Your ref
Our ref:  02/R34/4
Date    17 March 2003

Dear Sir,

ENVIRONMENTAL PROTECTION ACT 1990, SECTIONS 111 AND 112:  
CONSENT TO RELEASE GENETICALLY MODIFIED ORGANISMS

1. The Secretary of State for Environment, Food and Rural Affairs hereby grants consent 
to Syngenta Seeds Ltd, Jealott’s Hill International Research Centre, Bracknell, Berkshire, 
RG42 6EY, for application 02/R34/4 to release genetically modified organisms in 
accordance with the limitations and conditions set out in the Schedule to this consent.

2. Insofar as they relate to the protection of human health and safety, the terms and 
conditions of this consent have been agreed with the Health and Safety Executive.

3. The explanatory memorandum attached to this consent explains the limitations and 
conditions set out in the Schedule and contains important information on the general 
conditions and other provisions to which the consent is subject, but does not form part of 
the consent.

Yours faithfully

Dr L Smith
Joint Regulatory Authority

By authority of the Secretary of State for Environment, Food and Rural Affairs.
Schedule to Consent to release Genetically Modified Organisms Application Reference 02/R34/4

LIMITATIONS AND CONDITIONS


Condition 2. The release authorised is in accordance with the limitations and conditions set out in Annex 1 to this Schedule, those set out in condition 3 below and the conditions which are implied in the consent by virtue of section 112 of the Environmental Protection Act 1990.

Condition 3. The holder of the consent shall notify the following information to the Secretary of State at the times specified:

a. The general condition specified in section 112(5)(b) of the Environmental Protection Act 1990, as substituted by Regulation 29 of the Genetically Modified Organisms (Deliberate Release) Regulations 2002 (the 2002 Regulations), shall have effect subject to the specific condition that the consent holder shall submit a report on the effects of the release authorised by the consent for the assessment of any risks there are of damage to the environment from the genetically modified organism concerned:

   (i) by one month after the date of termination of the release or by 30 November 2003 whichever is the sooner and;

   (ii) by one year after the date of termination of the release or by 30 November 2004 whichever is the sooner to cover post-trial monitoring for the assessment of the effectiveness of measures to control volunteers and;

   (iii) by two years after the date of termination of the release or by 30 November 2005 whichever is the sooner to cover post-trial monitoring for the assessment of the effectiveness of measures to control volunteers and the re-evaluation of the post trial monitoring requirements.

Condition 4. The holder of the consent shall notify the Secretary of State when information regarding the genetically modified organism, as detailed in appendix 1 of Annex 1 to this Schedule, has been published and such notification shall be given as soon as practicable after the date of publication of those details.
EXPLANATORY MEMORANDUM

This memorandum explains but does not form part of the consent. It contains important information on the general conditions and other provisions to which the consent is subject.

Schedule

The Schedule to the consent sets out the limitations and conditions which are relevant to the particular release applied for.

Condition 2 sets out the scope of the release authorised by the consent. It includes everything set out in the application for consent except for any aspect which has been varied as a result of consideration of the case by the Secretary of State (including changes made on the recommendation of the Advisory Committee on Releases to the Environment and accepted by the Secretary of State).

Condition 3a specifies the information and time which the consent holder is required to notify to the Secretary of State in particular circumstances.

Condition 4 applies in all cases where an applicant has declared in the application for consent information which is commercial in confidence. Publication could involve a patent or scientific paper.

General conditions and other provisions to which the consent is subject.

Certain general conditions are implied in every consent for the release of genetically modified organisms. These conditions are set out in section 112 of the Environmental Protection Act 1990 and are summarised below.

Subject to the extent and manner of the release authorised by the consent and any particular limitations or conditions specified in the Schedule, the holder of the consent is required:

a. to take all reasonable steps to keep himself informed (by reference to the nature of the organisms and the extent and manner of the release) of any risks there are of damage to the environment being caused as a result of their being released (section 112(5)(a));

   Note: Condition (a) makes consent holders responsible for keeping themselves informed (e.g. by appropriate monitoring) of any risks to the environment which may arise at any time after the release has been carried out.

b. to notify the Secretary of State forthwith of -

i) any new information which becomes available with regard to any risks there are of damage to the environment being so caused, and

ii) any unforeseen event, occurring in connection with a release by him, which might affect the risks there are of damage to the
environment being caused as a result of their being released (section 112(5)(b) as substituted by Regulation 29(3)(a) of the 2002 Regulations);

Note: Condition b.(i) requires consent holders to notify the Secretary of State of any new information about the risks which becomes available. Condition b.(ii) is, in effect, a requirement for the consent holder to report to the Secretary of State on the outcome of the release and any unforeseen event(s), particularly in relation to any proposals to conduct further related work which may result in the marketing of a product.

c. to take such measures as are necessary to prevent damage to the environment being caused as a result of the release or, as the case may be, the marketing of the organisms (section 112(5)(c) as substituted by Regulation 29(3)(b) of the 2002 Regulations); and

d. notify the Secretary of State of the measures (if any) taken as a result of new information becoming available or an unforeseen event occurring as described in paragraph (b)(ii) above (section 112(5)(d) as substituted by Regulation 29(3)(c) of the 2002 Regulations); and

e. in a case where new information becomes available or an unforeseen event so occurs, revise the information contained in his application for a consent accordingly and supply the revised information to the Secretary of State (section 112(5)(e) as substituted by Regulation 29(3)(c) of the 2002 Regulations);

f. to take all reasonable steps to keep himself informed of developments in the techniques which may be available for preventing damage to the environment as a result of the release (section 112(7)(a)); and

g. to notify the Secretary of State forthwith if it appears at any time that any better techniques are available than is required by any of the conditions listed in the Schedule to the consent (section 112(7)(b)).

Note: Conditions c. d. e. f. and g. are all designed to ensure that the consent holder uses best available techniques not entailing excessive cost (BATNEEC) to control the effects of the release in relation to the protection of the environment, to inform the Secretary of State of those changes and to supply a revised copy of the application. If better techniques become available than those implied by the consent conditions specified in the Schedule, the conditions attached to the consent may be varied as appropriate.

Under section 111(10) of the 1990 Act, any consent granted by the Secretary of State may be revoked or varied:

The Secretary of State may at any time, by notice given to the holder of the consent, revoke the consent or vary the consent (whether by attaching new limitations and conditions or by revoking or varying any limitations and conditions to which it is at any time subject).

Note: Revocation or variation of a consent may be as the result of enforcement action, but is more likely to arise following information notified to the Secretary of
State under the terms of the consent (e.g. after the completion of a particular stage in a programme of work and before proceeding to the next stage).
PART A1: INFORMATION REQUIRED UNDER SCHEDULE 1 OF THE 2002 REGULATIONS

PART I

GENERAL INFORMATION

1. The name and address of the applicant, and the name, qualifications and experience of the scientist and of every other person who will be responsible for planning and carrying out the release of the organism and for the supervision, monitoring and safety of the release.

Name & address of Applicant:

Syngenta Seeds Ltd
Jealott's Hill International Research Centre
Bracknell
Berkshire
RG42 6EY

2. The title of the project.

The purpose of the release is to compare the pathogen infestation level and mycotoxin level of wheat modified to express an enhanced resistance to Fusarium pathogens with existing non-modified varieties, grown under standard agronomic conditions.
PART II

Information relating to the parental or recipient plant

3. The full name of the plant

(a) family name  Gramineae
(b) genus  Triticum
(c) species  aestivum
(d) subspecies
(e) cultivar/breeding line  Offspring of a conventional breeding line of UC 703 wheat into which the genes of interest have been introduced will be tested. 6 transformation events (FR001, FR003, FR005, FR006, FR007 and FR008) will be tested, along with unmodified controls.

(f) common name  Spring wheat

4. Information concerning -

(a) the reproduction of the plant:

   (i) the mode or modes of reproduction

Wheat is an allohexaploid (2n=6x=42), annual, monoecious, self-fertile plant that reproduces sexually with seed production. The inflorescence (ear) of wheat is composed of a set of spikelets arranged on a central axis (rachis) that provides a base for the cleistogamous flowers enveloped by glumes. The flowering of the ear is not synchronised, it is spread over two to three days. The first spikelet to flower is usually located in the upper third of the ear. Flowering subsequently progresses on both sides, and more rapidly towards the top. On the inside of each spikelet, flowering also occurs in a staggered manner.

   (ii) any specific factors affecting reproduction

Wheat flowers display characteristics that do not favour cross-pollination. Under natural circumstances the pollination of wheat relies mainly on self-pollination (average level of 97-99%). The flowers’ degree of openness is considered as a varietal characteristic, but environmental conditions at the time of the anthesis may blot out the differences among genotypes (Hucl, 1996). Compared to other allogamous grasses, the production of pollen by a wheat ear is very limited, 10% and 2.5% compared to that of rye and maize inflorescence respectively (de Vries, 1971). Wheat pollen can be dispersed by the wind (David
& Pham, 1993) but dispersal is limited because the pollen is heavy (de Vries, 1971, Anand and Sharma, 1974).

Within the specific context of hybrid wheat production, viable pollen dispersal studies have been conducted, in the absence of any pollen competition, using male sterile wheat plants (Porter et al 1966, 1967, Wilson 1968, Stroskopf and Rai 1972, Anand and Beri 1971, de Vries 1974). The dispersal of pollen was assessed over distances of up to 30 metres, by measuring the percentage of grains formed on the male sterile plants. A considerable amount of variability was observed among the results obtained, probably due to the differences in experimental design (genotypes, size of the pollinator plots, environmental factors etc.) leading to very variable degrees of pollen loads on the female flowers’ stigmata. Wheat pollination has been observed on male sterile plants at distances of around 30 metres from the pollen emitting source.

Wheat pollen is sensitive to environmental conditions; its viability varies from 1 min to approximately 30 min in optimal field conditions of 20°C, 60% relative humidity (Poehlman 1987). The longest life expectancy (2 hours) was observed under optimal lab conditions of 5 °C and a relative humidity of 60% (D'Souza, 1970).

(iii) generation time

Spring wheat is an annual crop and the generation time from sowing to harvesting the grains can be estimated at 5-7 months. The sowing in the south of England usually takes place from February with a harvest in August/September.

(b) the sexual compatibility of the plant with other cultivated or wild plant species, including the distribution in Europe of the compatible species.

Soft wheat or *Triticum aestivum* is a member of the *triticeae* grass tribe and is made up of 3 genomes (ABD, 2n = 6x = 42). All the species of this tribe, which includes, amongst others, the *Aegilops*, *Agropyron*, *Secale*, *Haynaldia*, *Hordeum*, *Elymus* and *Elytrigia* genera, have a basic number of 7 chromosomes. In each of these genera there are some species that can hybridise with wheat, however embryo rescue techniques are required (Johnson 1966, Sharma and Gill 1983, Zhao et al, 2000). Intergeneric hybrids can also be obtained by growing embryos in vitro (Lu et al 1990, Tabaeizadeh et al 1990, Cox et al 1991). Usually most interspecific or intergeneric hybrids obtained with wheat display a very high level of male sterility. They can produce offspring by means of pollination by the parental species or by a closely related species (Mann, 1987).

There are a small number of *Aegilops* species that have the potential to hybridise with wheat in the UK under natural conditions; *Ae. biuncialis*, *Ae. cylindrica*, *Ae. neglecta*, *Ae. peregrina*, *Ae. speltoides and Ae. triuncialis* (Kimber & Feldman,
1987, Van Slageren, 1994, Raybould and Brown, 2001). All species of Aegilops are annuals, often growing in dry, open habitats, not on managed agricultural land. The species described are most prevalent in Southern Europe, and are not indigenous in the UK. Additionally it is likely that the frequency of any spontaneous hybrid would be very low and these natural hybrids would be highly likely sterile (David et al 2000, Claesson et al 1990). In a search of the County Flora Data (from Kent to Devon) none of the Aegilops species were recorded (Davy, personal communication).

Wild Triticum species, T. monococcum and T. turgidum, are also potential candidates for cross pollination, but have not been recorded in the UK. They are native in Mediterranean countries.

Agropyron ssp. is widespread in the UK and particularly Agropyron repens, also known as couch grass, which is one of the most common weeds. Even though Agropyron repens is in constant contact with wheat, the existence of spontaneous hybrids capable of survival and multiplication in nature have not been reported. A hybrid was described by Comeau et al, 1985; this hybrid was obtained by means of treatments after the fertilization combined with rescue of the embryos by means of in vitro culture.

Barley (Hordeum vulgare), rye (Secale cerealis) and triticale are plants cultivated in the UK.

All attempts to hybridise wheat and barley remained fruitless until 1973 (Fedak, 1992), which is when the first Barley x Wheat hybrid was described in literature (Kruse, 1973). This hybrid was obtained artificially by treatment with gibberillic acid, followed by rescue of the embryos by means of in vitro culture.

Manual cross-breeding between wheat and rye, resulting in a progeny called triticale, is carried out in order to combine the high grain yield and protein quality of wheat with rye’s disease resistance and tolerance to poor soil conditions. There are only a few reports on natural hybridisation between wheat and rye (OECD, 1999). For example spontaneous hybridisation between wheat and rye occurred in 1918 with wheat cultivars exhibiting anemophilic flower characters under dry continental conditions.

It is possible for wheat pollen to fertilise triticale plants and this is often a technique used by plant breeders (Bernard, 1992). However, it is still an intensively managed process and does not occur spontaneously.

5. Information concerning the survivability of the plant:

(a) its ability to form structures for survival or dormancy
Spring wheat is an annual crop that reproduces by means of seed. Seeds remaining on the soil before or during harvest may overwinter and germinate the following spring. However seed is unlikely to survive longer than 2 years due to lack of dormancy (Field agronomist, personal communication) and volunteers are easily controlled under standard agricultural conditions.

(b) any specific factors affecting survivability

The survivability of the plant will be dependent on a number of factors including climatic conditions, the variety of wheat and agronomic conditions (pests, diseases, etc.). Wheat is a cereal of temperate climates. It does not compete or establish itself outside of the agricultural environment. Wheat plants are not competitive against UK native flora because of non-brittle spikes leading to lack of seed dispersal.

6. Information concerning the dissemination of the plant:

(a) the means and extent (such as an estimation of how viable pollen and/or seed declines with distance where applicable) of dissemination

The most obvious route for dissemination is by pollen dispersal using wind, or through seed.

Dissemination by pollen has been addressed in Section 4(a)(ii).

Dissemination of seed is rare under natural conditions as wheat has non-brittle spikes leading to lack of seed dispersal. However dissemination of wheat seed may occur by birds or small mammals eating the seed.

(b) any specific factors affecting dissemination

*Triticum aestivum*, being a self-fertile plant, does not produce large amounts of pollen (David and Pham, 1993). The flower morphology does not favour insect pollination but pollen can be dispersed by wind (anemophily). However this is likely to be limited because wheat pollen is heavy and therefore cannot move over great distances. In addition, wheat pollen does not remain viable for long periods (see 4(a)(ii)).

In the course of its domestication, wheat lost its capacity to disperse its seeds and now entirely depends on mankind for its cultivation (Feldman *et al*, 1995).
7. The geographical distribution of the plant

Wheat depends on mankind for its geographical dispersion. This is the world’s most cultivated crop and the main agricultural crop in the UK. The cultivation of wheat tends to be localised in both hemispheres between the 40th and 60th parallels. Wheat is practically absent from equatorial and tropical areas. Thus, the main production areas are located in Europe, from South East England to the Ukraine, in northern India, in the plains of northern China, the plains of North America, the Argentine Pampa, the extreme south of Africa and Australia. The annual worldwide production of wheat is approximately 540 million tons and wheat crops occupy in the region of 225 million hectares spread across the 5 continents. In the UK approximately 2 million hectares of *T. aestivum* are cultivated per year, of which 1.9 million in England.

8. Where the application relates to a plant species that is not normally grown in the United Kingdom, a description of the natural habitat of the plant, including information on natural predators, parasites, competitors and symbionts.

Not applicable

9. Any other potential interactions, relevant to the genetically modified organism, of the plant with organism in the ecosystem where it is usually grown, or elsewhere, including information on toxic effects on humans, animals and other organisms.

Certain pests (mainly including eelworms, slugs, wireworms, wheat bulb flies, cereal leaf roller, aphids and gall midges) and certain fungal (fusarioses, rust fungi, mildew, leaf spots, foot rot, etc.) or viral diseases (mosaic disease, yellow dwarf virus, nanism, etc.) can infest the crop. Ectomycorrhizal and arbuscular mycorrhizal fungi are mutualistic symbionts found on the majority of cultivated plants. They do not use mycotoxins in the establishment of their interactions with plants.

With regard to human health, two potential interactions should be considered; (i) gluten intolerance and coeliac disease, (ii) pollen allergies.

(i) The cells of the grain’s endosperm are filled with starch granules fitted tightly in a network of proteins. When mixed with water, these proteins, gliadins and glutenins, form gluten. Some people cannot digest gluten and thus develop wheat flour intolerance or a related allelopathy, known as coeliac disease. This intolerance only becomes apparent upon ingestion, and may go undiagnosed due to
vague symptomology. Those known to suffer avoid gluten containing foods throughout their lifetime.

(ii) Wheat pollen, like the pollen from all other plants, can cause allergic reactions in certain susceptible people when inhaled.
Part III
Information relating to the genetic modification

10. A description of the methods used for the genetic modification

Transformation procedures using the biolistics method, were carried out at Syngenta Biotechnology Inc, Research Triangle Park, North Carolina, USA. (See Appendix 1 Confidential Business Information).

After transformation, the cells were transferred onto the selection medium containing mannose. In the cell clusters that develop on this medium, the presence of the fungal resistance gene (FRG) is screened for using PCR.

11. The nature and source of the vector used

The plasmids used for the transformation were derived from the pUC19 plasmid described by Yanisch-Perron et al (1985).

<table>
<thead>
<tr>
<th>TABLE 1 : Specification of the plasmids:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequences</strong></td>
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<tr>
<td>-------------</td>
</tr>
<tr>
<td>Promoter</td>
</tr>
<tr>
<td>Gene</td>
</tr>
<tr>
<td>Terminator</td>
</tr>
<tr>
<td>Intron</td>
</tr>
<tr>
<td>Ampicillin resistance gene controlled by a prokaryotic promoter sequence.</td>
</tr>
</tbody>
</table>

Prior to plant transformation, each plasmid was cut using restriction enzymes in order to release the insert containing the gene of interest from the vector sequences (including the fragment carrying the bacterial marker that confers resistance to ampicillin). Following digestion, the fragments containing the genes of interest were separated from the backbone plasmid fragments using gel purification methods.

<table>
<thead>
<tr>
<th>TABLE 2: Specification of Restriction Enzymes Used</th>
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<tbody>
<tr>
<td><strong>Plasmid</strong></td>
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<td>----------------</td>
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</tbody>
</table>

13
12. The size, intended function and name of the donor organism or organisms of each constituent fragment of the region intended for insertion

The maps of the donor plasmids pZMLR 14 and pZMLR 69 are shown in figures 1 and 2 below.

**Fig 1: Plasmid map of pZMLR 14**

**Fig 2: Plasmid map of pZMLR 69**
Table 3 below describes the nature and source of the DNA sequences within pZMLR 14 and pZMLR 69.

**TABLE 3 : Vector components of plasmids pZMLR14 and pZMLR69**

<table>
<thead>
<tr>
<th>Coding sequence</th>
<th>Size</th>
<th>Function and origin of the sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubi</td>
<td>2000 bp</td>
<td>Promoter of a maize ubiquitin gene together with the 1st exon and the 1st intron of the gene (Christensen et al, 1992).</td>
</tr>
<tr>
<td>FRG</td>
<td>1380 bp</td>
<td>cDNA isolated from a fungal species, coding for an enzyme capable of conferring a <em>Fusarium</em> tolerance characteristic. (See Appendix 1 Confidential Business Information)</td>
</tr>
<tr>
<td>PMI</td>
<td>1103 bp</td>
<td>Phosphomannose Isomerase Gene isolated from <em>Escherichia coli</em> (Miles and Guest, 1984). This gene is used as a marker for the transformation, and allows positive selection on mannose (Bojsen et al 1994, Joersbo et al 1998, Reed 1999, Negrotto et al 2000).</td>
</tr>
<tr>
<td>Intron #9pepc (I-9)</td>
<td>107 bp</td>
<td>Intron isolated from the maize phosphoenolpyruvate carboxylase gene (pep-c) (Hudspeth and Grula, 1989); its role is to stimulate the expression of the PMI gene.</td>
</tr>
<tr>
<td>35S term</td>
<td>77 bp</td>
<td>Termination sequence of the cauliflower mosaic virus gene.</td>
</tr>
<tr>
<td>Nos term</td>
<td>250 bp</td>
<td>Termination sequence of the nopaline synthase gene, isolated from <em>Agrobacterium tumefaciens</em> (Depicker et al 1982, Bevan et al 1983)</td>
</tr>
</tbody>
</table>
Part IV

Information relating to the genetically modified plant

13. A description of the trait or traits and characteristics of the genetically modified plant which have been introduced or modified.

6 individual transformation events will be used in this trial. They have integrated:

- a gene coding for a protein capable of conferring a *Fusarium* fungal resistance characteristic (See Appendix 1 Confidential Business Information).

- a gene, used as a marker gene for selection, which allows the plant to grow on a medium containing mannose. Mannose tolerance is conferred by the *PMI* gene isolated from *Escherichia coli*. In plants and other organisms, mannose intervenes in the intermediary metabolism of the glycolytic pathway; it is phosphorylated into mannose-6-phosphate by a hexokinase. Naturally, most plants are not capable of using it as a carbon source, it accumulates and the cells stop growing. Mannose-6-phosphate is known to inhibit phosphoglucose isomerase, thus blocking glycolysis (Goldsworthy and Street, 1965). In addition, its synthesis depletes cells of orthophosphate that is required for ATP production (Goldsworthy and Street, 1965, Loughman 1966, Joersbo et al., 1998). The *PMI* gene codes for a protein, Phosphomannose Isomerase, which converts mannose-6-phosphate into fructose-6-phosphate, a metabolisable sugar, which enters glycolysis directly. Plants containing the *PMI* gene are capable of transforming mannose into fructose-6-phosphate. After transformation, the cells and seedlings are submitted to positive selection, by culture on regeneration medium containing mannose as the sole carbon source; only regenerated plants expressing the *PMI* gene are thus capable of growth. Non-transformed tissue either stops growing or dies due to starvation (Wright et al., 2001).

14. The following information on the sequences actually inserted or deleted:

(a) The size and structure of the insert and methods used for its characterisation, including information on any parts of the vector introduced into the genetically modified plant or any carrier or foreign DNA remaining in the genetically modified plant

See Figures 1 and 2 for the plasmid maps and Table 3 for vector components.
Molecular analysis was used to detect the presence of the fungal resistance and PMI genes.

Events FR001, FR003 and FR005 were characterized by Southern blot technique on foliage samples taken from homozygous plants. Results described in Appendix 3 show that: (i) the profile obtained on Southern blot for the fungal resistance gene indicated a complex insertion with several copies for FR001 and FR003 and a truncated copy for FR005 and (ii) the profile for PMI indicated that several copies were inserted in FR001 and FR003, whereas no copy was detected in FR005.

Events FR006, FR007 and FR008 have been subject to preliminary analysis by Southern blot techniques on foliage sample. These initial studies suggest that there is one copy of the FRG and one copy of PMI gene in each event. The intactness of the inserts and the presence of additional fragments from the FRG or PMI gene are being further investigated using Southern analysis.

The method used for the transformation was biolistics. Prior to co-transformation into the plant, the DNA fragments (containing either the FRG or the PMI gene) were separated from plasmid backbone DNA using standard gel purification methods. It is therefore unlikely that vector backbone will be contained within the plant.

(b) the size and function of the deleted regions

Not applicable.

(c) the copy number of the insert

Copy number determined with the data obtained so far.

<table>
<thead>
<tr>
<th>Transformation event</th>
<th>FR001</th>
<th>FR003</th>
<th>FR005</th>
<th>FR006</th>
<th>FR007</th>
<th>FR008</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of copies of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fungal resistance</td>
<td>&gt;4</td>
<td>2</td>
<td>1 truncated</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>gene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of copies of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMI gene</td>
<td>&gt;3</td>
<td>&gt;5</td>
<td>Not detected</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

(d) the location or locations of the insert or inserts in the plant cells (whether it is integrated in the chromosome, chloroplasts,
mitochondria, or maintained in a non-integrated form) and the methods used for its determination.

The inheritance of the novel genes, during sexual crosses follows the mendelian laws of genetics. Therefore, it is concluded that the novel genes are integrated into the nuclear chromosomes.

15. The following information on the expression of the insert:

(a) information on the developmental expression of the insert during the lifecycle of the plant and methods used for its characterisation.

The fungal resistance gene is under the control of the ubiquitin promoter isolated from maize. Its expression is expected to fit the expression pattern of the Maize ubiquitin promoter in wheat, which is constitutive (Christensen et al 1992, Stoger et al 1999, Rooke et al, 2000). It is therefore likely to be expressed through the plant at all stages of development.

Expression of the PMI and FRG genes transferred has been verified by:

PMI
- selection of the modified plants on a medium containing mannose,
- germination on mannose of T1 seeds of some events, resulting from self-pollination of primary transformant

FRG
- measurement of enzyme activity in leaf tissue,
- visual assessments showing increased resistance to Fusarium.

(b) parts of the plant where the insert is expressed, such as roots, stem or pollen.

See above 15 (a).

16. Information on how the genetically modified plant differs from the parental or recipient plant in the following respects:

(a) mode or modes and/or the rate of reproduction
Wheat reproduces sexually by the production of seed. As the intended effect of the modification is to enhance resistance to fungal attack, there is no evidence or reason to believe that the modification will affect the mode or modes of reproduction. The lines described here have been through a number of sexual cycles. They did not differ compared to non-modified lines in terms of flowering or seed-set. Evidence from previous field trials suggests that the genetically modified lines do not differ from the recipient plant in mode or rate of reproduction.

(b) dissemination

The most likely routes of dissemination are through the seed and pollen. The genetically modified wheat lines have been through several generations of self-pollination. The plants of each generation flowered normally and produced seed. Evidence from greenhouse and field trials suggests that the genetically modified lines do not differ from the recipient plant in mode or rate of dissemination.

As the intended effect of the modification is to increase tolerance of the plant to *Fusarium* pathogen, thereby decreasing mycotoxin levels, it is not envisaged that the genetic modification will affect the dissemination of the plant.

(c) survivability

The survival structure and dispersal agent of wheat is the seed. Plants of the genetically modified lines to be used have been grown to maturity in the glasshouse over several generations. They flowered normally and produced seed. There is no evidence from previous field trials in the USA, Canada, Argentina and UK to suggest the modification affects the survivability of the plant.

17. The genetic stability of the insert and phenotypic stability of the genetically modified plant.

The genetically modified plants were produced in 1998 and in 2000. Since that time the seed has been through three generations (FR006, FR007 and FR008) or four generations (FR001, FR003 and FR005) of self pollination with stable expression of the transgene effect. Therefore it is likely that the inserts have been stably integrated into the plant’s genome.

18. Any change to the ability of the genetically modified plant to transfer genetic material to other organisms.
The most obvious route for the transfer of genetic material to other organisms is through pollen dispersal. Wheat is a predominantly self-pollinating crop. It produces relatively small amounts of pollen (only 2.5% of that produced by a maize plant, de Vries, 1971). Wheat pollen is sensitive to environmental conditions and has a short viability. In the case of pollen dispersal, cross-pollination occurs by wind (anemophily) and wheat pollen, which is fairly heavy, is not dispersed over great distances. As a result outcrossing normally occurs only with adjacent plants. Field experiments carried out by Wilson (1968) found 10 percent seedsetting on male sterile wheat plants that were 30m from the pollen donor plants. Wheat does not exist as a wild plant, other than as volunteers in a managed agricultural environment.

Wheat has the potential to cross-pollinate with wild *Aegilops* and *Triticum* species. However according to a search of the County Flora Data (from Kent to Devon), (Davy personal communication), none of the *Aegilops* species have been recorded in the South of Britain. No wild *Triticum* species able to cross-pollinate with wheat have been found in the UK (Raybould and Brown, 2001).

The intended effect of the modification is to increase the plants tolerance to *Fusarium* infection. In previous field trials, genetically modified plants developed and flowered normally and no evidence for changes in flower morphology could be detected.

To our knowledge there is no evidence to suggest that gene transfer from plant to bacterium occurs under natural conditions. Several studies, reviewed by Bertolla and Simonet (1999), have been conducted to assess whether transgenes from genetically modified plant have the potential to be transferred to soil or plant associated microorganisms. None of these studies have shown a transfer of non-homologous DNA from plant to bacteria. In optimised *in vitro* conditions *Acinetobacter calcoacticus* has been transformed with homologous plant DNA but the frequencies remained quite low (Gebhard and Smalla, 1998). In the soil the steps required for the DNA to be transferred from a plant to a recipient bacterium involve a succession of very selective barriers (stage of competence, adsorption and efficient uptake of DNA, chromosomal integration via recombination). Therefore if such transfer occurs at all *in situ*, it occurs at extremely low frequencies.

There is no evidence or reason to believe that the modification will change these characteristics.

No products from the trial will enter the human food or animal feed chain.

19. Information on any toxic, allergenic or harmful effects on human health and the environment arising from the genetic modification.

There is no reason to believe that there will be any toxic, allergenic or harmful effects on human health and the environment arising from the genetic
modification, since the intended effect is enhanced resistance to *Fusarium* pathogens. Field trials carried out previously in the USA, Canada, Argentina and UK have shown no adverse effects to humans or the environment.

No products from the field trial will enter the human food or animal feed chain.

Two novel proteins are produced by the modified wheat, FRG and PMI. There is no evidence in the literature of toxic effects of the proteins synthesised by the introduced genes. Both protein sequences show no homology to known toxins or allergens at the 6 amino acid level. Additionally *in vitro* digestibility tests demonstrated a rapid breakdown of the proteins at 37°C with pepsin. This indicates that the proteins are unlikely to be allergens.

Further information on the FRG is given in Appendix 1 Confidential Business Information.

The *PMI* gene, isolated from *Escherichia coli*, codes for a protein, phosphomannose isomerase, which allows the reciprocal conversion of mannose-6-phosphate into fructose-6-phosphate. This gene is omnipresent in nature, except for non-leguminous plants. Initial safety studies on this enzyme have been conducted (Privalle *et al* 2000). These tests include a toxicity test and a study on the impact on agronomic characteristics and nutritional composition:

- An acute toxicity study conducted on mice showed that no clinical symptoms of toxicity were detected at a dose of 3030mg PMI/kg bodyweight. Based on the lack of adverse findings, the LD50 value was determined to be greater than this level.

- The comparison between the glycoprotein profile of genetically modified maize and beet plants expressing the *PMI* gene and that of control maize and beet plants (that do not possess this gene) did not show any changes.

- A study on maize did not show any impact of the expression of the gene on the plant’s agronomic characteristics or on the nutritional composition of the grains, under the experimental conditions applied.

20. **Information on the safety of the genetically modified plant to animal health, particularly regarding any toxic, allergenic or other harmful effects arising from the genetic modification, where the genetically modified plant is intended to be used in animal feedstuffs.**

This is a small scale research trial and no products from the field trial will enter the human food or animal feed chain.
21. The mechanism of interaction between the genetically modified plants and target organisms.

The target organisms are the *Fusarium* isolates present in the test region, particularly *Fusarium poae*, *Fusarium graminearum* and *Fusarium culmorum*, which are pathogenic fungi of cereals (wheat, barley and maize) and very widespread worldwide (Reid and Hamilton, 1996). These fungi form part of the *Fusarium* complex responsible for the disease called fusariosis, which causes considerable damage to the ears. In the UK, the disease is encountered on wheat cultivated throughout England and Wales and a recent survey has shown that the incidence of *Fusarium* head blight in winter wheat was above average in 2001 (CSL report 2001).

By enhancing the plants capability to modify the fungal toxin, the objective of the field release is to assess the damage to wheat ears due to infection by *Fusarium* and to assess the levels of the main mycotoxins likely to be affected by the expression of this gene.

(See Appendix 1 Confidential Business Information)

22. The potential changes in the interactions of the genetically modified plant with non-target organisms resulting from the genetic modification.

Non-target organisms, which may interact with the trial plants are fauna feeding on the plant or seed or insect herbivores which may visit the plant. As the intended effect of the modification is to increase the tolerance of the plant to *Fusarium* pathogen, it is unlikely that this will have an effect on non-target organisms. Non-target organisms already live in close proximity to fungi e.g. *Fusarium* species that contain this enzyme. No significant ill-effects have been reported. In addition, plants of the genetically modified lines to be used have been grown in the field previously and no adverse effects on human health or the environment were observed.

The inserted genes are from naturally occurring organisms, the fungal resistance gene derived from a fungal species and a marker gene encoding phosphomannose isomerase (*PMI*) from *E.coli*. Both are commonly found in the environment.

Other non target organisms are ectomycorrhizal and arbuscular mycorrhizal fungi that live in symbiosis with plants. They do not use mycotoxins in the establishment of their interactions with plants and therefore the insertion of the gene of interest into wheat should not affect these fungi.
Therefore the expression of the FRG and PMI gene are not anticipated to change the interactions of the genetically modified plants with non-target organisms.

23. The potential interactions with the abiotic environment.

No effects on biogeochemical processes arising out of interactions between the modified wheat and the target *Fusarium* fungal pathogens are anticipated. The modification is specifically designed to detoxify the mycotoxin produced by the fungal disease on infection, the expression product of the introduced gene is not acting directly on the pathogen, but on the toxic component that it produces and biogeochemical processes are not expected to be altered in any way by this.

Effects on biogeochemical processes as a result of effects on non-target organisms are likely to be the same as those effects resulting from cultivation of non-modified wheat.

*There is no indication that either protein breakdown or foliar decay will be altered by the modified wheat plant material. The introduced genes already occur naturally in the environment. These genes are therefore unlikely to affect biogeochemical processes.*

No adverse effects were noted from wheat plants with the same genetic modification released in the field in the USA, Canada, Argentina and UK.


The modified plants can be identified by several techniques:

- in tissue culture, explants will regenerate on medium containing mannose.
- PCR and genomic Southerns can be used to demonstrate the presence of the inserted DNA
- enzyme assays to show the activity of the protein encoded by the FRG.

25. Information about previous releases of the genetically modified plants.
The transformation events were planted in the field in the United States and Canada between 2000 and 2002. Additionally some of these events were released in Argentina and in UK in 2001 and 2002 respectively.
Part V

Information relating to the site of release

26. The location and size of the release site or sites.

The release will take place at Syngenta’s research site at Jealott’s Hill International Research Centre, (Grid Ref SU 874 738) approximately 6km away from the nearest town, Bracknell. There have been releases of genetically modified plants since 1989 at the site. There are many farm premises and hamlets surrounding the research site.

The field trial is located within the research centre, where the responsible scientist can supervise all field aspects of the trial.

The total size of the release plot will be no more than 35m x 40m. The proposed trial plan is shown in Appendix 2. The genetically modified wheat along with non-modified conventional lines will be sown into an area no more than 20m x 25m. The border will be comprised of at least 5m of unmodified wheat plants and these are included in the total trial size. The border rows are included to minimise plot edge effects.

27. A description of the release site ecosystem, including climate, flora and fauna.

The field trial will be carried out in a closely controlled agricultural ecosystem and records of previous crops and treatments are available for the trial site and the environs.

In 2003 a range of crops including cereals will be grown on the site. The nearest wheat crops will be planted at least 50m away from the transgenic trial site. These are trial crops for agrochemical research and development purposes only. The flora and fauna is typical of a lowland pasture grazing arable ecosystem.

A series of surveys of wild plants species occurring on the site have been carried out over the years and details of the local flora and fauna are available. Between 1965-2002, in excess of 1200 species were noted on the Jealott’s Hill Site. The survey is extensive and can be supplied on request.

The climate in Berkshire is temperate.

28. Details of any sexually compatible wild relatives or cultivated plant species present at the release sites.
In 1978, between April and September, a survey was carried out across the entire site at Jealott’s Hill International Research Centre. A list of all wild plant species observed was compiled. The site was divided into 250 survey squares and plant frequencies estimated for each. Subsequent surveys of selected areas on the site were carried out in June 1989 and between May and June in 1990. The last survey was carried out between May and October 2002 across the entire site. No wild representatives of the genus *Triticum* L. or *Aegilops* species were recorded in the surveys.

Whilst wheat plants may be present at the release site, they will be at least 50m away from the transgenic trial site. These crops on Syngenta land are being grown as part of agrochemical field trials, for research and development purposes only. There is potential for wheat, rye and triticale crops to be planted on non-Syngenta land at a distance of no less than 100m from the trial.

**29. The proximity of the release sites to officially recognised biotopes or protected areas which may be affected.**

There are no environmentally sensitive areas but BBONT (Berks, Bucks and Oxon. Naturalist Trust) have a site at Chawridge Bank which is approximately 3km away from the proposed release site. Further details can be found in a previous field release application, 93/R1/1.

The site at Chawridge Bank is unlikely to be affected by the trial, due to the 3km distance involved and the lack of wild relatives that could potentially cross-pollinate with wheat in the South of England.

In addition, local interest groups such as beekeepers and organic crop farmers will be kept informed.
Part VI

Information relating to the release

30. The purpose of the release of the genetically modified plant, including its initial use and any intention to use it as or in a product in the future.

The purpose of the release is to compare the pathogen infestation level and mycotoxin level of wheat modified to express an enhanced resistance to *Fusarium* fungal pathogens with existing non-modified varieties, grown under standard agronomic conditions. The genes have been introduced into a research breeding line and therefore the genetically modified plants released in this field trial will not be used as a product in the future.

31. The foreseen date or dates and duration of the release.

Release is anticipated between February 2003 and end October 2003 at the latest.

Seed samples taken for laboratory analysis or seeds stored for further analysis will be clearly labelled as being genetically modified and will be stored and disposed of under contained conditions (as required by 98/81/EC). Any seed sent for analysis off the Jealott’s Hill site will be clearly labelled as being genetically modified and will be disposed of under contained conditions (as required by 98/81/EC, or equivalent).

32. The method by which the genetically modified plants will be released.

Genetically modified wheat seed may be sown mechanically using a seed drill, or by hand. The border of unmodified wheat seed will be drilled using a seed drill. Netting may be used to protect the seed/seedlings from environmental or fauna damage if necessary.

33. The method for preparing and managing the release site, prior-to, during and after the release, including cultivation practices and harvesting methods.

The release site will be prepared and managed in accordance with good trials practice for wheat. For this specific trial it means:
• The plot may, if necessary, be treated with a total vegetative herbicide prior to the release and emerging weeds will be removed from the plot prior to the release.

• The total size of the trial will be no more than 35m x 40m including the border rows. The genetically modified wheat seed along with non-modified conventional lines will be sown, into an area no more than 25m x 20m which will be surrounded by a border of at least 5m of unmodified wheat seed. The border plants will serve to minimise edge effects.

• Netting may be used to protect the seed/seedlings from environmental or fauna damage if necessary.

• The trial will be visited at least monthly for observations and to ensure that the appropriate action is taken to control weeds, pests and diseases. The border row plants will be treated with a suitable, selective herbicide should the need arise due to weed pressure.

• Samples of mature wheat ears will be harvested manually from the genetically modified and non-genetically modified plants for mycotoxin analysis. The ears thus harvested will be gathered in duly identified bags and stored under contained conditions on site (as required by 98/81/EC). Seed samples taken for laboratory analysis, will be clearly labelled as being genetically modified and will be stored and disposed of under contained conditions (as required by 98/81/EC, or equivalent).

• At the end of the trial, the remaining ears from the wheat plants in the plots will be harvested mechanically. Both will be bagged and incinerated. After harvest the mechanical harvester will be cleaned at the trial site and verified for the absence of seeds. A total vegetative control herbicide will then be applied to the trial area. Plant material remaining after treatment will be pulverised and incorporated into the soil, as soon as the agronomic and environmental conditions allow. This is typically within a month of treatment. To help with volunteer control prior to incorporation, any residual grain will be left on the surface of the trial plot and encouraged to germinate by rain or irrigation.

• All border plants will be treated in the same manner as transgenic at the end of the trial.

34. The approximate number of genetically modified plants (or plants per m²) to be released.
No more than 450 genetically modified seeds per $m^2$ will be sown in the trial. The genetically modified plants along with non-modified conventional lines will be released into an area measuring no more than 25m x 20m surrounded by a border of no less than 5m of unmodified wheat plants. The genetically modified plants will be released into no more than 56m$^2$ plots. The total size of the trial will be no more than 35m x 40m.

The proposed trial plan is shown in Appendix 2.
Part VII

Information on control, monitoring, post-release plans and waste treatment plans

35. A description of any precautions to -

(a) maintain the genetically modified plant at a distance from sexually compatible plant species, both wild relatives and crops.

Agropyron repens is a common weed of wheat. However there are no reports of the spontaneous production and survival of wheat x A. repens hybrids. There are a small number of Aegilops species in the UK that have the potential to be pollinated naturally by wheat (Raybould and Brown, 2001). Although hybridations are possible, the frequency of the appearance of hybrids under natural conditions remains low, and these natural hybrids are highly sterile, although grains have sometimes been found (Van Slageren 1994, Cook et al 1992, David et al 2000, Claesson et al 1990). These Aegilops species are found in Mediterranean regions and are not indigenous in the UK. In a search of the County Flora Data (from Kent to Devon) none of the Aegilops species were recorded (Davy, personal communication). In addition, surveys carried out across Jealott’s Hill International Research Centre did not detect any wild representatives of the genus Triticum L. or Aegilops species (see 28 above).

Whilst wheat species may be present at the release site, they will be at least 50m away from the transgenic trial site. These crops on Syngenta land are being grown as part of agrochemical field trials, for research and development purposes only. There is potential for wheat, rye and triticale crops to be planted on non-Syngenta land at a distance of no less than 100m from the trial.

A border of at least 5m of unmodified wheat plants will serve to minimise plot edge effects.

(b) any measures to minimise or prevent dispersal of any reproductive organ of the genetically modified plant (such as pollen, seeds, tuber).

Pollen dispersal:

Wheat pollen is heavy and remains viable during a very short time span, generally not for more than approximately 30 minutes (see 4 (a)(ii)). Pollen dispersal is a rare phenomenon as wheat is 97-99 % autogamous.
Minimum isolation distances recommended for the production of certified seed range between 0 – 100m. Levin and Kerster (1974) mention 1.5 – 3m as a minimum in order to maintain varietal purity in the seeds of *Triticum aestivum*.

- A border no less than 5m wide of conventional wheat will surround the test site. This border will be treated in the same manner as transgenic at the end of the trial.

- Any wheat, rye, barley and triticale species either on Syngenta land or on non-Syngenta land will be at least 50m away from the release site.

The trial plan is shown in Appendix 2

**Seed dispersal:** this can occur at the time of sowing or harvesting.

- After sowing, the seed drill, if used, will be cleaned and verified for the absence of any seeds.

- The ears from genetically modified plants as well as the ears from the conventional control plants for analysis will be harvested manually. The ears thus harvested will immediately be gathered in duly identified bags and stored under contained conditions on site (as required by 98/81/EC).

- The wheat ears remaining in the plots will be harvested by hand and the wheat grain from the border rows will be harvested mechanically. Both will be bagged and incinerated. After harvest the mechanical harvester will be cleaned at the trial site and verified for the absence of seeds. A total vegetative control herbicide will then be applied to the trial area. Herbicide treated plant material remaining after treatment will be pulverised and incorporated into the soil, as soon as the agronomic and environmental conditions allow. This is typically within a month of treatment.

To help with volunteer control prior to incorporation, any residual grain will be left on the surface of the trial plot and encouraged to germinate by rain or irrigation.

- Any ungerminated seed remaining after sowing or viable seeds produced as a result of the trial could produce volunteers. A crop other than cereal will be grown for two years after termination of the trial and will allow the clear identification of volunteers. Any wheat volunteers emerging will be removed and incinerated or destroyed by treatment with an appropriate herbicide.
36. **A description of the methods for post-release treatment of the site or sites.**

The trial will be harvested as described in 33 above. After harvest a total vegetative control herbicide will be applied to the trial area. Herbicide treated plant material remaining after treatment will be pulverised and incorporated into the soil, as soon as the agronomic and environmental conditions allow. This is typically within a month of treatment.

To help with volunteer control prior to incorporation, any residual grain will be left on the surface of the trial plot and encouraged to germinate by rain or irrigation.

Any seeds that remain viable may emerge as wheat volunteers. A crop other than cereal will be grown for two years after termination of the trial and will allow the clear identification of volunteers. Any volunteers emerging will be removed and incinerated or destroyed by treatment with an appropriate herbicide. The site will be monitored for two years post harvest.

37. **A description of post-release treatment methods for the genetically modified plant material including wastes.**

Samples of mature wheat ears will be harvested manually from the genetically modified and non-genetically modified plants for mycotoxin analysis. The ears thus harvested will be gathered in duly identified bags and stored under contained conditions on site (as required by 98/81/EC).

Seed samples taken for laboratory analysis will be clearly labelled as being genetically modified and will be stored and disposed of under contained conditions (as required by 98/81/EC, or equivalent).

At the end of the trial the wheat ears remaining in the plots will be harvested by hand and the wheat grain from the border rows will be harvested mechanically. Both will be bagged and incinerated. After harvest the mechanical harvester will be cleaned at the trial site and verified for the absence of seeds. A total vegetative control herbicide will then be applied to the trial area. Herbicide treated plant material remaining after treatment will be pulverised and incorporated into the soil, as soon as the agronomic and environmental conditions allow. This is typically within a month of treatment.
To help with volunteer control prior to incorporation, any residual grain will be left on the surface of the trial plot and encouraged to germinate by rain or irrigation.

All border plants will be treated in the same manner as transgenic at the end of the trial.

Any seeds that remain viable may emerge as wheat volunteers. A crop other than cereal will be grown for two years after termination of the trial and will allow the clear identification of volunteers. Any volunteers emerging will be removed and incinerated or destroyed by treatment with an appropriate herbicide. The site will be monitored for two years post harvest. If during the monitoring procedure, concerns arise as to the persistency of the plants the monitoring period will be extended.

38. A description of monitoring plans and techniques.

The release site will be prepared and managed in accordance with good trials practice for wheat. Monitoring plans and techniques are described in 33 above.

The trial will be visited at least monthly for observations and to ensure that the appropriate action is taken to control weeds, pests and diseases. The border row plants will be treated with a suitable, selective herbicide should the need arise due to weed pressure.

Any seeds that remain viable may emerge as wheat volunteers. A crop other than cereal will be grown for two years after termination of the trial and will allow the clear identification of volunteers. Any volunteers emerging will be removed and incinerated or destroyed by treatment with an appropriate herbicide. The site will be monitored for two years post harvest. If during the monitoring procedure, concerns arise as to the persistency of the plants the monitoring period will be extended.


If necessary the wheat plants can be easily destroyed using a herbicide. After application of the herbicide, the plot will be treated as described in 37 above and monitored as described in 38 above. If during the monitoring procedure, concerns arise as to the persistency of the plants the monitoring period will be extended.

In the event of small scale vandalism, individual plants uprooted or damaged will be removed and destroyed by incineration. A decision may be taken to replant if it does not compromise the scientific validity of the trial. In the event of large
scale vandalism where it is deemed the trial cannot continue, the trial will be terminated by the application of a herbicide and treated as described in 37 above.

A crop other than cereal will be grown for two years after termination of the trial to enable volunteers to be controlled effectively. Wheat volunteers will be removed and incinerated or destroyed by treatment with an appropriate herbicide.

The site will be monitored for two years post harvest. If during the monitoring procedure, concerns arise as to the persistency of the plants the monitoring period will be extended.

In the case of vandalism to the trial and if emergency response plans need to be implemented, Syngenta Senior Management and DEFRA will be informed immediately.

40. Methods and procedures to protect the site.

Netting may be used to protect the seed/seedlings from environmental or fauna damage if necessary.
Part VIII

Information on methodology

41. A description of the methods used or a reference to standardised or internationally recognised methods used to compile the information required by this Schedule, and the name of the body or bodies responsible for carrying out the studies.

The methods to produce information and support of this field trial application have been quoted throughout the document.
APPENDIX 2 WHEAT TRIAL 2003 PROPOSED FIELD PLAN

Border

Trial wheat

Proposed access to the

≤ 35 m

≤ 20 m

≤ 5 m
APPENDIX 3   SOUTHERN BLOT ANALYSIS

DNA EXTRACTION

Homozogous seeds from events FR001, FR003, FR005 and wild-type UC703 were surface sterilized in 20% Clorox for 30 minutes, washed several times in sterile water, and germinated between layers of germination paper in the dark at 26°C for 10 days. Root and leaf tissue from positive plants was used for genomic DNA extraction. DNA was extracted according to Dellaporta et al., (Dellaporta S, Freeling, M. and Walbot, V. eds (1994) The Maize Handbook, Springer-Verlag). An additional RNase treatment was added, followed by phenol, phenol/chloroform, and chloroform extractions before the final DNA precipitation. DNA was quantified using a Shimadzu UV-1601 spectrophotometer and a small sample run on a gel to confirm high-molecular weight status.

SOUTHERN BLOT

Fungal resistance gene

Method

Five micrograms of genomic DNA was digested with the following restriction enzymes at 37°C for approximately 22 hours. Restriction enzymes SphI and SpeI were used to indicate promoter:gene intactness. Restriction enzyme Nhel was used to indicate copy number. One-half microgram of plasmid pZMLR69 was also digested with SphI and SpeI for control purposes. The digested genomic DNA, plasmid control, and molecular weight markers (Novagen Perfect DNA Markers) were run at constant current on a 0.8% TAE agarose gel overnight. The gel was photographed, denatured in 0.3 N NaOH, 1.5 M NaCl for 30 minutes. The DNA was transferred by capillary action to Zeta-probe GT nylon membrane overnight using 0.3 N NaOH, 1.5 M NaCl. The membrane was washed in 2X SSC, air dried, and cross-linked by UV exposure in a Stratagene UV Stratalinker 1800. The membrane was treated with Zeta-Probe prehybridization solution (0.25M NaPO₄, pH 7.2 and 7% SDS) at 65°C for several hours. Fresh solution was added along with randomly primed ³²P labeled 669 bp Fusa1 PCR fragment. The membranes were hybridized overnight at 65°C and washed as follows: 20mM NaPO₄, pH 7.2, 5% SDS - 2 times 30 minutes at 65°C; 20mM NaPO₄, pH 7.2, 1% SDS - 30 minutes at 65°C. The membrane was exposed to autoradiography film at -80°C.
Southern Analysis

Southern analysis shows the following: Event FR001 contains at least 4 copies of the fungal resistance gene. Some of those copies are intact, others are truncated. The intensity of the intact copy band is approximately equal to the 3 copy control band. Event FR003 appears to have 2 intact copies as judged by the intensity of the intact band and the number of insertion sites. Event FR005 has one truncated copy.
Annex 1 to the Schedule to consent 02/R34/4 issued on 17th March 2003

A: Novagen MW markers, (kb)

Intactness  B: UC 703 cut with SpeI and SphI
            C: FR001 cut with SpeI and SphI
            D: Data from a line not described in this application
            E: FR003 cut with SpeI and SphI
            F: Data from a line not described in this application
            G: FR005 cut with SpeI and SphI
            H: pZMLR69 cut with SpeI and SphI; 1 copy (expected size- 3447 bp)
            I: pZMLR69 cut with SpeI and SphI; 3 copies
            J: pZMLR69 cut with SpeI and SphI; 5 copies

Copy number  K: UC 703 cut with Nhel
              L: FR001 cut with Nhel
              M: Data from a line not described in this application
              N: FR003 cut with Nhel
              O: Data from a line not described in this application
              P: FR005 cut with Nhel
Q: Novagen MW markers, (kb)

PMI gene

Method

Six micrograms of genomic DNA was digested with the following restriction enzymes at 37°C for approximately 22. Restriction enzymes HindIII and PvuII were used to indicate promoter:gene intactness. Restriction enzyme BamHI was used to indicate copy number. See plasmid map for enzyme placement. One-half microgram of plasmid pZMLR14 were also digested with HindIII and PvuII for control purposes. The digested genomic DNA, plasmid controls, and molecular weight markers (Novagen Perfect DNA Markers) were run at constant current on a 0.8% TAE agarose gel overnight.

The gel was photographed then denatured in 0.3 NaOH, 1.5 M NaCl for 30 minutes. The DNA was transferred by capillary action to Zeta-probe GT nylon membrane overnight using 0.3 NaOH, 1.5 M NaCl.

The membrane was washed in 2X SSC, air dried, and cross-linked by UV exposure in a Stratagene UV Stratalinker 1800. The membrane was treated with Zeta-Probe prehybridization solution (0.25M NaPO4 pH7.2 and 7% SDS) at 65°C for several hours. Fresh solution was added along with random-primed 32P labeled 514 bp PMI PCR fragment. The membrane was hybridized overnight and was washed as follows: 20mM NaPO4 pH 7.2, 5% SDS - 2 times 30 minutes at 65°C; 20mM NaPO4 pH 7.2, 1% SDS - 30 minutes at 65°C. The membrane was exposed to autoradiography film at -80°C.

Southern Analysis

Southern analysis shows the following: Event FR001 has at least one insertion site and probably contains at least 3 copies of the PMI gene based on the intensity of the intact band. Event FR003 has at least three insertions and five or more copies of the PMI gene based on intensity of the intact copy. FR005 has no detectable PMI.
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