedling vigor, and stand establishment, and in other casual observations.

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

Means of Plant Disposition:
Following harvest, any remaining seed in the field were destroyed by cultivation. The site at Merced County, California will be harvested in mid-November. Any remaining seed in the field will be 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.

AgrEvo®

Field Trial Termination Report for LibertyLink® Rice Transformation Events

Date of Report: October 15, 1998
Notification Numbers: 98-083-05N, 98-071-67N
Dates of Release: May 1998
Dates of Terminations: September 1998
Number of States and Sites: Louisiana (2)

Purpose:
Evaluation of rice plants containing the *bar* gene, LibertyLink® rice, for tolerance to Liberty® herbicide (glufosinate-ammonium). Produce seed for nutritional and compositional analysis. All plots were treated with standard herbicide treatments to control local weeds.

Plot types -

1) Transgenic plots treated with two applications of glufosinate (Liberty Herbicide) at 0.45 lb.ai. /A (0.5%), once at the 2 to 4 leaf stage, and once at the 2 to 3 tiller stage of growth.

2) Non-transgenic counterpart plots with no Liberty application.

Results:
Glufosinate tolerant lines of rice were identified for advancement in the Liberty Link™ rice breeding program. Seed of selected lines was harvested for continuation in the breeding program. In addition, rough rice samples were harvested for nutritional and compositional analysis. No red rice was observed, as expected.

Observations:
The plots were visited on at least a weekly basis during the duration of the release. The area planted to transgenic rice was 2 acres.

T_2 generation seed were planted as panicle rows May 8, 1998 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 for variety development.

Also planted on May 5, 1998 were T_1 seed of additional lines. Plants were evaluated for agronomic characteristics and tolerance to herbicide application in the LSU breeding nursery. Seed of the survivors was held in reserve, in case the more advanced candidates did not continue to meet commercial quality criteria.

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

Insect Susceptibility:
Several insect species were observed in both the parent and transgenic plots. On the rice plants were observed grasshopper and stink bugs (immature and mature forms). Rice with anthers extruded were visited by honeybees observed to be foraging for pollen. Moving in the ducksalad (aquatic vegetation) in the alley ways between the plots were lepidopteran larvae and ants.

Disease Susceptibility:
The same range of diseases was noted in both parent and transgenic; panicle blight, sheath blight and stem rot. The panicle damage observed is very typical for the variety Bengal, when it is planted a bit late in the season, as was the case for these plots.

Weather Related Conditions:
It was a typical season for southwest Louisiana.

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 nontransgenic parent plots, the transgenic rows spanned a range of 10 days later to 5 days earlier in maturity than the parent did. 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 nontransgenic 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.

AgrEvo®

Field Trial Termination Report for LibertyLink® Rice Transformation Events

Date of Report:	October 15, 1998
Notification Numbers:	98-089-03N
Dates of Release:	May to June 1998
Dates of Terminations:	September to October1998
Number of States and Sites:	California (1)

Purpose:
To evaluate LibertyLink® rice plants for tolerance to glufosinate herbicide in the breeding program, seed increase of advanced and replicated yield tests of advanced lines. All plots were treated with standard herbicide treatments to control California weeds, Ordram and Lonax.

Plot design for Yield Test:
Randomized complete block design, 36 entries, two blocks, spray and no-spray treatments.

Plot types -

1) Transgenic plots treated with two applications of glufosinate (Liberty Herbicide) at 0.45 lb.ai. /A (0.5%), once at the 2 to 4 leaf stage, and once at the 2 to 3 tiller stage of growth.
2) Non-transgenic counterpart plots with no Liberty application.
3) Transgenic plots with no Liberty application.

Plot design for Seed Increase and Line Selection:
T_3 generation seed were planted as panicle rows in isolated blocks representing each event. Fifty panicle rows were planted per line, each event in a separate check, 20 ft between events.

Results:
Glufosinate tolerant lines of rice were identified for advancement in the Liberty Link™ rice breeding program. Seed of selected lines was harvested for continuation in the breeding program and production of pure seed. In addition, rough rice samples were harvested for nutritional impact studies.

Observations:
The plots were visited on at least a weekly basis during the duration of the release. The area planted to transgenic rice was 2 acres. All the parameters necessary for

plant variety protection rights application were collected. Statistical analysis of the agronomic parameters and ranking statistics of the plant confirmation and other non-parametric data were completed to identify the best commercial candidates. In addition, lines with desirable characteristics for entry into breeding programs to create varieties targeted for other uses and regions of adaptation were identified.

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

Insect Susceptibility:
The primary insect pest of rice grown in California is rice water weevil. Slight numbers of water weevils were observed in both the transgenic and non-transgenic rice plots.

Disease Susceptibility:
Common diseases to California rice production are sheath blight, rice blast, stem rot, and aggregate sheath spot. As expected, some disease symptoms were observed, however, applications of fungicide held the disease in check and there were no differences between the transgenic and non-transgenic plots.

Weather Related Conditions:
The cool, wet El Nino weather pattern delay planting by almost one month. The summer months were hot and dry, which is normal for the Northern California Sacramento River Valley. Cooler weather and rains were experienced in late September. The late planting and cool fall delayed the harvest until early October.

Physical Characteristics:
Rice plants were observed from emergence through maturity. No differences were observed between transgenic and nontransgenic rice in emergence, seedling vigor, and stand establishment, and in other casual observations.

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

Means of Plant Disposition:
Seed were harvested by hand and combine, cleaned, dried to 12% moisture and placed into storage in cloth bags. Following harvest, any remaining seed in the field were 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.

AgrEvo®

Field Trial Termination Report for LibertyLink® Rice Transformation Events

Date of Report: October 15, 1998
Notification Numbers: 98-112-08N
Dates of Release: May to June 1998
Dates of Terminations: September to October1998
Number of States and Sites: California (3)

Purpose:
Evaluation of LibertyLink® rice plants for tolerance to glufosinate herbicide, weed spectrum efficacy for Liberty Herbicide, and replicated yield tests of advanced lines.

Plot design for Yield Test:
Randomized complete block design, 36 entries, two blocks, spray and no-spray treatments. All plots were treated with standard herbicide treatments to control California weeds, Ordram and Lonax.

Plot types -

1) Transgenic plots treated with two applications of glufosinate (Liberty Herbicide) at 0.45 lb.ai. /A (0.5%), once at the 2 to 4 leaf stage, and once at the 2 to 3 tiller stage of growth.
2) Non-transgenic counterpart plots with no Liberty application.
3) Transgenic plots with no Liberty application.

Plot design for Efficacy:
Randomized complete block design for Liberty herbicide treatments and lines (transgenic and non-transgenic).

Liberty Herbicide treatment -
Transgenic plots treated with two applications of glufosinate (Liberty Herbicide) at 0.45 lb.ai. /A (0.5%), once at the 2 to 4 leaf stage, and once at the 2 to 3 tiller stage of growth.

Results:
Weeds were controlled with Liberty herbicide treatments. Yield and agronomic characteristics were measured.

Observations:
The plots were planted on May 29, June 1, and June 3. The plots were visited on at least a weekly basis during the duration of the release. The area planted to transgenic rice was 1 acre.

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

Insect Susceptibility:
The primary insect pests of rice grown in California is rice water weevil. Slight numbers of water weevils were observed in both the transgenic and non-transgenic rice plots.

Disease Susceptibility:
Common diseases to California rice production are sheath blight, rice blast, stem rot, and aggregate sheath spot. As expected, some disease symptoms were observed, however, applications of fungicide held the disease in check and there were no differences between the transgenic and non-transgenic plots.

Weather Related Conditions:
The cool, wet El Nino weather pattern delay planting by almost one month. The summer months were hot and dry and the growing seasons were typical for the Northern Sacramento River Valley. Cooler weather and rains were experienced in late September. The late planting and cool fall delayed the harvest until early October.

Physical Characteristics:
Rice plants were observed from emergence through maturity. No differences were observed between transgenic and nontransgenic rice in emergence, seedling vigor, and stand establishment, and in other casual observations.

Weediness Characteristics:
Growth rate and habit were identical in both transgenic and nontransgenic 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 were 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.

AgrEvo®

Field Trial Termination Report for LibertyLink® Rice Transformation Events

Date of Report:	October 15, 1998
Notification Numbers:	97-206-02N (final), 98-225-04N (interim)
Dates of Release:	September 1997 to May 1998
Dates of Terminations:	January 1998 to May 1998
Number of States and Sites:	Puerto Rico (1)

Purpose:
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 LibertyLink® rice breeding program.

Observations
The plots were visited on at least a weekly basis during the duration of the release. The area planted to transgenic rice was 0.5 acre. There were four plantings. In September and December 1997 the planting density was ~1,500 plants per acre and, in January 1998, the density was ~500,000 rice plants per acre. The fourth planting was in September 1998 and this trial will continue under 98-225-04N.

Panicle rows of T_1 events were evaluated in the Puerto Rico nursery in the September 1997 planting. Panicles were harvested from plants that survived the herbicide application. T_2 panicle rows were planted in January 1998 to create a number of populations for evaluation. Application of Liberty herbicide has been used to score the rows for segregation for glufosinate resistance. Rows considered to be homozygous (no sensitive plants) were harvested as independent populations and were advanced for variety evaluation in CA.

Panicle rows of additional T_1 events were planted in the December of 1997. Of those plants, which survived the herbicide application, T_2 seed was harvested for further evaluation in the Spring season at LSU.

Liberty Herbicide Tolerance:
Transgenic rice plants treated with Liberty Herbicide exhibited tolerance to the 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 nontransgenic rice in emergence, seedling vigor, stand establishment, lodging or shattering, 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 nontransgenic 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. The 4th planting will be handled in the same manner.

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. Following the harvest of the 4th planting monitoring and destruction of volunteers will be performed in the same manner.

Appendix 2. Background and Summaries of Related Studies

2.a. Long term dormancy study of red rice and rice

2.b. Seed bank dynamics of red rice

2.c. Protocol for rice seed dormancy evaluations

2.d. Outcrossing and introgression study for cultivated rice and red rice: Common Garden Study

2.e. Outcrossing study of transgenic rice and red rice: Interplanted Study

2.f. The *bar* gene in red rice background

2.a. Long term dormancy study of red rice and rice

Goss, W.L. and Brown, E. 1939. Buried Red Rice Seed. J. Am. Soc. Agron. 31: 633-637.

Design:

Seed representing five samples of red rice (three Southern and two Californian samples) and two cultivated rice varieties were buried in three rice growing locations (Beaumont TX, Stuttgart AR, and Biggs CA). Seed samples (200 seed) were mixed with dry soil and placed in clay pots for burial 4 inches under the soil to expose the seed to the moisture and temperature of each locale. Two treatments were imposed; natural rainfall (non-irrigated) and following the irrigation practices typical of the region (irrigated). Seed samples were also buried in sealed, glass jars for exposure to temperature, but not moisture. Samples were retained in the lab under dry storage conditions (a dry afterripening treatment). The seed were buried in the fall of 1930. Samples were exhumed in the spring of 1931, 1932, 1935 and 1937. The samples were shipped to Washington, D.C. for germination testing.

Findings:

After six months of dry storage, all the seed lots had germination of 90% or greater. By the spring of 1931, the commercial rice seed in the field ranged in viability from 0% (non-irrigated conditions) to ~20% (Stuttgart, irrigated pit). By the following Spring, all of the cultivated rice seed were no longer viable. Data from the first exhumation in spring of 1931, show all the red rice samples contained viable seed. The Southern red rices displayed a greater proportion of viable seed. Seed exhumed from the Stuttgart, irrigated pit had the highest viability of all the treatments. The California red rice was 17% viable, the three Southern red rice lots ranged from 67 to 87% viable. Viable seed remained in the Stuttgart, irrigated treatment through the last sample taken in the Spring of 1937. For this last sample, the California red rice lot was 3% viable, the three Southern red rices ranged from 2.5 to 20% viable. Seed viability in the Stuttgart, non-irrigated pit ranged from 0.5 to 1.5% in the last year of the study. At the Beaumont site, only two of the Southern types had remaining viable seed, 2.5% (irrigated) and 0.5% (non-irrigated). At the Biggs site, one of the Southern red rices had 0.5% (1 seed in 200) viable seed in the irrigated treatment at the last exhumation. Both of the California red rices were non-viable at the Biggs site by 1935. Figure 2 compares the loss of viability in the seed bank for three of the red rice lots under irrigated conditions.

Key points:

1. Dormancy can vary with the red rice type and environmental conditions.

2. Under the most conducive conditions of this study, red rice seed remained viable and the seed bank was not depleted after 6.5 years (78 months).

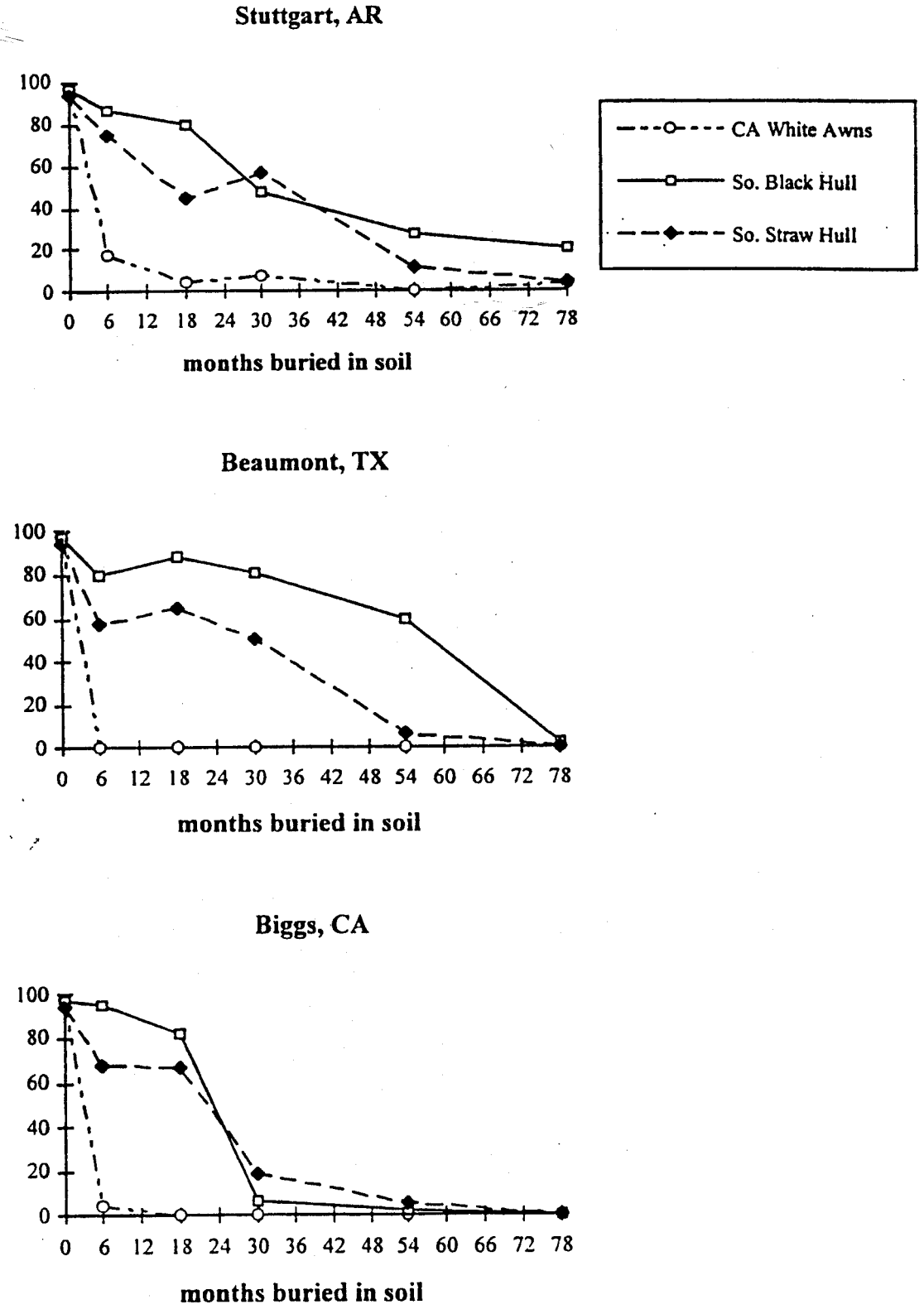
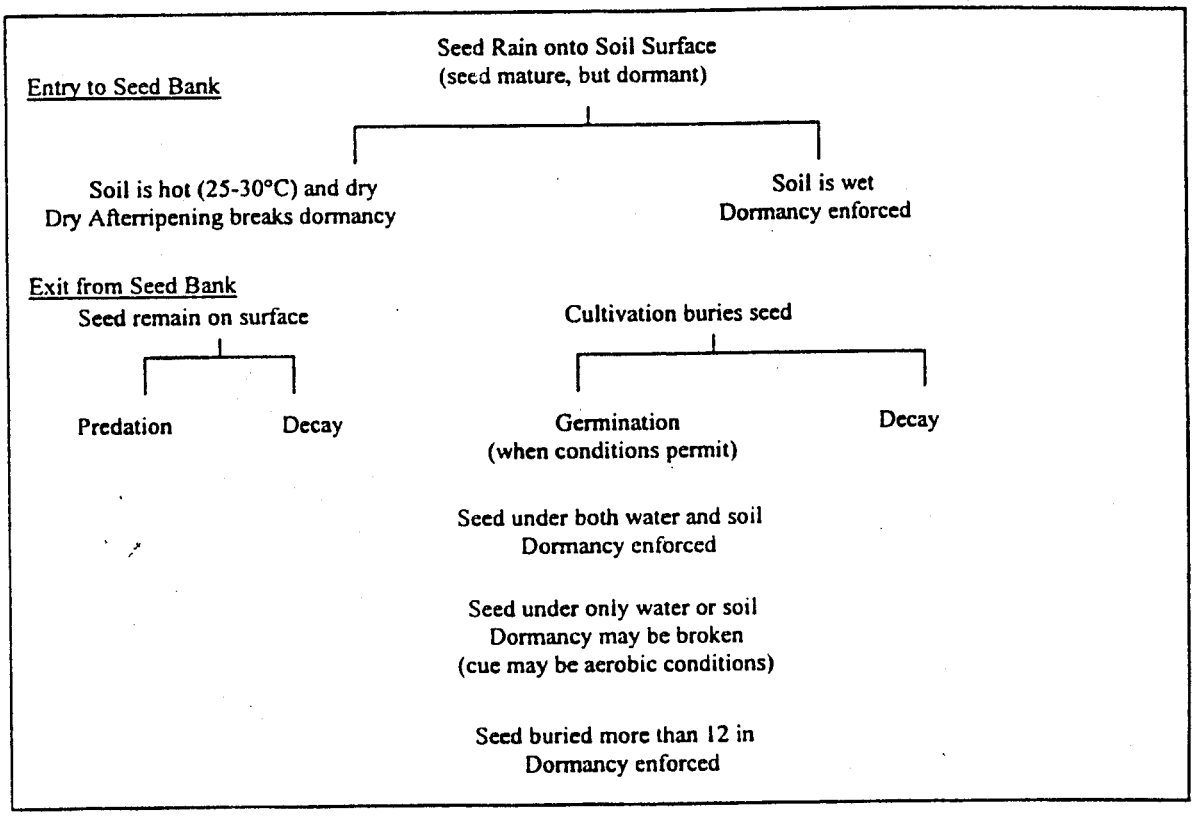


Figure 1.5. Seed bank decay of three red rices buried under conditions of typical irrigation practice (After Goss and Brown, 1939)

2.b. Seed bank dynamics of red rice

Seed banks of red rice are established as seed mature and shatter onto the soil surface. Under cultivated rice field conditions, shattering can occur either while the field is still under flood or as the field is dried in preparation for harvest. Often soil surface conditions at harvest are wet. If the seed remains in moisture, it will likely be dormant. If the seed is dried on the soil surface, it will undergo afterripening and dormancy will be broken. Surface seed may exit the seed bank by germination or predation. Birds digest the seed and are not a factor for red rice seed dispersal.

Figure 3. Seed bank dynamics in red rice



Seed are incorporated into the soil seed bank by cultivation. Red rice seed that is not dormant, can emerge from depths of 12 in.; cultivated rice can only emerge for 4 inch depth (Helpert and Eastin, 1979). When buried in flooded soil, seed can remain viable (dormant) for years. The length of dormancy is effected by both the genetics of the red rice (biotype) and the environmental conditions (personal communication, Mark Cohn, Louisiana State University, Goss and Brown, 1939).

Some of the seed bank characteristics can be used to control red rice and to reduce the resident seed bank. For example, the germination of red rice can be

suppressed by flooding. Agricultural practices, like water-seeded rice, that establish the cultivated rice stand before red rice can germinate have been developed. Red rice can germinate if the flood is lost. Irrigation management practices have used land leveling to allow "pinpoint" flooding , which can adjust the water level as the rice plants elongate, always keeping the soil surface flooded (University of Arkansas, 1992).

Post crop agricultural management practices also use knowledge of the seed bank. Red rice flushes of germination can be stimulated by cultivation, rolling of the soil surface and rainfall. Under fallow conditions, two years of repeated cultivation can deplete the seed bank of red rice (Huey and Baldwin, 1979). Two 3-year rotation cycles (rice, soybean, soybean, rice, soybean, soybean) can also deplete the seed bank of red rice to allow economic rice production from a field in which the red rice infestation was so severe that the red rice competition had prevented any harvest of rice (Sonnier, 1979). Management practices for seed bank depletion must adjust to the agricultural system, weather conditions and dormancy of the red rice.

Key points:

1. Red rice can exhibit prolonged dormancy if seed is buried and undisturbed.

2. Red rice seed under water and soil will not germinate and will either remain dormant or exit the seed bank via decay.

2.c. Protocol for rice seed dormancy evaluations

Dormancy of field-produced seed can be measured under controlled, laboratory conditions. Study designs for the measurement of dormancy in red rice have been developed by Marc Cohn, at Louisiana State University. Physiological studies have shown that dormant red rice seed can be hydrated, and remain viable but not germinate.

Characteristics of dormant and nondormant red rice seed in the process of germination (after Footitt and Cohn, 1992)

	Non-dormant seed	Dormant seed
Phase 1 24 hr/30°C	hydration, water inbibition pH drops	hydration, water inbibition pH remains stable
Phase 2 up to 7d/30°C	metabolic activation, embryo pH decrease, moisture content increase (12 hr) with splitting of pericarp and aleurone	moisture content constant and embryo pH stable
Phase 3	cell expansion of the radicle leads to germination	no activity

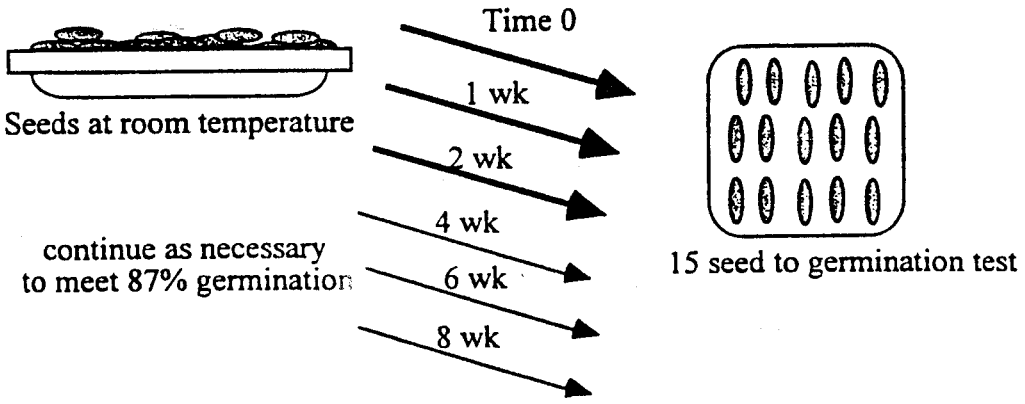
At the time of physiological maturity, all rice seed has some dormancy, that is, it will not germinate until dry afterripened. Harvest of the seed must be accomplished in a manner that does not compromise the dry afterripening process. The dormancy profile will define the duration of dry afterripening required to break dormancy and the proportion of seed that remain dormant or decay rather than germinate.

Seed dormancy profile elements

Dormancy Profile	Parameter to measure
Class 1. Dry afterripening requirement breaks dormancy	Dry afterripening required for seed germination
Class 2. Dormant and likely to germinate in a subsequent season	Non-germinated seed that remains firm in survival stress test
Class 3. Dormant but likely to exit the seed bank by death, not germination	Non-germinated seed that became soft in either the germination test or survival stress test

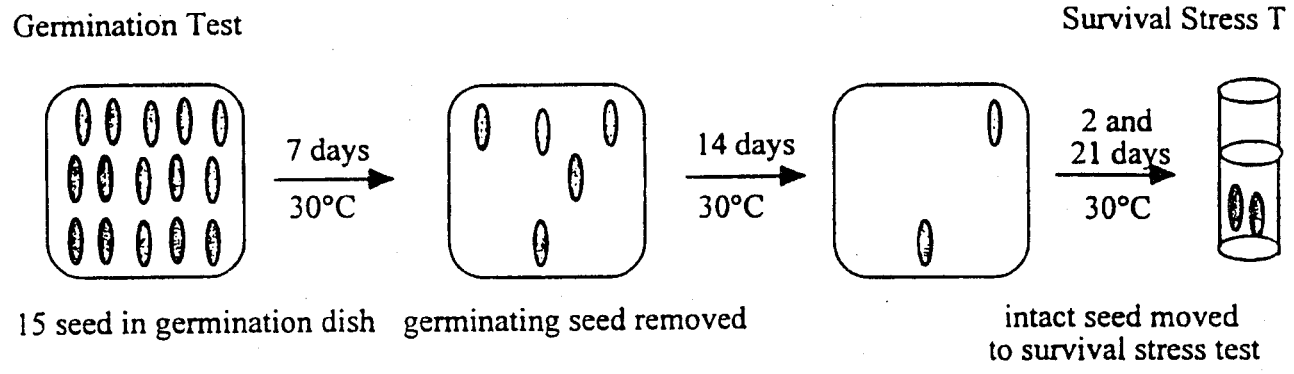
Afterripening test:

Remove seed from the panicle by hand. Do not disturb the seed coat. Remove awns, if present. Transfer seed to containers for dry afterripening. A subsample of 15 randomly selected seeds from each sample was taken for the Time 0 the germination test. The samples remain on the lab bench for dry afterripening treatments of 1 wk, 2 wk, 4wk, 6wk and 8wks. At each time interval, a subsample of 15 seed was removed and tested for germination and dormancy.



Survival stress test protocol:

Seed that have not germinated but remain firm will be transferred into glass vials. Completely submerge the seed with deionized water, the vials will be labeled and returned to the 30°C chamber. Seed will be evaluated after 2 days for firmness. If seed remains firm, the incubation will continue for 3 weeks.



After a final evaluation for seed firmness, the seed will be destroyed or cut into to break dormancy. If the seed can germinate, it will be considered as dormant but able to exit the seed bank via germination. If the seed cannot germinate, it will be considered as dormant and likely to exit the seed bank via death.

2.d. Outcrossing and introgression study for cultivated rice and red rice: Common Garden Study

Langevin, S.A., Clay, K. and Grace, J.B. 1990. The Incidence and Effects of Hybridization between Cultivated Rice and its Related Weed Red Rice (*Oryza sativa* L.). Evolution 44:1000-1008.

Objectives:

1) Determine occurrence and incidence of natural hybridization between cultivated rice and red rice.

2) Determine possible convergence of red rice with cultivated rice.

Design:

In a red rice weed control study at South Farm of the Louisiana Agricultural Experiment Station for Rice Research in Crowley, six rice varieties were planted with high red rice competition (three year study). Botany researchers, Langevin, Clay and Grace sampled the seed of red rice plants in the second year from each of the six variety plots. The red rice seed were germinated and grown out to evaluate for hybrid characters as evidence of introgression.

1985 inter-plant six varieties and red rice
1986 in the fall sample seed from red rice plants growing in fields of six rice varieties (100 plants from each of 4 replicates for each of six varieties, 2,400 plants sampled).
1987 germinate 5 seed from each of the red rice plant sample (100 plants from each of 4 replicates, 2,000 plants per variety)
transplant seedlings to common garden plots, back
evaluate for hybrid characteristics (morphological and isozyme)
red rice and varieties planted as parental controls for comparison

Findings:

1. Frequency of outcrossing

Hybrids formed between red rice and varieties in the field as measured by morphology and isozymes. The frequency of hybrids was highest where the flowering period of the cultivated and red rice had the greatest overlap.

Rice varieties	Days from planting to 50% anthesis	% hybrids
Early season varieties:		
Lemont		1.08%
Saturn		1.38%
Labelle	72-76 (early season)	2.50%
Mars		3.65%
Leah		7.36%
Nortai	82-96 (late season)	52.18%
Red Rice	74-99	

2. Opportunity for introgression

Heterosis was noted for all the hybrids of red rice x early season varieties, suggesting that these hybrids were first generation, F_1 hybrids. This was not the case with Nortai, the hybrids lacked of heterosis, but expressed hybrid isozyme banding patterns, suggesting backcrossed as well as F_1 hybrids were in the red rice seed population. The study suggests that F_2 individuals were identified in the Nortai hybrids, but not the other varieties. This is evidence of introgression of cultivated traits into red rice within 2 seasons of conspecific habitation (growing side-by-side in a cultivated field situation) with a late season variety. No introgression was noted for the early season varieties.

A gap in phenology was observed between the hybrids of the early varieties and red rice, but not for the hybrids made with the one late season cultivar, Nortai. The lack of opportunity for backcrossing in the F_1 population may explain the differences between the early season varieties and the one late season variety in this study.

Hybrids	Days from planting to 50% anthesis	% F_2 hybrids
Red Rice X 5 early season varieties	105-120	none found less than 0.004% (detection level 1 in 25,000)
Red Rice X Nortai	Stated to overlap red rice, but no data given	convergents were found no data given (sample of 2,000 plants)
Red Rice	74-99	

Key points:

1. The degree of outcrossing depends upon phenology (overlap of flowering).

2. Red rice / early season variety hybrids have a flowering period that did not overlap red rice, thus no or very slow introgression.

3. Red rice / late season Nortai hybrids flower coincident with red rice and thus, introgression has been observed within two seasons.

2.e. Outcrossing study of transgenic rice and red rice: Interplanted Study

Source of information:

Personal communication with the senior author, Dearl Sanders, Louisiana State University (LSU), who provided data and an abstract of a presentation made on March 2, 1998 (abstract provided in Appendix 3).
See also the termination and monitoring reports for Louisiana State University field permit 97-029-04R (Appendix 3).

A 1:1 seed mixture of transgenic Cypress and red rice was Interplanted. Germination was uniform and no delay or protracted period of red rice germination was noted. Plots were treated with glufosinate-ammonium at 0, 280, 430, 560 and 840 gm a.i./ha at preflood (3-4 leaf) and postflood (1st tiller) plant stages. Treatments were applied in a randomized complete block design with three replications. Red rice seed was collected from the non-treated and single application plots of the lowest rates. None of the other treatments produced red rice with seed, although some plants did survive.

Treatment with Liberty® Herbicide	%	Red Rice	Cypress	Red Rice hard seed
(gm a.i. / ha)	Control	height (in)	height (in)	per panicle
430 gm preflood	81.3	35.3	36.3	13.0
560 gm preflood	89.7	37.3	36.3	0
840 gm preflood	99.0	34.0	36.3	0
430 gm preflood 430 gm post	100.0	0	36.7	0
280 gm post	86.7	38.0	36.3	11.6
280 gm preflood 280 gm post	99.3	23.7	36.7	0
560 gm post	94.7	35.7	36.3	0
640 gm post	96.3	36.7	36.3	0
Untreated	0	51.7	33.3	44.3

In the untreated plots, the flowering time of the Cypress (50% heading, 7/31/97) and the red rice (7/27/97) overlapped, however the red rice plants were rated as earlier in maturity than the Cypress line. The 50% flowering date for the sub-lethal plots was 8/13/97. Seed were collected from red rice plants in the untreated plots and the sub-lethal plots over a four week period. The sub-lethal plots produced less red rice seed, 12 seed per panicle, compared to untreated red rice, 44 seed per panicle. Seed were dry afterripened until 80% germination was obtained. Plants were tested for herbicide resistance in greenhouse flats (Liberty® Herbicide at 560 gm ai/ ha applied at 2-3 leaf stage). Of the 1,940 seed that germinated and were tested, none survived glufosinate-ammonium application.

2.f. The *bar* gene in red rice background

Sankula, S., Braverman, M. P., and Oard, J. H. 1998. Genetic Analysis of Glufosinate Resistance in Crosses Between Transformed Rice (*Oryza sativa*) and Red Rice (*Oryza sativa*). Weed Technology 12:209-214.

Objective:

Inheritance of the *bar* gene in red rice genetic background.

Design:

Reciprocal crosses between two transformed rice varieties and red rice were made in the greenhouse using hand emasculations. F_1 plants were grown in the greenhouse, one tiller of each was transplanted and all were found to be resistant to 2.2 kg ai/ha glufosinate-ammonium. The main plant produced F_2 seed for testing in the next generation. Seedlings at the 2 leaf stage were treated with 1.1 kg ai/ha of glufosinate-ammonium under greenhouse conditions.

Segregation of glufosinate resistance in four F_2 populations fit a 3 (Resistant) to1 (Susceptible) ratio or 25% susceptible plants. The chi-square values for a 3:1 ratio is significant at the 95% confidence level.

Crosses	R	S	% susceptible	Chi^2 value
Gulfmont x Red	73	29	28%	0.47
Red x Gulfmont	90	30	25%	0.01
Koshihikari x Red	75	22	23%	0.17
Red x Koshihikari	69	21	23%	0.06

Key points:

1. All F_1 plants were resistant to 2.2 kg ai/ha glufosinate-ammonium.

2. F_2 segregation for glufosinate resistance was as predicted for a single locus, simple dominant inheritance. Direction of the cross did not influence outcome. Thus, the bar gene is functional in both red rice and cultivated rice genetic background.

Appendix 3. Expert Letters and Unpublished Reports

Expert Letters:
- Sanders
- Street

Unpublished reports:
- Linscombe, USDA termination reports
- Linscombe, Hybrid Fitness Report
- Rush, Disease evaluation

Abstracts obtained by personal communication:
- Oard, et.al., 1998
- Gealy and Gravois, 1998
- Sanders et.al., 1998
- Schwarzlose, et.al., 1998

Louisiana State University
Agricultural Center
Louisiana Cooperative Extension Service

Mailing Address: P. O. Box 2510(
Baton Rouge, LA 70894-510(

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(504) 388-414
Fax: (504) 388-247(

October 26, 1998

Dr. Sally Van Wert
AgroEvo USA Company
2711 Centerville Road
Wilmington, DE 19808

Dear Sally,

I am fully in support of any type of registration for Liberty on LL Rice. I have devoted a great deal of my time and resources the past five years to the development of Liberty Link Rice or anything that will give Louisiana rice growers some relief from red rice. Due to some unfortunate weather conditions combined with rather low commodity prices Louisiana has a worse red rice problem now than we did ten years ago. It currently is our number one (and probably our second, third, etc.) problem. Our LSU rice breeding program has brought out the most widely grown long grain variety and the second most widely grown medium grain variety in the U.S. We have virtually solved the rice disease problem with new fungicides. We even have finally received a replacement insecticide for Furadan. The bottom line is we have bred the right varieties that we can protect for diseases and insects, but our rice farmers cannot compete with growers from other states (state average yield numbers show this quite plainly). Red rice is currently the primary culprit in our inability to be competitive.

On our side of the project, we have an extensive data base on how, when, and why to use Liberty in Louisiana rice production. In reviewing just my own rather modest Extension program we have completed over 20 Liberty rice trials, both on LSU experiment stations and on growers' fields. We have refined the rates, timing, water management, fertility, pest management, drift potential and just about anything else that can be studied or measured in the use of Liberty on rice. I am particularly proud of our work on the potential for outcrossing, having finished two years of outcrossing trials and so far have found the potential not to be nearly as great as first assumed by many. In my twenty-five years at LSU I cannot think of a single research effort that has been so expansive or intensive. We have devoted the time, facilities, energy and money.

Our rice growers need the support of AgroEvo and the Environmental Protection Agency to bring this project to fruition. Many rice growers will not survive at projected prices and yields currently depleted by red rice. We appreciate your support.

Sincerely,

Dearl Sanders
Weed Specialist
Plant Science Division

DES/bij

Delta Research and Extension Center

Delta Branch Experiment Station
Mississippi Agricultural and Forestry Experiment Station
Division of Agriculture, Forestry, and Veterinary Medicine

Mississippi State University

October 28, 1998

To Whom It May Concern:

I am writing to express my support for the commercial clearance of Liberty Link rice varieties. I have been working with rice weed control research and extension for the past 18 years and Liberty Link technology will be a great boost to the rice industry. I have watched red rice spread throughout the rice growing area of Mississippi even though we were using all tools available to prevent its spread.

Although red rice infestations are not as serious in Mississippi as in other rice growing regions, it has become a serious problem on about 20 percent of our acreage. It occurs as a problem on a much wider area and is rapidly spreading. Most Mississippi rice growers rotate rice fields to soybean for two years to control red rice and this practice has helped reduce the spread of red rice, but it is not good enough. With the seed dormancy of red rice, the only effective control measure must be in both soybean and rice. Liberty Link technology offers the grower that option.

I have some concern for the potential outcrossing of the herbicide tolerant gene into red rice but that chance is very small. I believe even the possibility of outcrossing can be managed to greatly reduce the risk. Even if Liberty tolerant red rice developed, controlling red rice would be no more difficult than it is now.

The benefits of having Liberty Link rice varieties greatly outweigh the risks and I strongly support the clearance of this technology.

If I can provide any additional information concerning rice, please do not hesitate to call.

Sincerely,

Joe Street

Joe E. Street, Ph.D.
Rice Specialist

cc: Chip Morgan
Delta Council

P. O. Box 197 • Stoneville, MS 38776 • (601) 686-9311 • Fax (601) 686-7336

Louisiana State University
Agricultural Center
Louisiana Agricultural Experiment Station

Rice Research Station
Post Office Box 1429
Crowley, LA 70527-1429
(318) 788-7531
Fax: (318) 788-7553

April 3, 1998

Ms. E. Dianne Hatmaker
Chief, Biotechnology Permits
USDA-APHIS-BBEP
4700 River Road, Unit 147
Riverdale, MD 20737-1237

Dear Ms. Hatmaker:

RE: Field Release Reports

In compliance with the provisions of CFR Part 340.4, amended on May 2, 1997, the following field test report is submitted for your review.

APHIS reference number: 97-029-04r (renewal of 93-088-01r).

Methods of observation:
For the breeding nursery sites; plant growth stage, disease incidence and yield component measurements were recorded. For the fitness studies; plant life stage, reproductive traits, disease incidence, fecundity, seed shattering and dormancy were measured. For the red rice control study; herbicide efficacy, flowering delay and outcrossing were accessed.

Resulting data and analysis regarding all deleterious effects on plants, nontarget organisms, or the environment:
In no case were deleterious effects noted. In naturally occurring infestations of Sheath Rot and Leaf Scald, no effect of the transgene was observed.

A listing of field sites is provided in Appendix I. Citations for published reports are provided in Appendix II. A sumary report of the fitness studies is provided in Appendix III. Of particular relevance to the deleterious effects question are the data recorded for the fitness studies. A summary of those findings is provided in Appendix III Table 2.

Sincerely,

Steven D. Linscombe, PhD
Professor

Appendix I. Site locations and field release chronology

Site #1

LSU Breeding Nursery
LSU Agricultural Center
Rice Research Station
Post Office Box 1429
Crowley, LA 70527-1429
Plant: April 24, 1997
Harvest: August 30, 1997

Site #2

Arkansas Breeding Nursery
Rice Research and Extension Center
University of Arkansas
P.O. Box 351
Stuttgart, AR 72160
Plant: May 5, 1997
Harvest: September 19, 1997

Site #3

Eagle Lake Research Station
Texas A&M University
Eagle Lake, TX 77434
Plant: May 19, 1997
Harvest: September 9, 1997

Site #4

Herbicide Efficacy Study
LSU Rice Research Station - South Farm
P.O Box 1429
Crowley, La 70527-1429
Plant: April 30, 1997
Destroyed: June 12, 1997

Site #5

Fitness Study
Central Station- Ben Hur Farm
2410 Ben Hur Road
Baton Rouge, LA 70820
East Baton Rouge Parish, LA
Plant: May 7, 1997
Harvest: October 17, 1997

Site #6

Fitness Study
Southwest Research and Extension Center
362 Highway 174 North, Hope, AR 71801.
501-777-9702 (Extension 102)
Plant: May 13, 1997
Harvest: October 10, 1997

Site #7

Red rice control study
Central Station- Ben Hur Farm
2410 Ben Hur Road
Baton Rouge, LA 70820
East Baton Rouge Parish, LA
Plant: May 15, 1997
Harvest: October 10, 1997

Appendix II. Citations of published reports

Oard, J., Cohn, M., Papenberg, T.S., Linscombe, S., Sanders, D., Griffin, J, Jones,D., and L. Dohenrty. 1998. Louisiana Field Evaluation of Fitness Traits in Crosses Between Red Rice and Transgenic Glufosinate-resistant Rice Varieties. Proceeding of the 27th Rice Technical Working Group Meeting.

Sanders, D.E., Linscombe, S.D., Cohn, M.A. and R.E. Strahan. 1998. Outcrossing Potential of Liberty Link Rice to Red Rice. Proceeding of the 27th Rice Technical Working Group Meeting.

Gealy, D., and K. Gravois. 1998. Arkansas Field Evaluation of Fitness Traits in Crosses Between Red Rice (Oryza sativa) and transgenic glufoninate-resistant rice varieties. Proceeding of the 27th Rice Technical Working Group Meeting.

Linscombe, S. D., et al. 1997. Rice Breeding. Ann. Res. Rpt., Rice Res. Stn., Ls. Agri Exp. Stn., L.S.U. Agricultural Center (in press).

Appendix III. Fitness Study Report

Manuscript in preparation for submission to Molecular Ecology.

Study Title: Fate of Rice-Red Rice Hybrids

Goal: Measurement of fitness under field conditions in an agriculturally managed ecosystem.

Genetics and Breeding:	Steve Linscombe (LSU), Jim Oard (LSU), Kenneth Gravois (UA).
Seed Dormancy:	Marc Cohn (LSU) and David Gealy (USDA)
Agronomic Management	Jim Griffin
Study Location:	LSU Agricultural Center, Central Station - Ben Hur Farm, near Baton Rouge, Louisiana
Plant	May 7, 1997
Harvest	October 17, 1997
Agronomic Management	Kenneth Gravois and Roger Dunham
Study Location:	University of Arkansas Southwest Research and Extension Center, near Hope, Arkansas.
Plant	May 13, 1997
Harvest	October 10, 1997

Test case defined

1. Pollen containing the transgene for Liberty™ herbicide tolerance outcrosses into red rice with an overlapping flowering period.
2. Viable seed are set and shatters before the field is harvested.
3. Hybrid seed survive to produce F_1 plants which self pollinate, set viable seed and shatter creating a seed bank.
4. F2 populations are established in rice production and the trait is provided the opportunity to introgress into red rice.

Questions addressed

- Persistence of the trait in hybrid populations.
- Selective advantage of the trait.
- Weedy potential of the hybrid populations.

Crop-Weed Hybrid Study Chronology

Summer of 1996	Hand cross hybrids between red rice and domestic rice w/wo bar gene in greenhouse
Winter of 1996-7	Hybrid seed increase in greenhouse to produce F_2 populations
Summer of 1997	F_2 populations into field study - two locations, both outside rice production regions - collect relative fitness data - produce seed for dormancy evaluation
Winter of 1997-8	Relative dormancy lab studies

Relative Fitness Study Entries

- 8 F_2 families, 4 red rice and 6 commercial rice entries
- Randomized, complete block design at two locations
- 60 seed per plot, 4 replications per location, 2 locations
- 480 seed per entry

Entry	FEMALE	MALE
1 F_2 family	Transgenic Cypress	Red Rice, Strawhull/Awnless
2 F_2 family	Transgenic Cypress	Red Rice, Strawhull/Awnless
3 F_2 family	Transgenic Bengal	Red Rice, Black/Awned
4 F_2 family	Transgenic Bengal	Red Rice, Strawhull/Awnless
6 F_2 family	Red Rice, Strawhull/Awnless	Cypress
7 F_2 family	Red Rice, Black/Awned	Bengal
9 F_2 family	Bengal	Red Rice, Black/Awned
10 F_2 family	Cypress	Red Rice, Black/Awned
8 standard	Red Rice Black/Awned	
11 standard	Red Rice, Strawhull/Awnless	
12 standard	Red Rice, Strawhull/Awnless	
13 standard	Red Rice, Strawhull/Awnless	
14 standard	Transgenic Cypress	
15 standard	Transgenic Bengal	
16 standard	Transgenic Bengal	
5 standard	Transgenic Bengal	
17 standard	Cypress	
18 standard	Bengal	

- Observational plots for F_2 families with less than 480 seed were planted at Ben Hur. A number of reciprocal crosses were in the observational plots.

Parameters Measured

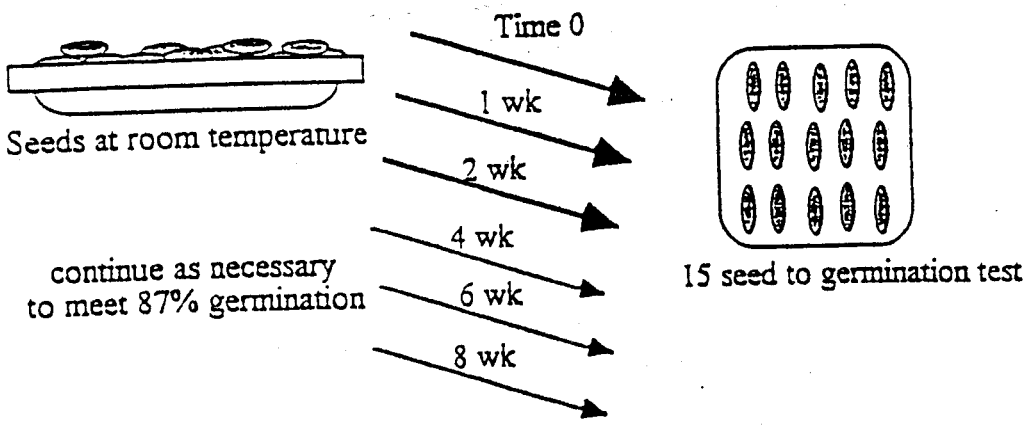
Fitness Trait	Measurements
Life Stage	Germination and stand establishment Rate of growth Vigor Height
Reproductive	Date of first flower Date of 50% flower Date of grain maturity
Harvest procedure	Harvest began when the grains of an entire panicle were mature (golden). A minimum of 4 panicles per plant harvested, 4 plants per replicate for the parameters; dormancy, shattering and fecundity.
Fecundity	Seed per panicle 100 seed weight Empty florets
Shattering	Shatter rating of mature panicles
Dormancy	Dry afterripening Germination rate Survival of imbibed seed (survival stress test)

Dormancy. Seed produced under field conditions is necessary to obtain a dormancy profile of the F_2 populations and the parent lines. At the time of physiological maturity, all rice seed has some dormancy, that is, it will not germinate until dry afterripened. Harvest of the seed must be accomplished in a manner that does not compromise the dry afterripening process. The dormancy profile will define the duration of dry afterripening required to break dormancy and the proportion of seed that remain dormant or decay rather than germinate.

Study evaluated two types of seed dormancy

1. Dry Afterripening

   Meet a required time in dry, warm conditions
   When seed imbibe water, they will germinate



2. Seed Dormancy

   Imbibed seed do not germinate but remain viable.

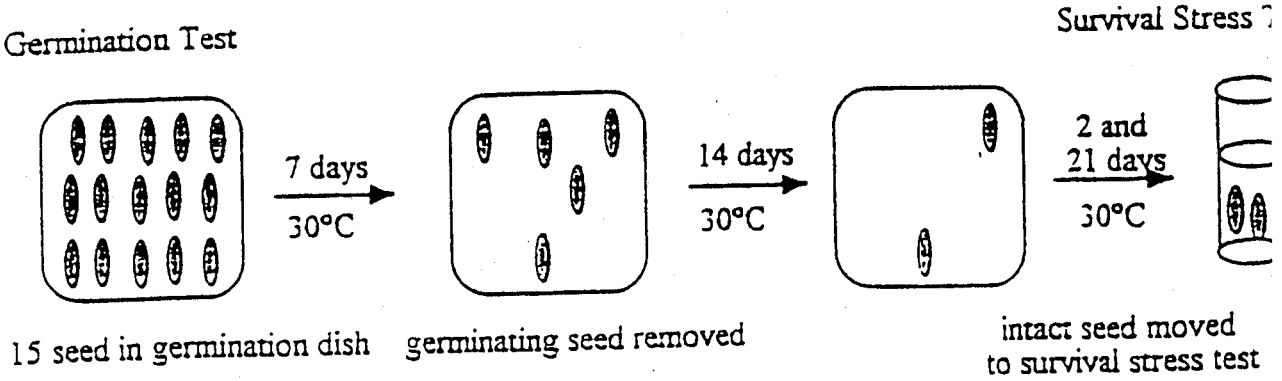


Table 1. Fitness Study Results Summary

Trait	Outcome
Life Stage	Hybrid populations cluster together for life stage traits. Commercial rice cultivars can be separated based upon some of the life stage parameters measured.
Reproductive	Hybrid populations have a wide maturity range which could provide more opportunities for overlapping flowering and outcrossing. Red rice and the hybrid populations had difficulty setting viable seed at the Ben Hur site.
Disease Resistance	For naturally occurring infestations of Sheath Rot and Leaf Scald, no effect of the transgene was observed
Fecundity	No differences observed.
Shattering	Shattering was high in any population with red parents. Mean shatter scores group red as highest, F_2 populations as intermediate and commercial rice in lowest group with little or no shattering.
Dormancy	Dormancy in the red and F_2 populations was higher than in the commercial varieties. Dry afterripening requirements were shorter for the commercial than the red and white-red hybrid populations. Commercial lines lacked dormancy, imbibed seed either germinated or rotted as the testing progressed. Seed of the red and white-red hybrid populations showed either germination or survival under prolonged, imbibed conditions.
Transgenic vs. Non-transgenic	In none of the fitness traits were we able to distinguish populations based on the presence of the *bar* gene, with the exception of tolerance to Liberty ™ herbicide.

Monitoring Plan

The two sites represent a unique opportunity to study the management of red rice in soil that has not been farmed to rice and does not have a preexisting red rice soil seed bank.

- Use best management practices to encourage germination and reduce interring of potentially dormant seed.
- Survey the fallow sites to collect data on volunteers.
- Liberty™ herbicide spray to identify herbicide resistant volunteers.
- Destruction of all volunteers to prevent seed production.

Louisiana State University
Agricultural Center
Louisiana Agricultural Experiment Station

Rice Research
Post Office
Crowley, LA 7
(318)
Fax (318)

October 30, 1998

Ms. Dianne Hatmaker
Chief, Biotechnology Permits
USDA-APHIS-BBEP
4700 River Road, Unit 147
Riverdale, MD, 20737-1237

RE: Field Release Monitoring Reports
APHIS reference number: 97-029-04r

Dear Ms. Hatmaker,

Submitted for your review are reports of our 1998 season monitoring efforts associated with glufosinate tolerant rice and red rice field studies completed in 1997. The studies were conducted on the Louisiana State University, Agricultural Center, Central Station-Ben Hur Farm located in East Baton Rouge Parish, LA and the University of Arkansas, Southwest Research and Extension Center located in Hope, AR.

In summary, best management practices were used to control the seed remaining at the field sites from all three rice types, red rice, hybrid populations and crop rice. Monitoring of the sites after each rainfall or irrigation that would be sufficient to stimulate germination recorded volunteers in the early spring. Some of the plants survived a Liberty® herbicide treatment and tissue samples were taken to confirm presence of the bar gene. Following the first germination flush, no subsequent volunteers were noted for the remainder of the 1998 growing season. After completing one year of monitoring the sites for persistance of crop/weed hybrid seed, we have observed no reason to prevent research in regions where outcrossing may occur.

Sincerely,

Steven D. Linscombe
Professor

Monitoring Report
Reference: USDA-APHIS 97-029-04r
Prepared by Dr. Steve Linscombe, LSU and Dr. David Gealy, USDA-ARS
October 30, 1998

Introduction

Management of potential gene flow of herbicide resistance genes from commercial rice into its companion weed, red rice was the topic of studies in 1997 located on the Ben Hur Farm and the Hope Research Station. The two sites represent unique opportunities to study the management of red rice in soil that has not been farmed to rice and does not have a preexisting red rice seed bank. Standard agronomic management practices were adapted for use in the small plots. Upon termination of the 1997 studies, the red rice study sites were subjected to best agronomic practices and monitored in 1998 for volunteers. In summary the plan was to:

1. Use best management practice to encourage germination and reduce interring of potentially dormant seed.
2. Survey the fallow sites to collect data on volunteers.
3. Use glufosinate ammonium herbicide spray to identify resistant volunteers
4. Destruction of all volunteers to prevent seed production.

Termination of the Fitness Study Site -LSU

The year following the field study of the rice-red rice hybrid populations allows the testing of best agronomic practices to control a "worst case" scenario, a high population of red rice tolerant to the herbicide, Liberty™ surviving harvest of a cultivated rice field. Dr. Steve Linscombe directed the monitoring study. Dr. James Griffin provided the agronomic management and weed science expertise.

1997- destruction of the plant material

The final harvest was made on October 17 and the remaining plants were cut with a cycle bar mower on November 25, when the weather permitted entry into the field with the equipment. All plant material, with the exception of the seed samples, remained at the site. The biomass was lightly incorporated into the soil. The levies remained intact to avoid any inadvertent seed burial and to allow standing water that may accumulate by the winter rainfall. The only seed removed from the site were those harvested by hand and placed in envelopes in the field plots. The envelopes were opened and seed processed in the laboratory inspected by the USDA and viewed as acceptable for transgenic seed work.

1998-observations

Field was irrigated with a single flush to stimulate germination of rice seed in June. Germination of rice was observed and the field was sprayed with glufosinate ammonium at the anticipated label rate (400 gm/ha). Survivors were observed in patches in the area above the levy and in a more continuous pattern below the levy. Red rice survivors were expected, seed shattering was observed in the F_2 families and seed remained in the field. Leaf samples were taken from five surviving plants from six of the patches. Samples were stored in a freezer and will be tested for presence of the bar gene. Best management practices were used to remove the surviving red rice plants before flower, shed pollen and/or set seed. Second irrigation applied in early July. The site was monitored in July, August and September. No additional rice volunteers were observed.

1999-management plan

Monitoring for rice volunteers and/or red rice will continue in the 1999 season following each significant rainfall or irrigation where volunteers may be expected to germinate. The field site will be planted to soybean using practices typical for crop rotation plans of rice and soybean production.

Termination of the Sub-lethal Liberty™ Herbicide Study Site

In this study, a 1:1 seed mixture of Liberty™ Link rice, cv. Cypress and red rice. Germination was uniform and no seed dormancy was noted. The flowering time overlapped for the crop (50% heading, 7/31/97) and the red rice (7/27/97). The chance of setting F_1 seed in the untreated check (UTC) plots was excellent. Seed were collected from the untreated, control plots and the sub-lethal plots for greenhouse germination and herbicide treatment. Of the 1,940 seed which germinated, none survived glufosinate ammonium application. The germination rate was 80%. The 50% flowering date for the sub-lethal plots was 8/13/97, a delay sufficient to prevent cross-pollination.

1997- destruction of the plant material

Following hand harvest of seed in selected areas, the study was mowed and the biomass left at the site. The site was treated as the adjacent fitness study plots.

1998-observations

As was the case for the adjacent fitness study plots, some rice and red rice seed did germinate following the June irrigation; however, none survived the glufosinate ammonium application. No additional volunteers were observed in the rest of the season.

Termination of the Fitness Study Site -Arkansas

The termination and monitoring for the Southwest Research and Extension Center in Hope Arkansas was managed by Dr. Mike Philips (center director) and Roger Dunham (research specialist). Dr. David Gealy, USDA-ARS Stuttgart AR coordinated the collection of monitoring information.

1997-98-management of the fallow site

Following the last seed collection for dormancy testing on October 10, 1997, the remaining plant material was cut and left in the field to dry. In order to enhance burning, some old hay was placed on top of the test site. On December 10, 1997, rice stubble at the test site was burned. The levees remained standing and the soil surface was undisturbed except for a light cultivation operation to stimulate germination in the early spring of 1998. The surrounding area is pasture used primarily for hay production. Therefore, the site can remain undisturbed and will be monitored in 1999 after each germination flush. The field will be sprayed with glufosinate ammonium herbicide and a census taken in each of the defined quadrants. The census data will quantify the depletion of the red rice seed in the field test site

1998-observations

On May 25, 1998, glyphosate was applied to remove weeds and allow rice seed remaining on the soil surface to germinate. On July 10, 1998, rice volunteers were observed and glufosinate ammonium was applied (Liberty at 1 pt/acre). Survivors were counted and leaf samples taken on July 15 and on July 17 all surviving rice plants were removed by hand. Survivors were noted in patches and leaf samples were taken from six

quadrants for analysis. These six quadrants (3-x 3-ft) were sprayed a second time on October 1 and no additional volunteers were noted.

Quadrant	Original Plot ID	Survivors following 7/15 spray	Survivors following 10/1 spray
1	104	6	0
2	302	9	0
4	305	6	0
5	603	10	0
6	606	9	0

Consequence of hybrids between transgenic herbicide tolerant rice and its related weed, red rice (*Oryza sativa* L.)

Data provided by

Dr. Steve Linscombe, Dr. Jim Oard, and Dr. Marc Cohn,
Louisiana State University Agricultural Center

Dr. David Gealy, USDA-ARS, National Rice Germplasm Center

Provided to AgrEvo for the sole use of reporting to the USDA

Statistical analysis by AgrEvo

October 30, 1998

Manuscript for publication in preparation.

Objectives of the Study

To address the potential consequence of gene flow from herbicide tolerant rice into red rice, this study was undertaken to measure the relative fitness of hybrid populations under the field conditions typical for rice production. The rice breeding program at LSU has developed several lines of rice containing the glufosinate tolerant gene, know as the *bar* gene (Oard, et al., 1996). Characterization of these lines has shown that if not properly managed, rice pollen carrying the bar gene may cross into red rice.

In this study fitness characteristics that could effect the persistence of crop genes in red rice were evaluated. The characteristics included life stage, fecundity, shattering and seed dormancy. To evaluate the fitness of red rice containing the bar gene, crosses to red rice were made and the resulting hybrid seed were increased under contained greenhouse conditions. F_2 seed populations were evaluated under field conditions for life stage fitness and seed characteristics.

Field sites in Louisiana and Arkansas were chosen to reflect the typical environment of rice production, but as a biological safeguard the sites were selected to be outside the commercial rice growing regions. Seed dormancy characteristics were measured under laboratory conditions. Field sites were monitored in the following year for occurrence of volunteers. Agronomic practices common for rice agriculture were successfully used to control hybrid volunteers.

Materials and Methods

Hybrid populations Greenhouse crosses were made in summer of 1996 with Bengal and Cypress (transgenic and the parental lines) and red rice lines collected from a local population. Cross combination were opportunistic and manual emasculations were performed in the early morning to prepare the female recipients before pollen shed. Fertile crosses were obtained and F_1 seed was planted for greenhouse seed increase over the winter of 1996-97. F_2 seed was harvested in the spring of 1997 and prepared for field planting (Table 1). All pollination and seed increases were carried out in the contained greenhouse facility at LSU Agricultural Center, Rice Research Station in Crowley, LA. Appropriate authorizations were obtained from the USDA-APHIS for all environmental releases (field planting) of the transgenic rice seed.

To reserve as much F_2 seed for planting as possible, a small lot germination test was performed at the Crowley station using standard 7 day germination test of 10 seed. Seed packets were prepared for planting the plots; each packet contained 60 seed. 18 entries were planted for the test at two locations with 4 replicated plots for each entry in a randomized, complete block design (RCBD). Each plot was planted with 60 seed (6 rows per plot, 10 seed per row). The plot dimensions were 2 by 1.33 meters. The seeding rate provided sufficient space between plants to allow individual plant measurements.

Eight entries represent seed from F_2 families of white and red rice crosses; four with transgenic white rice and four with non-transgenic white rice varieties. Two white rice varieties, Bengal and Cypress, both transgenic and non-transgenic, and two biotypes of red rice, which had the phenotypes; strawhull/awnless and black hull/awned were included. Table 2 provides a list of entries and the genotypes of parents used for the hybrid crosses. Entry 5 represents a transgenic derived line that was not selected from a population for agronomic characteristics, and thus was not included in the contrast analysis. Seed lots of lines for which insufficient seed (less than 480 seed) was available for the RCBD test were planted at one site for limited observational data.

Table 1. Crop-Weed Hybrid Study Chronology

Summer of 1996	Hand cross hybrids between red rice and domestic rice with and without bar gene in greenhouse
Winter of 1996-97	Hybrid seed increase in greenhouse to produce F_2 populations
Summer of 1997	F_2 populations into field study - two locations, both outside rice production regions - collect relative fitness data - produce seed for dormancy evaluation
Winter of 1997-98	Relative dormancy lab studies
Summer of 1998	Monitoring field sites for persistence of volunteers

Table 2. Entries planted for the Fitness Study

Provided are entry number and the parents of the seed planted.

ENTRY		FEMALE	MALE
1	F_2 Family	Transgenic Cypress	Red Rice, Strawhull/Awnless
2	F_2 Family	Transgenic Cypress	Red Rice, Strawhull/Awnless
3	F_2 Family	Transgenic Bengal	Red Rice, Black/Awned
4	F_2 Family	Transgenic Bengal	Red Rice, Strawhull/Awnless
6	F_2 Family	Red Rice, Strawhull/Awnless	Cypress
7	F_2 Family	Red Rice, Black/Awned	Bengal
9	F_2 Family	Bengal	Red Rice, Black/Awned
10	F_2 Family	Cypress	Red Rice, Black/Awned
8	standard	Red Rice, Black/Awned	
11	standard	Red Rice, Strawhull/Awnless	
12	standard	Red Rice, Strawhull/Awnless	
13	standard	Red Rice, Strawhull/Awnless	
14	standard	Transgenic Cypress	
15	standard	Transgenic Bengal	
16	standard	Transgenic Bengal	
17	standard	Cypress	
18	standard	Bengal	

Field site management The field release sites were located on the LSU Agricultural Center, Central Station - Ben Hur Farm, near Baton Rouge, Louisiana and the University of Arkansas Southwest Research and Extension Center, near Hope, Arkansas. As a safeguard, the locations were selected to be outside the commercial rice production areas of the respective states. The field sites were treated as managed, agro-ecosystems. Soil preparation, irrigation and weed competition management were typical for the rice agricultural systems of Louisiana and Arkansas. Both sites received a full complement of herbicide treatments applied at registered label rates and recommended timing.

The Ben Hur site was planted on May 7, 1997 and the Hope site on May 14, 1997. Bird netting was installed to enclose the site when the plots were at 50% heading . Harvest began when panicles were at maturity. The harvest window for Ben Hur was August 22 to October 17. At the Hope site two harvests were made; September 25 and October 10. The bird netting was removed after the final harvest.

The Ben Hur site was mowed on November 25; the first opportunity to enter the field due to wet conditions. All plant material, with the exception of the seed samples, remained at the site. The biomass was lightly incorporated into the soil. The levies remained intact to

avoid any inadvertent seed burial and to allow standing water that may accumulate by the winter rainfall. At Hope, the remaining plant material was cut and left in the field to dry on October 14, 1997. Dry hay was placed on top of the test site and on December 10, 1997, rice stubble at the test site was burned. The levees remained standing and the soil surface was undisturbed except for a light cultivation operation to stimulate germination in the early spring of 1998. After the first recorded germination of rice volunteer, six quadrants (1 meter square) were established to include patches of volunteers. The site remained fallow and undisturbed. After each germination flush, the field was sprayed with glufosinate-ammonium herbicide and a census taken in each of the defined quadrants. The census data will quantify the depletion of the red rice seed in the field test site. The plots will be monitored until volunteers are no longer observed.

Fitness Parameters Measured Parameters indicative of plant life stage and reproductive traits, disease incidence, fecundity, seed shattering and dormancy were measured. Table 3 provides a summary listing of the measured traits. Emergence counts were taken on at least two dates. Each plot was planted with 60 seed. All emerged seedlings in each plot were counted for each date. Vigor ratings were taken by Steve Linscombe at the Ben Hur site using plant breeding criteria, rating of 1 to 9, with 1 being the best rating. Plant height was measured from the soil line to the tip of the flag leaf on 4 plants in each plot at the Ben Hur site. The date of first tiller in each plot and date on which the plot reached 50% tiller were noted. For each plot, the date of first heading, the date of 50% heading and the date of last heading were recorded. Heading was defined as panicle is completely emerged from the boot. Disease ratings using a continuum scale of 1= no disease and 4= severe disease incidence were made by plant pathologist, Chuck Rush, for two naturally occurring infestations of sheath rot and leaf scald. Populations were evaluated for the segregation of two traits by Jim Oard, rice geneticist. Liberty® Herbicide resistance was tested by painting one leaf from 15 plants from each plot for each entry with 400 gm/ acre commercial formulation of Liberty® Herbicide. At the same time, the genetic character, leaf pubescence was scored. Leaf pubescence is a diagnostic character for red rice weed identification.

At physiological maturity, the kernel hull color of the top half of the panicle is devoid of green color and displays a yellowish cast and/or incipient shattering is observed. From plants judged to be at physiological maturity, two sets of samples were taken, one for seed dormancy and one for fecundity and shattering evaluation. Each sample set consisted of four mature panicles per plant and at least four plants were sampled per plot. Panicle samples were collected in the field, placed in bags and transported to the seed lab for evaluation. Special care was taken to hold sample bags under ambient conditions (e.g., any dry shady area) to minimize excessive weather- or seed-related heating until ready for transport to the laboratory.

In the procedure for shatter rating practiced at LSU, the four panicle sample was removed from its envelope, rapped against the side of a bucket and the a rating for shattering given based upon the percentage of seed remaining attached to the panicle. If no seed were shattered, the score was 0. A score of 1 indicated slight shattering, a score of 2 was indicative of 30 to 60% shattering and a score of 3 complete shattering, little or no seed remained on the panicle. At the seed lab in Stuttgart, the four panicle sample remained in the envelope and was rapped against the side of the lab bench. The panicles were removed and weighed. The seed envelope was weighed and a ratio of shattering was calculated. Following shatter evaluation, the panicles were stripped by hand into a bucket, all seed returned to the sample envelope and allowed to dry for three days at 50°C. The seed were then passed through a small seed lot thresher to separate the blank florets from the seed. Both empty floret and total seed weight were obtained. A sample of 100 seed was weighed and the total number of seed was calculated. A standard 100

empty floret weight was used to calculate the number of florets that did not produce seed. Observations were recorded for the genetic characters of hull color and awn presence.

Seed Dormancy Evaluation Using care not to disturb the seed coat, individual dispersal units (seed) were removed from the panicle by hand. In the cases where awns were present, the awn was removed. Seed samples were transferred to containers for dry afterripening. A subsample of 15 randomly selected seeds from each sample was taken for the Time 0 in the germination test. The samples remained on the lab bench for dry afterripening treatments of 1 wk, 2 wks, 4 wks, 6wks and 8wks. At each time interval, a subsample of 15 seed was removed 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. Seed were incubated at 30°C, high humidity and no illumination. Germination was scored at 7 and 14 days. Data collected at each evaluation included; 1) number of seed in dish, 2) number of seed germinating (at least 1 cm of root or shoot emerged from seed coat), 3) number of seed not germinating, and 4) number of non-germinating seed that are firm. Firmness was determined by a gentle touch by forceps across the breadth of the endosperm. If the seed yields, it is 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. Seed 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. The seed were completely submerged with deionized water and returned to the 30°C chamber. Seed were evaluated after 2 days for firmness. If seed remained firm, the incubation continued for 3 weeks and seed were tested for firmness again.

Statistics All life history trait analysis were completed on plot means or values. The minimum model used for all analysis of variance comprised entries and replications, with the entry*replication interaction used as the error term. Replications and the entry*replication interaction were considered random effects. Analysis of variance was completed using JMP® (SAS Institute Inc. 1994). The tests of the full germination data were done on individual plants, using SAS PROC GLM, using the repeated measures analysis. Contrasts between weeks (0-8) and days (7 & 14) were done using a polynomial function. Generally, replicate effects were not significant.

Table 3. Summary of parameters evaluated

Fitness Trait	Measurements
Life Stage	Emergence and stand establishment Rate of growth (first and 50% tillering date) Vigor Height
Reproductive	Date of first flower (heading) Date of 50% and last heading Date of grain maturity
Disease resistance	Severity rating (LSU only, sheath rot and leaf scald)
Harvest procedure	Harvest began when the grains of an entire panicle were mature (golden). A minimum of 4 panicles per plant harvested 4 plants per replicate for the parameters; dormancy, shattering and fecundity.
Fecundity	Seed per panicle 100 seed weight Empty florets
Shattering	Shatter rating of mature panicles
Dormancy	Dry afterripening Germination rate Survival of imbibed seed (survival stress test)
Persistence	Census of volunteers in subsequent season

Results

Emergence and Vigor Evaluation

Mean vigor rating for entries was correlated with that of June 12 emergence (r—0.79). Thus, the ANOVA for vigor rating was computed with the June 12 emergence as a covariate. With the adjustment for the June 12 emergence, differences among the entries were non-significant (p=0.34). The correlation between vigor and emergence is negative; so the lower the germination, the greater the plants were rated for vigor. Entry 5 is a clear outlier in the data, with very low germination (14%) and very high vigor rating (7.5). However, with entry 5 removed, the correlation remains quite low (-0.61) and statistically significant. With entry 5 removed from the ANOVA, adjusted differences among entries are marginally significant (p=0.033), with entries 15,17 and 2 having the highest adjusted vigor (6.9, 5.8, and 5.8, respectively) and entry 14 the lowest (0.85).

Final Height

As with vigor rating, heights were correlated with the June 12 germination (r=0.76). With the June 12 germination considered as a covariate in the ANOVA of height, none of the effects were significant.

Life History Traits

Tiller and heading date measurements were correlated, and thus were analyzed by a multivariate analysis of variance (MANOVA). Differences among replications and entries were significant (p=0.0002 and p <0.001, respectively). Differences among replications were based on the first and 50% tiller dates and primarily differentiated the first replication from the others. Differences among entries fell into two patterns: The first, canonical vector (Can Var 1) represents the first and 50% heading dates and, weakly, the 50% tiller date (Table 4). The second canonical vector (Can Var 2) represents the last heading date, moderately and negatively, the first tier date, and weak and negatively, the 50% tiller dates. This means that, as entry scores for the second vector increase, the last heading date increases, while the first and 50% tiller dates tend to decrease (see Figure 2).

Table 4. Correlations of entry means between original tiller and heading measures and scores of canonical variables 1 and 2

Variable	Can Var 1	Can Var 2
Can Var 1	1.0000	0.0084
Can Var 2	0.0084	1.0000
Tiller Date, first	0.1635	-0.6705
Tiller Date, 50%	0.3937	-0.4421
Heading Date, first	0.9704	-0.1653
Heading Date, 50%	0.9527	0.2644
Heading date, last	0.1694	0.9450

Eight contrasts were tested with these data; between transformed and nontransformed standard rice parents (entries 14, 15,16 vs. 17,18), between the Bengal and Cypress derived lines (entries 15, 16, 18 vs. 14, 17), between transformed and untransformed crosses (entries 1-4 vs. 6, 7, 9, 10), between crosses of Bengal and Cypress parents with red parents (entries 3, 4, 7, 9 vs. 1, 2, 6, 10) and two in which the Bengal and Cypress varieties were separated for the crosses as those transformed (entries 3, 4 vs. 1, 2) and not transformed (entries 7, 9 vs. 6, 10). In addition, two contrast groups were tested in which red and white rice were separated as non-crossed (entries 8, 11,12, 13 vs. 14,15, 16,17, 18) and crossed (entries 8, 11,12, 13 vs.1-4, 6, 7, 9, 10).

Table 5. Tested contrasts in the MANOVA of life history traits

Contrast	F	Num df	Den df	prob>F
Parents: Transformed vs. Untransformed	0.72	5	32	0.6155
Parents: Bengal vs. Cypress	13.25	5	32	<0.0001
Crosses: Transformed vs. Untransformed	14.24	5	32	<0.0001
Crosses: Bengal vs. Cypress	1.26	5	32	0.3030
Transformed crosses: Bengal vs. Cypress	1.31	5	32	0.2859
Untransformed crosses: Bengal vs. Cypress	2.42	5	32	0.0573
Red vs. White	12.79	5	32	<0.0001
Red vs. Crosses	28.30	5	32	<0.0001

The transformation had no effect on parental responses in this study (Table 5 and Figure 2). However, the Cypress parents (entries 14 and 17) headed later than the Bengal parents did (entries 15, 16, 18), but tended to tiller earlier. The life history responses for the Cypress standards tended to be similar to those of the red rice standards (entries 8, 11-13) (Fig. 1). Transformed and untransformed crosses were significantly different, in contrast to their domestic rice parents (Table 5): The transformed crosses tended to head earlier than the untransformed ones. Differences between crosses of the domestic rice parents were not significant (Table 5), due primarily to the heterogeneity of the untransformed crosses (Table 5). When the transformed crosses of the domestic rice parents were compared, these were significantly different (Table 5). Differences on the basis of the last heading date were similar to those of the original parents; those crosses made with Cypress parents headed later.

There was a distinct tendency for the F_2 populations, especially those from the transformed parents, to head early, yet the last heading was late. Variation in the populations was observed for maturity, and thus lower overall fecundity.

Disease

For naturally occurring infestations of sheath rot and leaf scald, no effect of the transgene was observed. There are significant differences in the disease ratings. The white rice tends to be above average in both traits; some of the hybrids and one of the reds tend to be below average for both. Most of the reds tend to have low scores for sheath rot and high scores (severe disease) for leaf scald.

Seed Shattering Analysis

Data from both locations were combined. The shattering ratios calculated for the Arkansas samples were grouped into four classes for comparison with the Louisiana data. The combined data was analyzed for correspondence (Figure 3). The commercial rice lines, both transgenic and nontransgenic (entries 5, 14,15, 16, 17, 18), were clustered about the zero class score, indicating no shattering, and located in the lower quadrants. Red rice lines (entries 8, 11,12, 13) are clustered in the upper right quadrant with the highest shattering rate (score of 3). Hybrid families (1-4, 6,7, 9, 10) are clustered in the upper left quadrant, with intermediate shattering scores (1 and 2). Strong correlation is shown between the three groups and the amount of shattering recorded. The probability that entries are the dominant effect is <0.000. These is also a location by entry interaction, but the relative shattering ranking remains the same for both sites.

Seed Dormancy Results

At the time of physiological maturity, all rice seed has some dormancy, that is, it will not germinate until the dry afterripening requirements are met. There are genetic and environmental components for seed dormancy. Thus, to obtain a dormancy profile of the F_2 populations and the parent lines, seed was produced under field conditions evaluated. The laboratory tests of the harvested seed samples describe the duration of dry afterripening required to break dormancy and the proportion of seed that remain dormant or will decay rather than germinate.

Table 6. Seed dormancy profile elements

Dormancy Profile	Parameter to measure
Class 1. Dry afterripening requirement breaks dormancy	Dry afterripening required for seed germination
Class 2. Dormant and likely to germinate in a subsequent season	Non-germinated seed that remains firm in survival stress test
Class 3. Dormant but likely to exit the seed bank by death, not germination	Non-germinated seed that became soft in either the germination test or survival stress test

Germination and Dry Afterripening Evaluation

The analysis of the germination data used a repeated measures analysis of variance on entries and replications. This method is suited to data in a time series of germination rates by date; these rates are thus correlated. The repeated measures analysis of variance is a multivariate analysis of variance, whereby differences among effects and the date by effect interactions are tested. The latter tests heterogeneity in germination rates for a given effect. Overall differences for entries were significant ($p<0.0001$), however replications were not significant ($p=0.18$).

Figure 1 illustrates some of the differing germination response curves observed at the Ben Hur Farm. The highest average germination rate was for entry 3, one of the hybrid families, while the lowest germination (and the greatest percentage of soft seed) was for entry 5, one of the transgenic lines. The interaction between date and entry is significant ($p<0.0006$), while the date by replication interaction is not significant (($p<0.08$). This interaction is illustrated again by entries 3 and 5; the germination rate for entry 3 is much greater than for entry 5. Had the main effect for entries been significant, but the interaction not, the means for entries 3 and 5 would have differed, but the response curves would be parallel. Another type of interaction is illustrated by entry 11 (a red rice standard) and 16 (a transgenic crop rice standard), whereby the forms of the respective response curves differ (Fig. 1).

Contrasts were tested under the date by entry interaction: 1) between transformed and nontransformed standard rice parents (entries 14, 15, 16, vs. 17, 18). 2) Between the Bengal and Cypress derived lines (entries 15, 16, 18 vs. 14, 17). 3) Between transformed and untransformed crosses (entries 1-4 vs. 6, 7, 9 10). 4) Between crosses of Bengal and Cypress parents with red parents (entries 3, 4, 7, 9 vs. 1, 2, 6, 10). In addition, two contrast groups were tested in which red and white rice were separated as non-crossed (entries 8, 11,12, 13 vs. 14,15, 16,17, 18) and crossed (entries 8, 11,12, 13 vs.1-4, 6, 7, 9, 10).

Thus, although germination rates among entries differed significantly, there were no consistent patterns by parentage and transformation. However, when red rice was included in the genetic background, the dry afterripening requirement and length of dormancy was always greater. Significant contrasts were found between red rice and the crop, white rice (Table 7).

Table 7. Contrast analysis for germination at Ben Hur Farm

Contrast	F	Num df	Den df	prob>F
Entry:				
Parents: Bengal vs. Cypress	38.4720	1	46	<.0001
Red vs. White	105.0930	1	46	<.0001
Red vs. Families	0.1166	1	46	0.7343
Week * Entry:				
Parents: Transformed vs. Untransformed	1.9921	5	42	0.0996
Parents: Bengal vs. Cypress	1.5326	5	42	0.2004
Crosses: Transformed vs. Untransformed	1.3630	5	42	0.2576
Crosses: Bengal vs. Cypress	4.6061	5	42	0.0019
Red vs. White	7.4367	5	42	<0.0001
Red vs. Families	4.4319	5	42	0.0025

For the seed produced at Hope, any entry with red background required at least one week of dry afterripening to reach 20% germination. All crop rice, transgenic or not, had in excess of 20% germination without any period of dry afterripening treatment in the laboratory. After two weeks of dry afterripening, the crop-weed hybrid populations have less germination than their crop rice parents. Red rice in the genetic background is the factor that accounts for seed dormancy, not the presence of the transgene.

The commercial rice lines (15, 16, 17, 18) have high germination and low dormancy. Commercial rice lines 5 and 14 have low germination and low dormancy. For the commercial rice lines, seed that does not germinate is more likely to be soft and thus non-viable than dormant (Class 3). Red rice and the hybrid families have a high number of individuals with dormancy requirements that are met by dry afterripening (Class 1) and a high number of seed that are likely to remain dormant and germinate in a subsequent season (Class 2). The F_2 families have dormancy characteristics similar to red rice, but no worse.

Table 8. Contrast analysis for germination at Hope

Contrast	Exact F	Num df	Den df	prob>F
Entry:				
Parents: Bengal vs. Cypress	22.3086	1	51	<.0001
Red vs. White	77.2711	1	51	<.0001
Red vs. Families	79.3772	1	51	<.0001
Week * Entry:				
Parents: Transformed vs. Untransformed	13.9097	5	47	<.0001
Parents: Bengal vs. Cypress	13.5098	5	47	<.0001
Crosses: Transformed vs. Untransformed	1.0202	5	47	0.4166
Crosses: Bengal vs. Cypress	2.4733	5	47	0.0454
Red vs. White	11.5863	5	47	<.0001
Red vs. Families	18.4398	5	47	<.0001

Persistence of the hybrid populations Best management practices were used to control the seed remaining at the field sites from all three rice types, red rice, hybrid populations and crop rice. The two sites were monitored after each rainfall or irrigation that was sufficient to stimulate germination and volunteers were recorded in the spring. Six, one meter quadrants were established, each containing volunteers. Glufosinate ammonium herbicide was applied to the volunteer plants in each quadrant. Of the 6 to 10 plants which survived herbicide treatment in each quadrant, tissue samples were taken to confirm presence of the bar gene via pcr analysis. Following the first germination flush, no subsequent volunteers were noted for the remainder of the 1998 growing season.

Summary of Findings

Likelihood of hybrid populations becoming established in agriculture

The avenue for hybridization between cultivated rice and red rice is the same for either transgenic or non-transgenic rice. We observed no preferential behavior in the success of hand-crossed pollinations or seed production in the greenhouse crosses. The glufosinate tolerant gene did express tolerance to glufosinate ammonium herbicide in a red rice background.

No correlation was observed between the presence of the bar gene, the glufosinate resistance phenotype, and any adaptive trait, except herbicide tolerance. The differences identified by statistical analysis can be attributed to the contribution of red rice vs. commercial crop rice genetic background. A summary of study findings is provided in Table 9. The principal factor for differences between the groups is crop vs. weed, not transgenic vs. non-transgenic. In every case, the hybrid populations were intermediate between the cultivated rice and red rice parents. Crosses with red rice produced taller

plants with little reduction in the number of panicles set, but some reduction in the number and weight of mature gains that developed. All F_2 families (transgenic or not) with red background expressed reduced shattering and less dormancy than red rice.

The second year of the study provided a unique opportunity to evaluate best management practices to control the crop-weed hybrid families produced by seed left on the soil surface from the previous season. Only a single germination flush was noted in the fallow fields. The volunteers were prevented from flowering with alternative herbicide application and cultivation practices.

Impact on agriculture of hybrid populations

We found that the best management practice currently in use for red rice control are effective for control of the hybrids. An important management practice is to never allow red rice to flower in a field of herbicide tolerant rice. Two applications of glufosinate ammonium herbicide may be advised to control any late germinating red rice in a field of glufosinate tolerant rice.

Herbicide tolerant rice can provide rice farmers with an additional tool to use within the context of present weed management programs.

After completing one year of monitoring at the field sites for persistence of the hybrid populations, we have observed no reason to prevent research in regions where outcrossing may occur. Such research will be important to define best management practices for each rice growing region to minimize the risk of outcrossing and control of hybrid populations, should gene flow occur.

References:

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Table 9. Fitness Study Results Summary

Trait	Outcome
Life Stage	Hybrid populations cluster together for life stage traits. Commercial rice cultivars can be separated based upon some of the life stage parameters measured.
Reproductive	Hybrid populations have a wide maturity range which could provide more opportunities for overlapping flowering and outcrossing. Red rice and the hybrid populations had difficulty setting viable seed at the Ben Hur site.
Disease Resistance	For naturally occurring infestations of Sheath Rot and Leaf Scald, no effect of the transgene was observed.
Fecundity	No differences observed.
Shattering	Shattering was high in any population with red parents. Mean shatter scores group red as highest, F_2 populations as intermediate and commercial rice in lowest group with little or no shattering.
Dormancy	Dormancy in the red and F_2 populations was higher than in the commercial varieties. Dry afterripening requirements were shorter for the commercial than the red and white-red hybrid populations. Commercial lines lacked dormancy, imbibed seed either germinated or rotted as the testing progressed. Seed of the red and white-red hybrid populations showed either germination or survival under prolonged, imbibed conditions.
Persistence	A single flush of germination was observed in the spring of the following year. Volunteers were removed by standard agricultural practices
Transgenic vs. Non–transgenic	In none of the fitness traits were we able to distinguish populations based on the presence of the *bar* gene, with the exception of tolerance to Liberty® Herbicide.

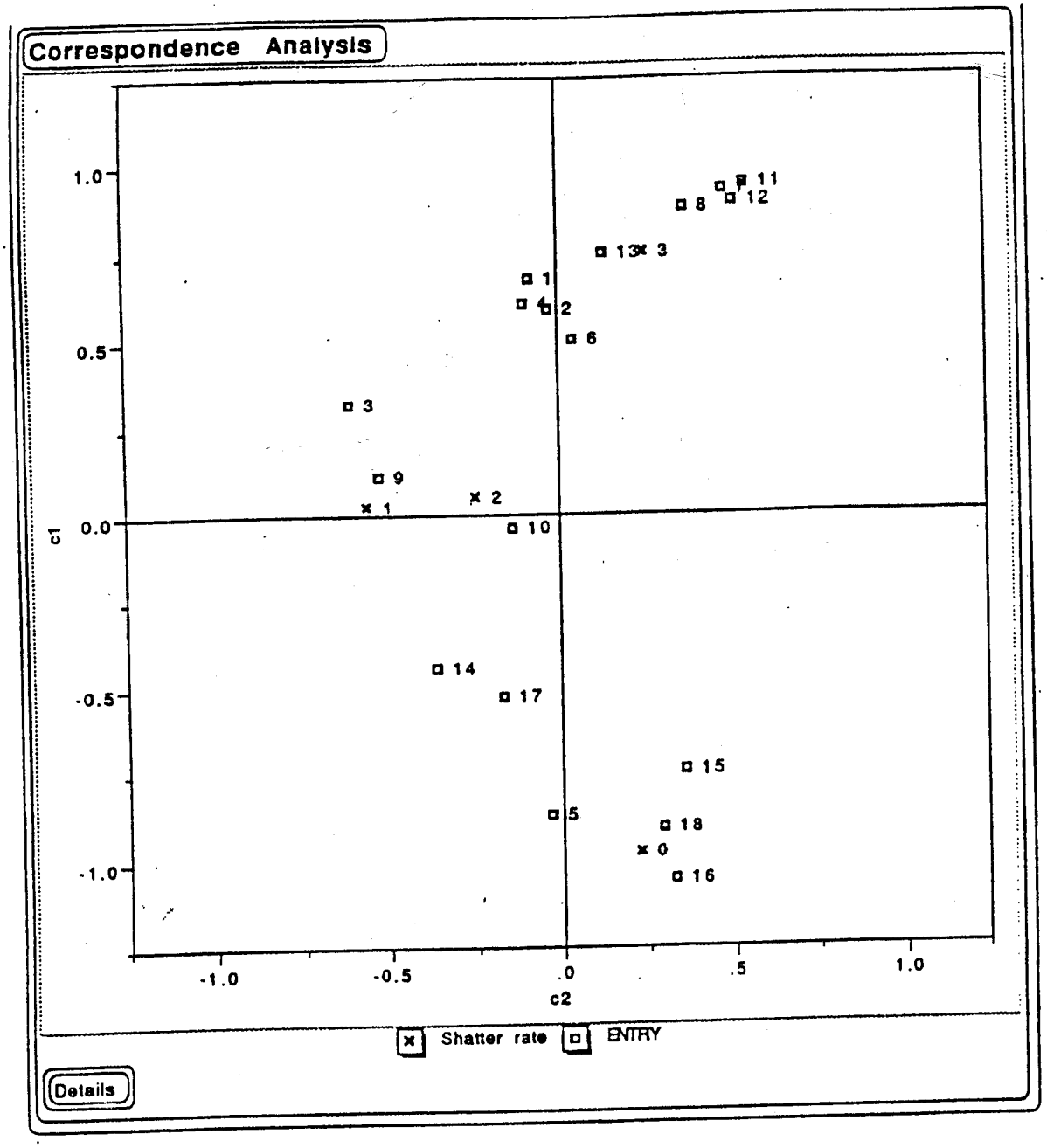

Figure 1. Seed shattering as defined by correspondence analysis for both locations. Commercial rice lines are clustered in the lower quadrants. Red rice lines are clustered in the upper right quadrant with the highest shattering rate. Red-crop hybrid families are clustered in the upper left quadrant, with intermediate shattering scores. Strong correlation is shown between the three groups and the amount of shattering recorded. The probability that entries are the dominant effect is 0.000. There is a location by entry interactions, but the relative shattering ranking remains the same for both sites.

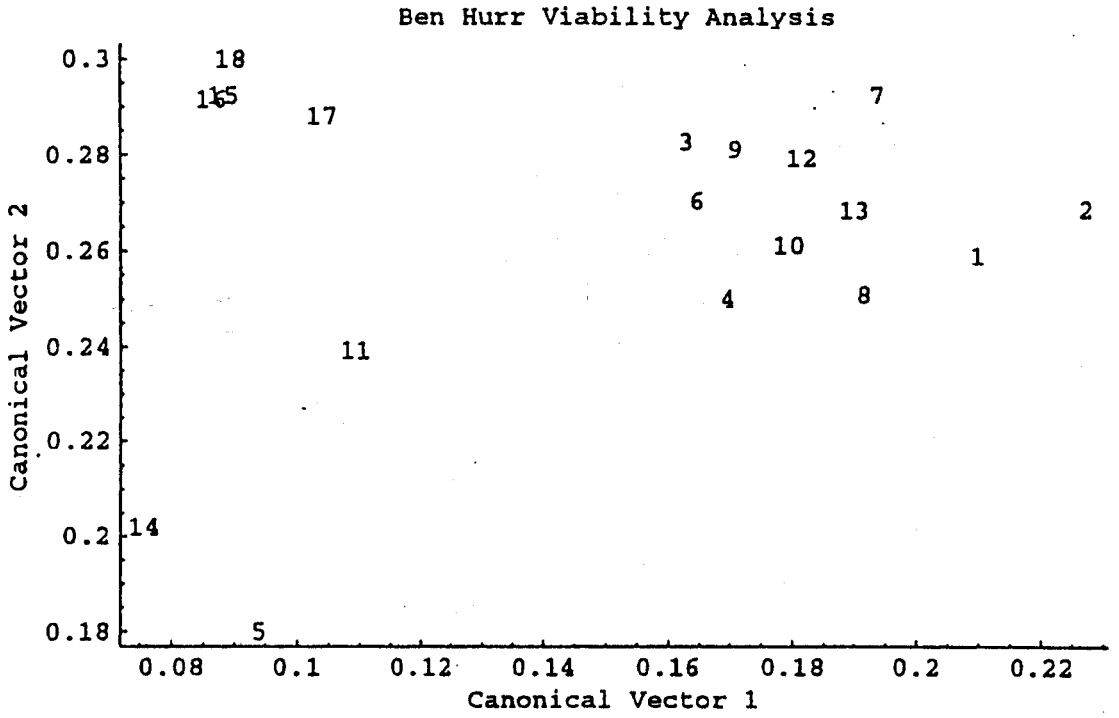

Figure 2. Seed dormancy analysis comparing the cumulative viable parameter (Canonical Vector 2) with seed surviving the stress test, the firm parameter (Canonical Vector 1).

The commercial rice lines (15,16,17,18) have high germination and low dormancy. Commercial rice lines, 5 and 14 have low germination and low dormancy. For the commercial rice lines, seed that does not germinate is more likely to be soft and thus, non-viable than dormant (Class 3). Red lines and hybrid families have a high number of individuals with dormancy requirements that are met by dry afterripening (Class 1) and a high number of seed that are likely to remain dormant and germinate in a subsequent season (Class 2).

DISEASE RATINGS OF SHEATH ROT AND LEAF SCALD - BEN HUR FARM
23 SEPT. 1997 RATINGS BY DR. CHUCK RUSH

ENTR.	SHEATH ROT REP1	REP2	REP3	REP4	MEAN			LEAF SCALD REP1	REP2	REP3	REP4	MEAN
1	1	3	3	2	2.25			2	4	3	4	3.25
2	3	2	2	2.5	2.37			2	3	4	3.5	3.12
3	2	2	1	4	2.25			1	1	1	2	1.25
4	3	2	2	2	2.25			3	3	3	2	2.75
5	3	3	3	3	3.00			4	4	2	3	3.25
6	1	1	1	2	1.25			1	3	3	3	2.50
7	2.5	1	1	1	1.37			1	3	3	4	2.75
8	1	2	1	1	1.25			2	2	2	2	2.00
9	1	1	2	2	1.50			2	3	2	2	2.25
10	3	1	2	2	2.00			3	2	2	2	2.25
11	1	1	2	3	1.75			3	4	4	4	3.75
12	1	1	1	3	1.50			2	3	3	4	3.00
13	1	2.5	1	2	1.62			3	4	4	4	3.75
14	4	3	3	3	3.25			3	1	2	2	2.00
15	3	2	3	3	3.25			3	3	2	3	2.75
16	4	2	3	3	3.00			3	3	3	2	2.75
17	3	3	2	3	2.75			2	3	2	3	2.50
18	4	3	3	4	3.50			2	3	2	2	2.25

Disease Rating: 1 = No Disease. 2 = Light Disease Incidence.
3 = Moderate Disease Incidence. 4 = Severe Disease Incidence.

c:\agrevo97\disease.ratings.wpd

Louisiana field evaluation of fitness traits in crosses between red rice and transgenic glufosinate-resistant rice varieties.

Oard, J., Cohn, M., Papenberg, T., S. Linscombe, S., Sanders, D., Griffen, J., Jones, D., and Doherty, L.

Transfer of glufosinate resistance from transgenic cultivated rice to red rice could occur under field conditions. A replicated field trial was conducted to evaluate several agronomic traits of F_2 progeny produced from crosses of transgenic glufosinate resistant varieties and red rice biotypes. A total of 9 parents (resistant or susceptible to glufosinate) and 12, F_2-derived populations were planted in a randomized complete block design with 4 replications in 1997 at the Louisiana Agricultural Experiment Station, Ben Hur Farm, Baton Rouge, Louisiana. This site occurs in a area where no commercial or experimental rice is normally planted. Data from over 600 individual plants in 84 plots were obtained, and more than 8,600 harvested seeds were plated in the laboratory for dormancy and germination studies. Various traits that were measured on an individual plant or plot basis included rate of seedling emergence, vigor, tillering, date of first and last heading, plant height, lodging, seed shattering, grain weight and number per panicle, seed germination, dormancy and glufosinate resistance. We looked for differences between the resistant and susceptible parents for the different characters that were analyzed. Differences between F_2 populations that did or did not segregate for glufosinate resistance were also evaluated. From the overall data, we concluded that segregation and expression of glufosinate resistance did not significantly enhance or decrease fitness levels for various traits measured in the transgenic parents or F_2 populations. Parents and progeny were also examined in replicated plots at Hope, Arkansas by Drs. Gealy and Gravois, University of Arkansas. Results from that trial will be presented at this me

Abstract to be published in:

Proceeding of the 27th Rice Technical Working Group Meeting.

Arkansas field evaluation of fitness traits in crosses between red rice (Oryza sativa) and transgenic glufosinate-resistant rice varieties.

Gealy, D. R. and Gravois, K. A.

A cooperative study was conducted by University and USDA-ARS researchers in Arkansas and Louisiana to evaluate fitness traits of red rice-rice crosses that potentially may arise from an unintended hybridization of herbicide-resistant rice cultivars and red rice grown in close proximity to one another. F2 plants derived from reciprocal crosses made in Crowley, LA between red rice lines and two rice cultivars ('Bengal' and 'Cypress') that had been transformed to be resistant to glufosinate, were grown in field plots near Hope, AR in the summer of 1997. Some of the fitness traits of these segregating populations such as shattering, production, size, and dormancy of seeds, were determined in tests in late 1997 and early 1998. F3 seeds from reciprocal crosses of all red rice and rice cultivars tested were highly dormant following dry afterripening periods of at least seven days after harvest in 1997, but were no more dormant than were the red rice parents. Initial dormancy levels of all transformed and nontransformed rice cultivars were much lower than for the red rice- rice crosses. Interestingly, initial dormancy levels of the transformed rice cultivars were about half those of their nontransformed counterparts. Dormancy levels following extended afterripening periods are currently being evaluated over the winter months of 1997-1998. All crosses appeared to be as prone to shattering as were their red rice parents and were significantly more prone to shattering than were the rice parents. Hundred seed weights and total seeds per panicle are being determined and will be presented. The fitness and potential agressiveness of populations of red rice- transformed rice crosses relative to their individual red rice and rice parents will be discussed. Because these data are preliminary, it has not yet been possible to determine the degree of segregation in these populations for any of the fitness parameters evaluated.

Abstract to be published in:

Proceeding of the 27th Rice Technical Working Group Meeting.

Outcrossing Potential of Liberty Link Rice to Red Rice

Sanders, D.E., Linscombe, S.D., Cohn, M.A. and Strahan, R.E.

Previous reports of the outcrossing potential of commercial white rice to red rice have varied widely. With the development of herbicide resistant rice the longevity of the ability to control red rice is dependent on when and how often outcrossing occurs in the field and the development of management strategies to stop or limit the outcrossing of herbicide resistant genes into red rice. All previous studies with Liberty Link rice grown in rice infested fields were terminated prior to heading to eliminate potential outcrossing at those sites.

A study was undertaken in 1997 at the Central Stations Research Farm to determine the actual rate of outcrossing between Liberty Link Rice (cv Cypress) and a Louisiana strain of red rice (straw hull). Included in the study was a determination of the effect of sublethal doses of Liberty on the outcrossing potential of red rice. This was undertaken to determine if red rice that survives a Liberty treatment can flower, outcross, and produce Liberty resistant red rice.

A 50:50 ratio of Liberty Link Cypress and strawhull red rice was planted in 1.5 x 3.6 m plots. Rates of Liberty were applied at 0, .43, .56 and .84 preflood; .28 preflood fb .28 postflood and .43 preflood fb .43 postflood; .28, .56 and .84 kg a.i./ha postflood. Preflood treatments were applied at the 3-4 lf. stage. Postflood treatments were applied 7 days after permanent flood (1 tiller). Treatments were applied in a RCB arrangement with 3 replications.

Percent control, plant heights and maturity (days to 50% heading) were recorded. Samples of mature red rice seed were taken on 4 days over a 4 week period from the untreated checks and the single treatments of .43 kg a.i./ha preflood and .28 kg a.i./ha postflood. All other treatments produced no red rice seed over the 4 week harvest period even though there was a small percentage of surviving red rice plants. Also noted were abnormalities in the red rice plant, panicle or seed. Seed from the red rice plants were ripened for 60 days at 22 degrees C and low humidity. Seed germination was determined periodically and when germination exceeded 60 percent seed were planted by plot number in seedling trays in the greenhouse at the rate of 200 seed per plot. The red rice seedlings were sprayed twice with .56 kg a.i./ha Liberty at the 2-3 lf. stage and then again 6 days later. The number of surviving red rice plants were counted.

Red rice plants in the untreated checks were 14 days earlier in maturity than the Liberty Link Cypress. Gernination of the red rice seed taken from these plots averaged 65% in the incubator and the greenhouse. To date no plants have survived the Liberty treatments in the greenhouse. Red rice in the sublethal treatments (.43 kb a.i. preflood and .28 kg a.i. postflood) were 7-14 days later in maturity than the Liberty Link Cypress. Most surviving plants never produced a panicle. Those producing a panicle produced only 10-20 mature seed compared to 130-160 seed for the plants in the untreated check. Germination of the seed from the sublethal treatments was much lower than the untreated checks. To date no plants have survived the Liberty treatments in the greenhouse.

It appears that in this study the rate of outcrossing as represented by the Liberty Link gene being transferred into red rice is lower than previously reported. Greenhouse studies will continue until all of the harvested seed is exhausted. In addition to the greenhouse studies the integrity of the test location has been maintained. This site will be disced and flushed in the spring of 1998 and treated with the same rates of Liberty as in the greenhouse study. Surviving plants will be counted.

Abstract to be published in:

Proceeding of the 27th Rice Technical Working Group Meeting.

1998 Proc. Southern Weed Sci. Soc. 51 abs. 108

ABSTRACT

EVALUATION OF LIBERTY IN RICE. G. L. Schwarzlose, M. H. Ehlhardt, P. N. Odom, T. L. Smith, and W. F. Strachan. AgrEvo USA Company, Spring Branch, TX, Chico, CA, Collierville, TN, Madison, MS, and Collierville, TN.

Liberty (glufosinate-ammonium) is a non-selective, post-emergent herbicide that will control many annual and perennial weeds. It belongs to a new class of herbicides, the phosphorylated amino acids. Through biotechnology, crops of economic importance, including corn, soybeans, and rice, have been genetically engineered to be resistant to Liberty Herbicide. Because Liberty Herbicide is a broad spectrum herbicide with excellent activity against almost all grass and broadleaf weeds, it is ideally suited for use in herbicide resistant crops. Results from testing over the past five years in corn and soybeans and over the past two years in rice have shown that broadleaf and grassy weeds in a vegetative growth stage were effectively controlled with post emergence applications of Liberty Herbicide at .25 - .50 lbs. ai/acre. Rates, application timings, and program approaches with commercially available herbicides were evaluated. Liberty and Liberty Link crops will give farmers a tool to better manage weeds that have developed resistance to other types of herbicides, or help prevent the development of weed resistance to their standard herbicides. The introduction of rice tolerant to Liberty will allow producers the ability to use a non-selective herbicide on their crops to deliver fast-acting post-emergence control on virtually all their grass and broadleaf weed problems.

CBI Deleted Copy
98-329-01p

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Amendment 1 to Petition 98-329-01p

Provide a clear description of the pedigree which explains how the generations T1, T2, T3 were developed from the T0 material. This information might fit well early in Section III on the "Transformation System and Plasmid Used".

Page 43. At the top of the page, there is a reference to "lines adapted for the California medium grain market (LLRICE06)". What relationship do these lines bear to the generations (T1, T2, etc.) of LLRICE06 described elsewhere in the petition? Clarify how you intended for data obtained on these lines to be used to support the petition for nonregulated status of LLRICE06.

Transgenic generation convention

T_0 plants are regenerated from the transformation experiments. The T_0 plants are grown to maturity in a greenhouse where T_1 seed is produced by selfing. T_0 plants are hemizygous for the inserted gene. Their T_1 progeny are segregating for the gene 3:1, tolerant to sensitive. Panicles are harvested from the tolerant T_1 plants. These panicles contain T_2 seed produced by selfing. Nine panicles from each T_1 plant are grown out and sprayed to evaluate for segregation. These T_2 panicle rows, which have 100% survivors, are homozygous for the *bar* gene. Seed from homozygous rows is harvested (T_3 seed) for further evaluation and advancement. The T_3 seed is produced by selfing. The seed resulting from a T_2 single panicle is considered to be a **line** and is named and evaluated as such.

Pedigree

The parent line of LLRice06 is M202. The parent line of LLRice62 is Bengal. See page 18, Section III.B for description of the parent lines.

Transformation event; LLRICE06 was created in cv. M202, a rice cultivar adapted for the growing conditions of California. The homozygous T_3 lines were evaluated for agronomic characteristics in California, the region in which the parent cultivar is adapted. The previous two generations, T_1 and T_2 were grown in winter nursery (Puerto Rico) and evaluated for tolerance to glufosinate. From the T_2 homozygous panicle rows, lines were selected for advancement based upon uniformity, maturity, heading, height, disease resistance, lodging resistance, fertility, plant type and general vigor.

Transformation event; LLRICE62 was created in cv. Bengal, a rice cultivar adapted for the growing conditions of Delta region of the USA. The T_1 generation was grown in winter nursery (Puerto Rico) and evaluated for tolerance to glufosinate. Panicle rows of the T_2 generation were planted in Louisiana and evaluated for tolerance to glufosinate. From the homozygous rows, lines were

1/20/99

selected for advancement based upon uniformity, maturity, heading, height, disease resistance, lodging resistance, fertility, plant type and general vigor.

Please see Section IV, p. 23-24 and Section V, p 41-43 for a description of the generations and the selection of lines for advancement in the breeding program.

Nomenclature
To define the use of the term "line," AgrEvo references the taxonomic convention for cultivated plants as described by Jack Harlan (1992) Crops and Man, "Classification of Cultivated Plants" American Society of Agronomy, Madison WI, pp. 110 -112.

'Species, Subspecies, Race, Subrace, Cultivar, Line (a selection from the cultivar), Genotype (an individual plant or homozygous line selected from a cultivar) and Clone (an individual plant selected from the cultivar and propagated asexually by cutting, tissue culture, apomixis, etc.).'

Page 16. For personal communications cited here and throughout petition, please provide a written letter from the person describing the information.

Please see attached letters from Dr. Steve Linscombe (petition pages 16, 17, 51, 56), Dr. Roy Smith (petition page 17), Dr. Marc Cohn (petition pages 52, 58) and Dr. David Gealy (petition page 56). Please see attached meeting manuscript for Dr. Günter Donn (petition page 56).

Page 17. In Section II D, it is stated that "Roy Smith has never seen a red rice population... growing in non-irrigated crops, like wheat or oats". What is meant by non-irrigated crops? Clearly, red rice is a weed in soybeans (you cite the work by Nastasi and Smith (1989)). Is soybean an irrigated crop? How do you distinguish irrigated crops from non-irrigated crops, and where does the rice crop fit?

As a note of clarification Dr. Smith is referring to crops grown in non-irrigated production systems. Dr. Smith has not seen red rice populations growing in non-irrigated wheat and oat crop production systems (often called "dry land"). Many crops can be grown in either irrigated or no-irrigated production systems. Red rice can be a weed in soybean production, which is often grown using irrigation, especially for the germination and stand establishment growth stages. Rice is generally grown in an irrigated production system.

Page 17. In Section II E, it is stated that "any of the Oryza species containing the AA genome can intercross and produce hybrid seed", and in Table II.3 you list six species with the AA genome, two (O. rufipogon and O. longistaminata) of which are listed as federal noxious weeds. Even though O. longistaminata is not found in the US and O. rufipogon is found in only one location outside of the rice growing region in the US, more information should

be provided about the potential for the AA species to form viable hybrids with O. sativa. For example, which, if any, of the reproductive barriers which you cite from Morishima (1984) are present in hybrids with these species? Discussions of outcrossing should address all relevant species, not just those found in the United States.

Members of the genus *Orzya* with the same genomic designation have complete pairing of their chromosomes in meiosis and can produce fertile hybrids. *O. sativa* is classified as AA genome as are *O. rufipogon* and *O. longistaminata* (see Table II.3, page 16). Section VI.A.1. (p. 50-52) provides a discussion of outcrossing with wild and weedy relatives found in the USA. In this section, we cite *O. rufipogon* as included on the federal noxious weed list. We did not list *O. longistaminata*, as it is found in Africa and not in the USA. However, if either of these species were present in the United States and grown conspecific (in the same field) with rice, *O. sativa* and if flowering occured at the same time, and depending upon the extension of the stigma (which is related to the amount of self-pollination), that some degree of outcrossing would likely occur. If we assume that the developing hybrid embryos are viable and hybrid seed are set, the ecological consequence depends upon the direction of the cross. If the resulting hybrid seed were produced with *O. sativa* as the maternal parent, the seed would not shatter and likely be harvested with the crop (shattering is inherited from maternal parent). If one of the wild species was the maternal parent, the hybrid seed would likely shatter and fall to the surface of the soil prior to harvest of the crop rice. The hybrid seed would likely express the dormancy of the maternal parent. If the hybrid seed received the proper germination cues to break dormancy, hybrid plants may be produced. Depending upon the adaptive characteristics of the hybrid plants, a population may be established. For example, *O rufipogon* is adapted to swamp habitats (Vandiver, 1992) and a hybrid population would likely require a similar habitat for survival.

As noted in the petition, hybridization between rice cultivars and red rice, and between rice cultivar/red rice hybrids and red rice, depends upon overlapping in flowering time (phenology). Later maturing cultivars are more likely to introgress with red rice. This was a critical conclusion of the study you describe in Appendix 2, p9-11, and you list it as a botanical barrier on page 72 of the petition, and you refer to it in other places as well. Yet, you present no data on the phenology of the transformation events or their parents compared to red rice. Did you collect any data on the days to 50% anthesis or days to flowering with your agronomic performance data? You do show in Table V.3, p.43 that some of the advanced lines are later maturing. Does this translate into later flowering? Do you have any evidence that these transformation events will be likely to overlap with red rice or with red rice x rice hybrids in flowering time?

Active introgression between rice and red rice occurs in the field (Langevin et.al, 1990). Red rice populations do vary for maturity (see Linscombe letter), so no

matter what the maturity of our transgenic lines, there will be opportunity for outcrossing into red rice, if red rice is allowed to flower. The major point of our resistance management program is to control (remove) red rice early in the season before flowering occurs.

On pages 9-11 of Appendix 2, you have summarized the published work of Langevin et al., by stating in the second table (unlabelled) that the number of days to anthesis *of red rice x 5 early season varieties as 105-120 days and red rice x Nortai as - no data given. Actually, in that study, the authors reported that 105-120 days is the average of all red rice x cultivar hybrids, including Nortai. The study proposed that the overlap of the late season Nortai with the red rice x rice hybrids was the factor which facilitated introgression.*

We do not disagree with APHIS's interpretation and believe that the table provided in Appendix 2, p. 9-11 may be misleading.

Pages 17 & 18. It is not clear which statements are attributed to cited literature. For example, "populations of red rice are diverse genetically" (bottom of page 17).

Red rice in not a native species (Craigmiles, 1978), but rather an introduced weed which inhabits only agricultural-eco systems (Roy Smith, personal communication).

Rice-red rice, crop-companion weed hybridization is common, and given overlapping flowering, outcrossing is to be expected (Section II. B and C, pages 14-15, Section VI. A. page 50-52). Populations of red rice are diverse genetically (Noldine, 1998, Linscombe, personal communication; Craigmiles, 1978).

Page 18. There is no citation here or elsewhere for the method used for "direct gene transfer".

Rice transformation was made by direct DNA transfer. [**CI DELE**

] No organisms were used to mediate the DNA transfer.

Page 20. Table III.1 uses the term "complement" for both the CaMV regulatory sequences and the bar gene. Please explain the use of this term for some of the genetic elements but not for others.

The position in vector was given for all sequences in the ascending bp order. When given in ascending order for some sequences the DNA strand given is complementary to the strand that provides the described function. For example for the *35S* terminator: the position given from bp 2205-2398 is on the strand complementary to the functional strand (bp 2398-2205).

Pages 24 & 25. Was glufosinate (Liberty) applied at the same application rate for all of the Mendelian inheritance testing? Does the application rate (concentration of glufosinate) influence the apparent incidence of resistance to the herbicide? Is this a factor in interpreting the results? Some of the test results were not included in Table IV.3 on page 25 ("data not shown"), but there is no indication of how many additional panicle rows were evaluated and how the results compared to the predicted results. Please include at least a summary of these data that are not shown and indicate how closely the data fit the expected values. This table also has a superscript "a" on the column for Chi square, but there is no corresponding footnote entry (the footnote has "b").

Page 25. Further clarification for the Mendelian inheritance data - Is 23% (second sentence) an average of the plants that did not survive only from the panicle rows that fit the expected ratio? How many panicle rows did not fit the expected ratio? If there were only a few, could you present the data for those with their X2 results in Table IV.3? If there are too many, could you state how many, and perhaps give an average of their ratio and X2?

The glufosinate (Liberty) application rate is the same for all Mendelian inheritance testing. The rate used was 1600 gm active ingredient per hectare, which is 4 x the likely single application rate to be registered with the EPA for use on LibertyLink rice.

We acknowledge that the footnote below Table IV.2 should read "a" not "b."

Of the four populations, 211 T_2 panicle rows were planted and 69 rows contained no sensitive plants (see Table IV.2, and text on page 24). The remaining 142 panicle rows were segregating. To make this assessment a cone planter was used so the seed were spaced evenly over a fixed distance. As the number of seed per panicle varies, each row contains a different number of plants. In a plant breeding program, the rows with no dead plants are identified (Table IV.2) as homozygous and advanced in the program. A sampling of the panicle rows that were segregating was censused for both surviving and dead plants. These counts are difficult to make as the surviving plants are often densely planted and individual plants can be difficult to identify.

Not all of the 142 segregating panicle rows were counted, as there was no reason to do so. Please recall that only seed of the plants from the homozygous rows continued in the breeding program. Further work was carried out with segregating panicle rows from populations three and four (Table IV.2.) to confirm the expected segregation of 3:1 for selfed seed from the heterozygous panicles. Partial results are given in Table IV.3.. 23% (page 25) is the average of the sensitive plants from panicle rows listed in Table IV.3. The average number of sensitive plants from panicle rows that did not meet the chi square criteria and are not included in the table is 15%.

Pages 27 & 28. There does not appear to be sufficient evidence to support the conclusion that "the weakly hybridizing 16 KB fragment is the result of partial digestion of the genomic DNA" or the vague interpretation offered that "the larger EcoRI fragments might represent incomplete and/or rearranged and/or partially digested copies." This degree of remarkable uncertainty suggests that further analysis is warranted. This type of vague interpretation of results continues on the bottom of page 28 through to the top of page 29. Please clarify.

The digests in question have since been repeated several times, and each time we observed the same bands. From this we conclude that the bands in question could represent incomplete and/or rearranged digested copies.

Page 28. Based on the data presented, it is not clear why you have stated that the apparent 1000 bp NcoI fragment represents an "incomplete copy." It is also unclear why you state that a minimum band size of 1373 bp is expected when cutting with NcoI. Couldn't the observed 1000 bp band indicate an upstream plant-transgene fragment? Please clarify the basis for your conclusions.

The minimum band size of 1373 bp comes from digestion of the transforming DNA (1501 bp HindIII/PvuI fragment described on page 19) with NcoI (see Figure IV.1. and text on page 28). The 1000 bp fragment could come from an upstream plant-transgene fragment (but a weaker hybridization signal would be expected as only a small portion of the hybridizing fragment would have homology to the probe). It is more likely that the 1000 bp fragment comes from an incomplete cassette insert.

Page 31. The method for the ELISA technique should have just been referenced with only those modifications of the technique spelled out in the text. There should be information on antibody production, especially regarding the immunogen that was used to develop the antibodies (e.g., the source, purity, etc.). PAT levels in the grain do not provide information on specific activity in leaf tissues during the growing season, the time when the plant is likely to encounter the herbicide. For Table IV.9 on page 32, indicate the sample sizes selection of samples (from heads, panicles, already harvested, maturity of grain, etc.).

No phytotoxicity was observed when the events were sprayed in the field with 4 x the likely single application rate of glufosinate (Liberty) or with two 1 x application rates at growth stages determined best for weed control. This indicates that the LLRICE events express sufficient amounts of the PAT protein when they are likely to encounter Liberty herbicide.

The immunogen used for antibody production was PAT protein purified from fermentation of *Streptomyces hygroscopicus*. For the PAT ELISA data reported in Table IV.9. one bulk sample of grain was harvested in the field at maturity for each plant type reported. The analytical laboratory received approximately 125 grams of each sample. A 12 gram sample was randomly taken from each and ground. Two 1 gram ground samples from each were extracted. Each extract was assayed twice (2 wells on an ELISA plate). The reported numbers are an average of the reading from 4 wells (2 replicate extracts each assayed twice).

Pages 34-38. The figure legends indicate that 10μg DNA was used for the restriction digests, but it doesn't say how much of these digests were loaded in each lane of the gel. Please clarify.

Entire restriction digests were loaded, so the lanes contain approximately 10μg DNA.

Southern gel figures. Is there some reason why some of the molecular weight marker bands light up and others don't? This does not seem to be consistent from one gel to the next.

There was no λ probe used in the hybridizations. All of the hybridizing λ bands are from cross hybridization with the probes used for each blot. Some probes will always hybridize with the same limited number of λ-DNA PstI fragments. Some probes never hybridize with λ-DNA PstI fragments. Some probes sometimes hybridize with distinct λ-PstI fragments. There are a number of plausible explanations for this, however, the hybridization is due to short GC regions of the probes that are homologous to some λ PstI fragments. In probe synthesis many small fragments may be generated. These can hybridize non-specifically with a large amount of plasmid or phage DNA, but won't give a visible non-specific hybridization with plant genomic DNA since the genomic DNA is present as a continuous smear across the gel lane as compared to the distinct phage bands (an overexposure of the blot may reveal the non-specific hybridization in the form of a smear). Sometimes there is even a homologous region between probe and MW marker. This is for instance the case with the Promega RNA MW marker that we use and the *bla* probe.

Pages 41-47. Section V. B.& C. Most of this information is presented on LLRICE06, but very little is provided for LLRICE62. Are similar data available for LLRICE62? If yes, before compiling these data, please consider the points made below. Many of the studies and conclusions do not address issues relevant to the determination of nonregulated status. For many of these studies, it is not clear how the experimental designs and results support the conclusions that have been made.

There is no additional data available for LLRICE62. This event is not as far advanced as the other in the breeding process so AgrEvo completed the studies

deemed most relevant to risk assessment with the limited seed we had available. These evaluations included seed germination, dormancy, shattering, and dry afterripening requirement; disease and pest susceptibility; and composition as this could be relevant to non-targets which may eat rice seed. In every case the event was compared directly to the parent cultivar grown in the same field and in the region of adaptation to the parent.

On the bottom of page 43, you state that "comparisons were also made to determine the possibility of reduced yield for transformation events (Table V.4)." Such yield studies have little or no relevance to APHIS, assessment of the potential for the transformation events to pose a plant pest risk if they are no longer regulated articles. In addition, the test was not designed to reflect the degree of variation among transformation event "lines." To do this the test should have included "lines" of the parental variety M202. It is not clear what point you are trying to make with these results. This confusion continues to the explanation on the top of page 44. In addition, it is not clear what relevance these results have in evaluating the similarity or dissimilarity when one considers the sample size, experimental design, and the fact that the data are from a single growing season (and perhaps a single location, also). The petition does not make clear why these types of data are provided for LLRICE06 but not LLRICE62.

The comparisons reported in Table V.4. are between the parent cultivar M202 and several transgenic lines (line = a selection from the cultivar). Table V.4 is based upon yield tests (34 lines, 4 replications, and with and without glufosinate herbicide treatment at 2 of the 3 locations). It is common plant breeding practice to compare lines with the parent cultivar. The M202 seed used for comparison in all these tests was certified seed, and thus representative of the cultivar. Such yield evaluations are normal activities of plant breeders to be ascertain whether the inserted gene has had an obvious effect on cultural performance. The best performing lines with the desired crop characteristics are advanced.

Page 48. The compositional data on food quality (Table V.8) are not required for APHIS, assessment. However, if these data are to be presented, there should be adequate information on sample sizes, replication, statistical analysis of results, etc.

A single sample was analyzed as a preliminary screen for assurance that no gross changes occurred as a result of the DNA insertion. Since a single sample was analyzed statistical analysis is not practical. The analysis showed that all parameters tested were in an acceptable range so no further analysis was done at the time.

The samples had the same origin as those used for the PAT ELISA. For minerals and nutrients (Table V.8) a 60 gram sample of each event and non-

transgenic counterpart was analyzed. For anti-nutrients (Table V.9) a 30 gram sample was analyzed.

Page 69. Does AgrEvo have any data to address whether hybrids of LibertyLink rice X red rice are resistant to Liberty at the approved or other application rates? What, if any, effect do these observations have on weed management practices for such hybrids?

To address your question concerning the tolerance of Liberty Link rice X red rice hybrids to glufosinate, please see Appendix 2f, page 13 for a summary of Sankula, Braverman and Oard, 1998. We predict that rice X red rice hybrids will be tolerant to glufosinate as this was shown by these studies. The weed management practices were described in this petition because we know that outcrossing can occur and we need to manage resistance development (see introduction to Section VI, page 50). It is important to understand that weed control methods to control red rice (see Section IV.E.2, pages 65-67) may also be used to control Liberty Link rice X red rice hybrids, should any develop.

Pages 71-74. Section F. "Conclusion of Environmental Impact Assessment" discusses three main issues, but does not elaborate very much on the "key question" raised on the bottom of page 71 and the top of 72, namely: "Will the transfer of herbicide tolerance to wild and weedy rice relatives exacerbate problems of weed control and thus lead to decreased rice production yields." In the following "Impacts of agriculture and rice productivity" under Issue 2: consequences of Gene Flow" the items listed do not address the consequences of gene flow, but instead focus on factors associated with using LibertyLink rice in agriculture. Previously in Issue 2, the "Assessment" states that "the fitness of crop-weed hybrids is not greater than that of red rice," but this conclusion is based on relatively little data. There is no reiteration of the point that there are still many well established weed control practices that will continue to be effective in controlling any crop-weed hybrids that might arise. This point is made very briefly in the end of the summary paragraph on page 74.

Thank you for pointing out that we did not sufficiently emphasize this key point. Indeed, it is important to note that there are many weed control methods for the control of LibertyLink rice X red rice hybrids or LibertyLink rice X other rice species hybrids, should any develop anywhere in the United States. Even if the weed management practices AgrEvo recommends to growers are not followed and glufosinate resistant LibertyLink rice X other rice species hybrids arise there are other well-established methods to eliminate these from the environment.

Page 18. The correct designation is cauliflower mosaic virus (no caps).

Page 19. The designation "bar" needs italics.

Misspellings of "alan" instead of alanyl (p.21), "phrenology" instead of phenology (p.51), "stewartship" instead of stewardship (p.73).

Page 45. Typographical error (I assume) "access" instead of "assess."

Thank you for pointing out these discrepencies.

Page 32. In the text, Table IV.9 is referred to incorrectly as "Table VI.9." Check elsewhere for this same error.

We acknowledge that Table IV.9 on page 32 was incorrectly listed as Table VI.9. We found no other instance of this error.

Pages 27-30. Presentation of Tables IV.5-8 would have been clearer if columns which indicated only "no hybridization signal" had been replaced with a single summary statement in the footnote. This would have allowed for the recommended column which indicates which Figures illustrate the experimental results (Southerns). This comment is for future reference; don't bother changing these Tables.

Page 33. Figure IV.1 would have been clearer and more consistent with the nomenclature used in the text if the &bar cassette8 were labeled. There is no need to change this now.

Please note: Errors and inconsistencies in the numbering of pages makes it extremely difficult for APHIS to provide accurate photocopies of the documentation to the public. These are not trivial considerations when we consider the amount of time necessary to ensure that complete and accurate copies are made. For this reason, APHIS asks for complete and accurate pagination of petition submissions. Likewise, the Table of Contents must be complete (indicate each Appendix and the page numbers where they can be found). One acceptable approach would be to indicate the pages of an Appendix A as &A-1, A-2, A-3,...8, then the pages of Appendix B would be &B-1, B-2, B-3...8 Alternatively, the entire document could be paginated with a single continuous number series. Either of these approaches makes it possible to spend a reasonable amount of time for APHIS to ensure that the photocopies we make are complete and in order. APHIS will not reject the current petition submission this time because of the faulty pagination of the appendices and incomplete table of contents, but future submissions should be more carefully prepared.

Appendix 1. Pages not numbered.
Appendix 2. Page numbering; Tables and Figures not labeled.

Acknowledged

Louisiana State University
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Louisiana Agricultural Experiment Station

Rice Research Station
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December 18, 1998

Ms. Donna H. Mitten
AgrEvo USA - Rice Biotechnology
414 Fourth Street, Suite A
Woodland, CA 95695

Dear Donna:

In answer to your questions concerning the adaptation of rice and red rice, in my career working as a rice breeder in the irrigated rice production regions of the United States, I have never observed either rice or red rice growing outside of cultivated agriculture.

To address your questions concerning the genetic diversity of red rice populations in Louisiana, it is my observation that distinctive homogenous populations are rare in cultivated rice fields. In a single field, red rice plants can be found that vary for such traits as culm coloration (blackhull or strawhull), plant height, flag leaf angle, and may range in awn length from long to awnless. Homogenous populations of red rice exist only in weed science programs where the well characterized biotypes are important for research.

To address your questions concerning native resistance to Liberty herbicide, I have observed some degree of differential sensitivity to low levels of glufosinate in rice varieties.

As I have offered in the past, the reviewers of the deregulation petition at USDA may call me if there are further questions. I can be reached at 318 788 7531.

Sincerely,

Steve Linscombe, Ph.D.
Professor

/dmr

USDA

United States Department of Agriculture

Research, Education and Economics
Agricultural Research Service

December 21, 1998

Donna H. Mitten
AgrEvo USA - Rice Biotechnology
414 Fourth Street, Suite A
Woodland, CA 95695

Dear Donna,

In answer to your questions concerning the adaptation of rice and red rice, red rice in not a native species, but rather an introduced weed which inhabits only agricultural-eco systems. In my 35+ -year career as an agronomist conducting research on control and biology of rice weeds in the southern United States, I have never seen a red rice population growing in non-agricultural land nor one growing in non-irrigated crops, like wheat or oats.

Sincerely,

Roy J. Smith, Jr.

Roy Smith, Ph.D.
Professor, retired

ars

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January 4, 1999

Ms. Donna H. Mitten
AgrEvo USA - Rice Biotechnology
414 Fourth Street, Suite A
Woodland, CA 95695

Dear Donna:

In answer to your questions concerning the length of dormancy found in red rice, we have observed in lab tests of submerged seeds or seeds continuously buried in flooded soil at 30C, that red rice will remain viable and dormant for 4 to 6 years. Anecdotal evidence from farmers indicates survival for up to 20 years in soil (e.g., old levees knocked down in reworking previously abandoned fields.

Concerning the dormancy of the seeds harvested from the crop-red rice hybrid study, seeds of the red and white-red hybrid populations either germinated or remained firm under prolonged, imbibed high temperature conditions. Testing of the firm seeds found many of the hybrids did not germinate when cut. This indicates that the hybrid seeds were not able to respond to a common germination cue (removal of the husk and cutting the seed in half; a common test for cereal seed viability).

The reviewers of the deregulation petition at the USDA may call me if there are further questions. I can be reached at 225-388-1464 or 225-388-1322.

Sincerely,

Marc Alan Cohn, Ph.D.
Professor of Crop Physiology
Editor-in-Chief, *Seed Science Research*
<MCOHN@LSUVM.SNCC.LSU.EDU>

USDA

United States Department of Agriculture

Research, Education and Economics
Agricultural Research Service

December 21, 1998

Donna H. Mitten
AgrEvo USA - Rice Biotechnology
414 Fourth Street, Suite A
Woodland, CA 95695

Dear Donna,

For your preperation of the USDA deregulation petition of glufosinate resistant ('LibertyLink') rice, I described our work in screening a collection of red rice biotypes for sensitivity to various herbicides. Included in our collection was the biotype identified by Dr. Noldine while at Texas A&M as expressing some tolerance to glufosinate herbicide. We found that none of the biotypes, including this Texas biotype, survived the recommended application rates and timings of glufosinate. Many biotypes did not survive the 1/4 lb. rate and only the most tolerant biotype survived the 1/2 lb. rate. Even at these low rates, surviving plants did not produce viable seeds due to excessive delays in plant maturity. None of the biotypes survived the one-time, full application rate (1 lb./A) used in these studies. These results are from ongoing work, part of which is the subject of a manuscript now being drafted by our group.

If there are further questions, I can be reached at 870-672-9300 ext. 226.

Sincerely,

David Gealy

David Gealy, Ph.D.

ars

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ANPP – DIX-SEPTIEME CONFERENCE DU COLUMA
JOURNEES INTERNATIONALES
SUR LA LUTTE CONTRE LES MAUVAISES HERBES
DIJON – 9,10 ET 11 DECEMBRE 1998

GLUFOSINATE SELECTIVITY IN TRANSGENIC MAIZE

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RÉSUMÉ:

D'abord développé pour des usages non sélectifs, le glufosinate est aujourd'hui proposé en usage sélectif sur maïs tolérants possédant un gène de résistance spécifique. Le glufosinate ainsi que les gènes de résistance pat et bar ont été isolés à partir de souches de bactéries du sol. Le mode d'action du glufosinate et le mécanisme de sélectivité introduit dans le maïs sont détaillés. Les grandes lignes de l'évaluation du risque du couple gène/herbicide pour l'environnement et pour la santé humaine sont présentées. Enfin la probabilité d'apparition spontanée d'un nouveau mécanisme de résistance au glufosinate par les adventices est une éventualité purement théorique et qui n'a aucune réalité dans la pratique. Cela constitue un atout pour le développement de l'usage sélectif de cette substance active.

Mots-clés : glufosinate, gènes pat et bar, protéine PAT et BAR, maïs tolérant au glufosinate, *Streptomyces hygroscopicus, Streptomyces viridochromogenes.*

SUMMARY:

Firstly developed as a non-selective herbicide, the glufosinate is now proposed as a selective herbicide on tolerant corn containing a specific resistance gene. The glufosinate and the resistance genes pat and bar were isolated from soil bacteria strains. The mode of action of glufosinate and the mechanism of selectivity introduced in the maize are detailed. An outline of risk assessment of the gene/herbicide couple for the environment and human health are presented. The probability of the spontaneous occurrence of novel glufosinate resistance mechanism is only a theoretical possibility with no practical relevance. This is an asset for the development of the selective use of that compound.

Key words: glufosinate, pat and bar gene, PAT and BAR protein, glufosinate tolerant maize, *Streptomyces hygroscopicus, Streptomyces viridochromogenes.*

1. Introduction

In this article an attempt is made to compile as a case history the background knowledge and technology developments which finally led to the development of glufosinate tolerant maize varieties.

2. Origin of glufosinate

In the late 1960's a team of microbiologists under the direction of Prof. Zähner at the University of Tübingen discovered on the search of new compounds with bactericide activity a Streptomyces strain, named *S. viridochromogenes*, which synthesized an unknown substance. In the coming years it became evident, that this compound was a tripeptide, consisting of 2 alanine molecules and a new non proteinogenic amino acid which was named phosphinothricin [Bayer E. et al, 1972]. The university group also described the mode of action of the new substance. In the 1970's the tripeptide and the phosphinothricin were tested for their bactericide properties. The free amino acid phosphinothricin showed a 1.000 to 10.000 fold reduced bactericide activity compared to the tripeptide. Because both compounds were not competitive in their bactericide activity, their use as research compounds in the pharmaceutical research labs was abandoned.

A sample of synthetic D,L-phosphinothricin, named glufosinate, was tested in the biological research department of the Hoechst Agricultural Division. There the general biological properties of test compounds was investigated. It turned out, that glufosinate showed no significant activity against insects, nematodes and fungi, but it had a strong herbicidal effect when sprayed on the foliage of the test weeds. Meiji Seika, a Japanese company, discovered the herbicidal properties of phosphinothricin-alanyl-alanin (bialaphos) produced by *Streptomyces hygroscopicus* and began to develop it as a herbicide for the japanese market.

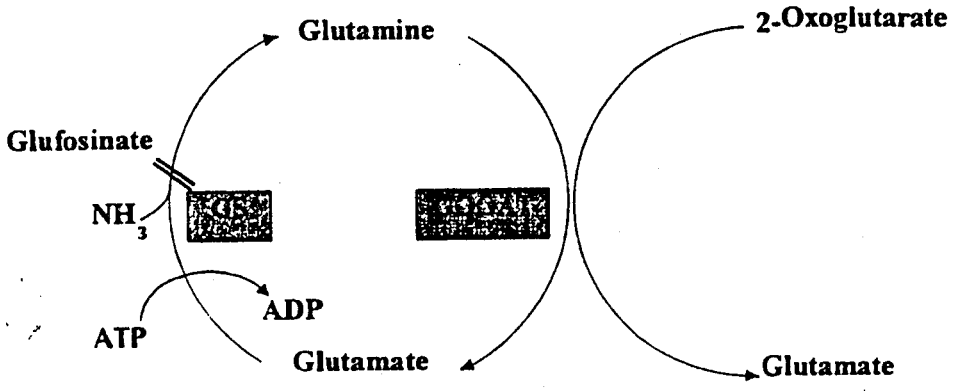
If sprayed as a preemergent herbicide on the soil, in which weed seeds were sown, glufosinate did not inhibit the germination of the weedy species. The selectivity test with different crops showed that the compound affects weeds and crops in the same manner, if a foliar spray is applied. Field trials were initiated at Hoechst to test the properties of glufosinate under agronomic conditions. Due to the lack of selectivity there was no chance to use the substance as a postemergent herbicide in field crops, but in orchards, vineyards and tropical plantations crops it revealed its broad spectrum activity . Due to the lack of soil activity it did not harm the perennial crops [Schwerdtle et al, 1981; Langelueddeke et al, 1982]

Chemists at Hoechst optimized the methods to synthesize glufosinate [Hoerlein et al, 1994]. Its toxicological and ecotoxicological properties were investigated, and finally in 1984 the ammonium salt named glufosinate-ammonium was introduced under the trade name Basta® as a new nonselective herbicide. Since then the product has been registered as a nonselective herbicide in more than 50 countries.

3. Mode of action of glufosinate

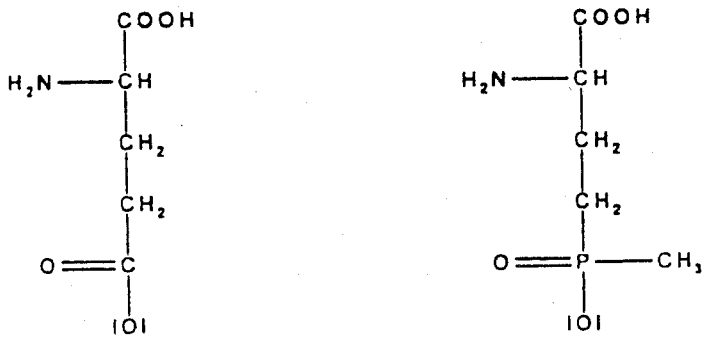
The target of glufosinate is the enzyme glutamine synthetase (GS). In terrestrial plants, the main source for ammonia comes from photorespiration [Keys at al, 1978] and its reassimilation and detoxification is completely dependent on a functional GS-GOGAT cycle. Glutamine synthetase (GS) converts ammonia into glutamine and then the second enzyme glutamine-oxoglutarate-aminotransferase (GOGAT) which is also called glutamate synthase, converts 1 molecule of glutamine and 1 molecule of oxoglutarate into 2 molecules of glutamate which then keep the cycle running or can be used for the synthesis of amino acids and nucleic acids.

Figure1 - The conversion of glutamate and ammonia into glutamine by the enzyme glutamine synthetase (GS) is blocked by glufosinate. As a consequence the glutamine pool in the plant cells is rapidly depleted and ammonia accumulates in photosynthetic tissues, because the vast majority of the ammonia in plant cells is released by a side reaction of photosynthesis (photorespiration) when 2 molecules of glycine are converted into serine. The GS-GOGAT cycle is essential for plants to detoxify ammonia and to generate glutamine and glutamate as precursor molecules for the biosynthesis of amino acids and nucleic acids.



Already the group at the University of Tübingen discovered that phosphinothricin is a structural analogue of glutamate and a strong inhibitor of glutamine synthetase.

Figure 2 - Glufosinate as an analogue of glutamate (glutamic acid)



Plants are especially vulnerable if glutamine synthetase is inhibited. For terrestrial plants the only efficient pathway to detoxify ammonia is through its conversion into glutamine with the help of glutamine synthetase. Aquatic plants and some animals are able to secrete the toxic ammonia into the water, as well as microorganisms, whereas most terrestrial animals detoxify ammonia as ureic acid (reptiles, birds and some in vertebrates) or urea (mammals), which then is excreted.

In the mid eighties it was shown that the interruption of the GS-GOGAT-Cycle via deficiency mutation is deleterious for plants. GOGAT-deficient barley mutants which grow well under conditions which inhibit photorespiration (low O_2-partial pressure and/or high CO_2-partial pressure) were isolated [Kendall et al, 1986]. As soon as these mutants are kept under normal atmospheric conditions they develop identical symptoms known from plants treated with a GS inhibitor like glufosinate (ammonia accumulation, inhibition of photosynthesis, collapse of chloroplasts, and finally death of the photosynthetic tissue). Similar GS deficiency mutants were described for barley [Wallsgrove et al, 1987] which show the same symptoms under photorespiratory conditions.

Under conditions where no photorespiration occurs such as in the dark or in the roots, the GS inhibitor glufosinate does not trigger a toxic accumulation of ammonia [Koecher et al, 1983] and such plant tissues are not effected by the herbicide in a lethal way.

4. Properties of glufosinate

The herbicidal activity is very broad. The compound affects annual and perennial grasses and broad-leaved weeds, but its soil activity is close to zero The amino acid is highly biodegradable. Within 3 to 10 days the active ingredient (the ammonium salt of the racemic mixture of D- and L-glufosinate) is completely converted into the non herbicidal α-keto-phosphinico-propionic acid, which is then degraded within 30 – 60 days to NH_4^+, CO_2, PO_4 and water. Despite the fact that glufosinate is an effective inhibitor of glutamine synthetase and that glutamine synthetase is an essential enzyme for all living cells, the toxicological and ecotoxicological profile of the substance clearly shows that at the rates which are of agronomic significance (0.3 – 1.0 kg a.i./ha) the substance is not harmful for non target organisms, including soil microorganisms, diverse animals and human beings [Dorn et al, 1992; Hack et al, 1994].

5. Attempts to create selectivity for glufosinate

In the 1980' several attempts were made to generate selectivity for glufosinate. Physical means to achieve selectivity, namely directed spraying of the herbicide on the weed canopy and physical protection of the crop by shielding was one solution to use glufosinate-ammonium in field crops. Due to the costs of the equipment and the necessity of significant size differences between crop and weeds as prerequisites to use such a system, it had limited practical significance. The chemical solution to generate selectivity, namely search for a safener compound which accelerates the metabolism of the herbicide in a crop species, but does not affect the herbicidal activity against the

weed species was unsuccessful. The biological challenge, the search for spontaneously occuring resistant individuals (mutants) either in breeding populations or on the cellular level in vitro also failed for glufosinate-ammonium. The only exception was the successful selection of a glufosinate tolerant alfalfa cell line which became resistant due to the amplification of a GS gene and consequently this cell line overexpressed GS 4 – 10 fold compared with the wild type cell line [Donn et al, 1984].

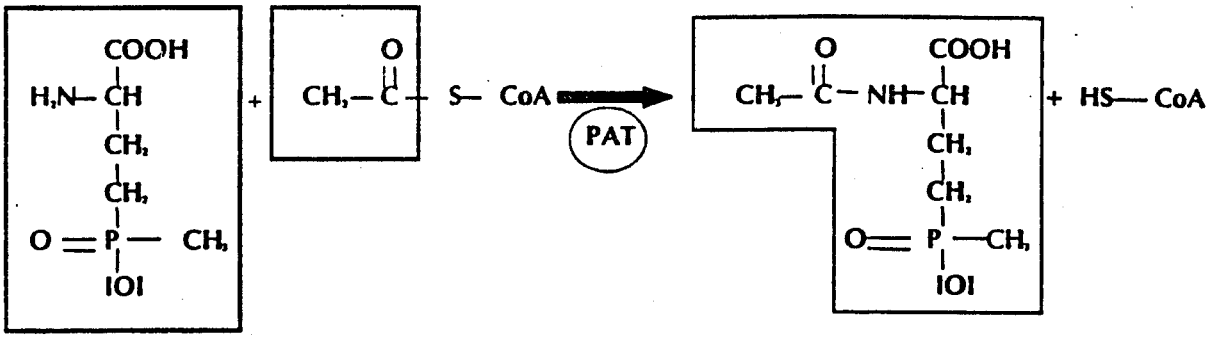
Different attempts to regenerate alfalfa plants from the GS-amplified cell line, including protoplast fusion experiments failed. Even the transfer of the gene of the target enzyme and its overexpression in transgenic tobacco plants did not lead to agronomically useful levels of glufosinate tolerance in the transgenic plants. The transgenic tobacco plants expressed the alfalfa GS at a very high level. Nevertheless the plants showed herbicidal symptoms after spraying with 2000g glufosinate/ha [Eckes et al, 1989].

6. Discovery of the resistance genes

Whereas Meiji Seika, a Japanese company producing bialaphos as a herbicidal compound, was interested to improve the bialaphos production capacity of their producer strain of *Streptomyces hygroscopicus* by molecular biological methods, Hoechst was interested to get access to the enzymes involved in the biosynthesis of phosphinothricin in order to evaluate the chance to use this knowledge for improvements of the chemical synthesis of glufosinate. Both companies cooperated in their research programs with external molecular biologists and biochemists. Meiji Seika supported a research program at Biogen whereas Hoechst supported a joint research effort at German universities, including Tübingen and Bielefeld. Both research programmes unexpectedly led to the discovery of bialaphos resistance genes in the gene cluster for the bialaphos biosynthesis related enzymes. When these bialaphos resistance genes were isolated, attempts were started at Plant Genetic Systems, to transfer the bialaphos resistance (bar) gene from *S. hygroscopicus* into crop plants [De Block et al, 1987]. Prof. Pühlers' group at the University of Bielefeld and Hoechst isolated and used the phosphinothricin-acetyl-transferase (pat) gene from *Streptomyces viridochromogenes* for their gene transfer experiments [Wohlleben et al, 1988].

Both pat and bar genes conferred a high level of glufosinate tolerance in the plants expressing the respective *Streptomyces* gene. The genes share a high degree of homology (~ 85 %). The amino acid sequence of the PAT protein and BAR protein are identical at 87 % of their amino acid positions [Wohlleben et al, 1988]. Both *Streptomyces* structural genes show a high percentage of GC pairs due to differences in the codon usage of *Streptomyces* and plants. Because cytosine can be methylated by methyltransferases and because changes in the methylation pattern of genes may affect the expression level, a synthetic pat gene was synthesized at Hoechst in which the codon usage was adopted to the codons preferred by higher plants [Strauch et al 1988]. Now in retrospect it can be said, that pat and bar genes, regardless if the latter is the natural or the synthetic gene, are equally suitable for plant transformation work and no phenotypic difference in the level of glufosinate tolerance of the plants can be observed.

Figure 3 - Inactivation of glufosinate
The enzyme phosphinothricin-acetyl-transferase (PAT) converts glufosinate in presence of Acetyl-Co-enzyme A into N-Acetyl-glufosinate. Proteinogenic amino acids are not acetylated by the enzyme. Due to the high substrate specificity, only trace amounts of PAT are sufficient to convert glufosinate into the biologically inactive metabolite.



L-glufosinate (= L-phosphinothricin) Acetyl-Co-enzyme A N-Acetyl-glufosinate Co-enzyme A

7. Transfer of the pat and bar genes into maize

For researchers interested in the development of methods for gene transfer into agricultural crops, the pat and the bar genes facilitated the selection of transgenic cells. The selection pressure in presence of appropriate glufosinate (or bialaphos) concentrations enables the selection of transgenic cells reliably especially in cereals where antibiotic resistance genes like nptII (conferring kanamycin resistance) or hptII (conferring hygromycin resistance) are either inefficient or lead to false positive nontransgenic cells and regenerants. Even a plant tissue which produces only trace amounts of the PAT enzyme is resistant against 100 – 200 mg glufosinate/l which is equivalent to 0,5 – 1 mM of the herbicide. In the late 1980 when corn transformation still was the major obstacle for the application of molecular biology for corn breeding purposes the use of the bar and pat genes revealed there superior property as a selectable marker. Within a few months independent groups proved with different transformation methods that maize transformation is routinely feasible with the pat or bar genes either by particle bombardment or by PEG (Poly-ethylen-glycol) mediated protoplast transformation protocols if the recipient corn cells are totipotent and can be regenerated into fertile plants. We transformed protoplasts of the highly embryogenic cell line HE 89 [Morocz et al 1990] with our synthetic pat gene in 1989 and were able to prove in 1990 that the transformants were glufosinate tolerant and transmitted the gene and the resistance phenotype in the T_1 and further generations [Donn et al 1990; 1992]. The segregation pattern of the pat gene inheritance followed mendelian segregation. Southern analysis revealed that the pat gene was inserted in the maize genome and was stably transmitted to the progenies. One transformant in which only one pat gene copy was integrated in the maize genome and which showed a stable and high pat gene expression over several generations of sexual propagation was used as the preferred candidate in breeding programs of several maize breeders. This transformation event is known as the T25 event.

Even though the protoplast transformation event was made with the pUC 18 plasmid carrying the p35S-pat gene construct, only a fraction of the plasmid was integrated in the maize genome. Namely the bla gene of the pUC 18 vector conferring ampicillin resistance is partly deleted. As approximately 25 % of the bla structural gene and the bacterial promoter in front of the gene are deleted, expression and functional transmission of ampicillin resistance cannot occur. Therefore in this transformant the remote chance that a functional antibiotic resistance gene can be transferred from the DNA of a transgenic plant either into soil bacteria or in bacteria of the digestion tract is completely eliminated due to the destroyed integrity of the bla gene.

Besides the glufosinate tolerance no other phenotypic changes became apparent during the different backcrossing programs based on the T25 event. Inbred lines and hybrids carrying the T25 insertion are presently under field evaluation and hybrid registration and the necessary steps for getting the full approval for hybrids based on T25 are initiated. In North America the T25 based hybrids are already commercialized after such hybrids got the full approval in 1997. The use and the properties of glufosinate-ammonium as a selective herbicide in T25 derived maize hybrids with Liberty Link® quality will be described in a separate paper [Tromas et. al 1998].

The glufosinate tolerance trait of the maize lines and hybrids with Liberty Link quality is based on the constitutive i.e. the continuous synthesis of PAT protein in all relevant plant tissues with the exception of maize pollen, where the expression level of the pat gene is below the detection level. Because the PAT protein is extremely specific, it converts no proteinogenic amino acid at measurable quantities into an acetylated product. Only phosphinothricin (L-glufosinate) and desmethyl-phosphinothricin (the precursor molecule of phosphinothricin in the biosynthetic pathways of the 2 known phosphinothricin producer strains) are accepted as substrates. The affinity to the substrate is very high [Wehrmann et al 1996]. This explains why trace amounts of the enzyme present in the transgenic cells are sufficient to convert the herbicidal active substance L-glufosinate completely into N-Acetyl-glufosinate within less than one hour. Therefore as fast as the herbicide penetrates into the cells of the transgenic crops, it is converted into the non herbicidal metabolite. The high specificity of the PAT proteins encoded from bar and pat genes is the result of the evolutionary optimization of the enzyme specificity. This was a consequence of the selection pressure not to interfere with other amino acid metabolism pathways in the phosphinothricin producing strains. This precise fit characterizes the glufosinate and the PAT/BAR inactivation system as an unique example for tailor made herbicide inactivation in crops.

8. Properties of the PAT and BAR enzymes

Besides the high substrate specificity the 2 proteins have a range of similarities in respect of pH optimum, temperature optimum and further physicochemical properties [Wehrmann et al, 1996]. Extensive studies with purified PAT and BAR proteins in gastrointestinal fluids of several mammals revealed that both proteins are degraded within seconds in gastrointestinal fluids in presence of the indigenous proteases. The digestion of both proteins is already complete after 30 seconds. Only trace amounts of these proteins are present in the crop (0,1 % of the total protein in a T25 - derived

transgenic maize plant consists of PAT protein). For both proteins computer models discovered no sequence homology to known allergenic proteins. Known allergenic plant proteins fullfill at least 2 of the following criteria 1) slow digestibility by proteases [Astwood et al, 1996], 2) abundant occurrence and 3) homology to known allergenic proteins. PAT and BAR proteins fulfill none of these criteria. These are very strong indications that PAT or BAR protein do not have an allergenic potential.

9. Glufosinate tolerant crops and the risk for the development of glufosinate resistant weeds

From the beginning of the development of transgenic herbicide tolerant crops this issue is discussed extensively. Indeed a resistance principle which works perfectly in crop species may have the same efficacy if the resistance gene would get a chance to enter the gene pool of weedy species. The question is how big is the likelihood that a gene transfer of a resistance gene from a crop to a weedy species occurs under agricultural conditions.

For corn, mankind made unvoluntarily a world wide risk assessment study over more than 30 years. Corn has an inborn resistance mechanism which confers natural resistance against triazines, whereas other plants including a wide range of weeds are susceptible against this class of herbicides. After more than 30 years of extensive triazine use on more than 100 million hectares all the spontaneously developed triazine resistant weeds carry a point mutation in the gene of the target protein of triazine action. Not a single case is known, where the resistance mechanism of corn, an efficient triazine inactivating glutathion-S-transferase, was transmitted to other species.

The spontaneous occurrence of glufosinate resistant weed ecotypes in fields after reported glufosinate treatment is not yet observed, even in vineyards where since almost 20 years the glufosinate-ammonium was applied several times per year. The mode of action of glufosinate as discussed above does not allow the development of spontaneous resistance in weed populations carrying an insensitive glutamine synthetase. In vitro mutagenesis studies with E. coli strains carrying a deletion mutation in the bacterial GS-gene and expressing the alfalfa GS-gene (DasSarma et al 1986) revealed a low number of alfalfa GS mutants under glufosinate selection. All GS mutants which showed a reduced inhibitor binding affinity for glufosinate showed a similar reduction in GS activity for its natural substrate glutamate.

Up to now no efficient glufosinate inactivating enzymes are known from nontransgenic plants. Therefore the probability of the spontaneous occurrence of novel glufosinate inactivating pathways under selection pressure is only a theoretical possibility with no practical relevance.

Over the last 14 years extensive efforts were made in my lab to generate glufosinate tolerant crops either via mutant selection or via gene transfer. Within the last 9 years at least 10^{11} protoplast derived maize cells were put under selection pressure. None of the glufosinate tolerant calli which could be regenerated into plants in presence of glufosinate was a mutant. All surviving cell colonies and regenerants with a resistance level of agronomic relevance turned out to be transformants. For other herbicidal

chemicals, namely sulfonylureas and aryloxy-phenoxypropionic acids the same cell line enabled us to select within less than one year herbicide resistant mutants, from which plants with resistance levels of agronomic relevance could be regenerated. This indicates that for different herbicides with different modes of action and different modes of resistance the likelihood of spontaneous occurrence of resistant mutants differs in several orders of magnitude.

10. Conclusions

For maize it can be stated that glufosinate tolerant varieties offer an attractive option for postemergent weed control measures. For varieties of Liberty Link quality, the highly biodegradable but nevertheless broad spectrum herbicide is safe for the crop and non target organisms and can be applied independently from the developmental stage of the crop and the weed flora.

The properties of the active ingredient and the nature of the resistance mechanism reveal that the transgenic crop and the herbicide do not impose a risk for the environment and human health.

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