



**Australian Government**  
**Department of Health and Ageing**  
**Office of the Gene Technology Regulator**

**Risk Assessment and  
Risk Management Plan for  
DIR 067/2006**

**Limited and controlled release of  
waterlogging tolerant GM cotton**

Applicant: CSIRO

December 2006

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# Executive Summary

## **Introduction**

The Gene Technology Regulator (the Regulator) has made a decision to issue a licence for dealings involving the intentional release of genetically modified (GM) cotton lines which are tolerant to waterlogging, into the environment in respect of application DIR 067/2006 from the Commonwealth Scientific and Industrial Research Organisation (CSIRO).

The DIR 067/2006 licence permits the release of up to 30 GM cotton lines on a limited scale and under controlled conditions.

The *Gene Technology Act 2000* (the Act) and the *Gene Technology Regulations 2001* (the Regulations) govern the process undertaken by the Regulator before a decision is made on whether or not to issue a licence. The decision is based upon a Risk Assessment and Risk Management Plan (RARMP) prepared by the Regulator in accordance with the *Risk Analysis Framework* and in consultation with a wide range of experts, agencies and authorities and the public.

More information on the comprehensive assessment required for licence applications to release a genetically modified organism (GMO) into the environment is available from the Office of the Gene Technology Regulator (OGTR) (Free call 1800 181 030) or at <http://www.ogtr.gov.au/>.

## **The application**

CSIRO applied for a licence to release up to 30 GM cotton lines with tolerance to waterlogging into the environment under limited and controlled conditions. The trial is to take place at one site in the shire of Narrabri, New South Wales (NSW) on a maximum total area of 0.1 ha during each of the three summer growing seasons of 2006/07, 2007/08 and 2008/09.

The GM cotton lines contain a new gene (*AHb1*) derived from thale cress that encodes a protein (AHB1) expected to provide tolerance to waterlogging stress. A variant of the *AHb1* gene also occurs naturally in cotton as well as a number of other plant species and the AHB1 protein is common in many food plants such as barley, maize, wheat, rice, soybean and tomato.

The GM cotton lines also contain a bacterial gene (*nptII*, conferring resistance to the antibiotics kanamycin or neomycin) that was used to select successfully modified plants during initial research and development work in the laboratory.

The purpose of the trial is to conduct early stage ('proof of concept') research to measure the expression levels of the introduced gene; to evaluate the tolerance of the GM cotton plants to waterlogging stress under simulated conditions; and to conduct a preliminary assessment of their agronomic performance in the field. Cotton seed will be collected for further studies and possible future releases (subject to additional approvals). No products from the release will be used for human food, animal feed or for the production of fabrics and/or other cotton products.

CSIRO proposed a number of measures to limit the spread and persistence of the GMOs and the introduced genetic materials that were considered during the evaluation of the application.

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## **Risk assessment**

The hazard identification process considered the circumstances by which people or the environment may be exposed to the GMOs, GM plant materials, GM plant by-products, the introduced genes, or products of the introduced genes.

A hazard (source of potential harm) may be an event, substance or organism. A risk is identified when a hazard is considered to have some chance of causing harm. Those events that do not lead to an adverse outcome, or could not reasonably occur, do not advance in the risk assessment process.

Eighteen events were identified and assessed whereby the release of the GM cotton lines might give rise to harm to people or the environment.

These 18 events included consideration of whether expression of the introduced genes could result in products that are toxic or allergenic to people or other organisms, alter characteristics that may impact on the spread and persistence of the GM plants, or produce unintended changes in their biochemistry or physiology. In addition, consideration was given to the opportunity for gene flow to other organisms and its effects.

None of the 18 events are considered to give rise to an identified risk that requires further assessment. The principle reasons comprise:

- small scale of the trial that is limited in both area and duration
- containment and disposal measures proposed by the applicant to limit the spread and persistence of the GM plants
- none of the GM plant materials will be used for human food, animal feed or for the production of fabrics and/or other cotton products
- widespread presence of the same or similar proteins encoded by the introduced genes in the environment and lack of known toxicity or allergenicity from these proteins
- limited capacity of the GM cotton lines to spread and persist in the area of the release
- limited ability and opportunity for the GM cotton lines to transfer the introduced genes to other sexually related species.

Therefore, any risks of harm to the health and safety of people, or the environment, from the release of the GM cotton lines into the environment is considered to be **negligible**.

## **Risk management**

The risk management process builds upon the risk assessment to determine whether measures are required in order to protect people and/or the environment. As none of the 18 events identified and characterised in the risk assessment are considered to give rise to an identified risk that requires further assessment, the level of risk is considered to be **negligible**.

The Regulator's *Risk Analysis Framework* defines negligible risks as insubstantial, with no present need to invoke actions for their mitigation. However, containment and disposal measures have been imposed to restrict the release to the size, duration and location to those requested by the applicant, as these were an important part of establishing the context for assessing the risks.

The licence conditions require the applicant to limit the duration of the release to three summer cotton growing seasons (2006/07, 2007/08 and 2008/09) on a maximum total area of 0.1 hectares per season; prevent the use of the GMOs, or materials from the GMOs for any

other purposes; maintain physical isolation of the release site; and conduct post-harvest monitoring to ensure all GM plants are destroyed<sup>1</sup>.

### ***Conclusions of the RARMP***

The risk assessment concludes that this limited and controlled release of up to 30 GM cotton lines with tolerance to waterlogging in the shire of Narrabri, NSW, poses **negligible** risks to the health and safety of people and the environment posed by or as a result of gene technology.

The risk management plan concludes that these negligible risks do not require specific risk treatment measures. However, licence conditions have been imposed to contain the release to the size, duration and location requested by the applicant.

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<sup>1</sup> The licence for DIR 067/2006 is available on the OGTR website (<<http://www.ogtr.gov.au/gmorec/ir.htm#table>> via the link to DIR 067/2006).

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## Abbreviations

<i>adh</i>	Alcohol dehydrogenase
Agmobiol	Agricultural Molecular Biology Laboratory of Peking University
<i>Ahb1</i>	<i>Arabidopsis thaliana</i> non-symbiotic haemoglobin gene
AHB1	<i>Arabidopsis thaliana</i> non-symbiotic haemoglobin protein
ANZFA	Previous name for Food Standards Australia New Zealand
APVMA	Australian Pesticides and Veterinary Medicines Authority
AQIS	Australian Quarantine and Inspection Service
asp	asparagine
ATP	Adenosine triphosphate
CaMV	Cauliflower mosaic virus
CRC	Cooperative Research Centre
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DIR	Dealing involving Intentional Release
DNA	Deoxyribonucleic acid
EFB	European Federation of Biotechnology
ELISA	Enzyme-linked Immunosorbent Assay
EMBL	European Molecular Biology Laboratory
FAO	Food and Agriculture Organization of the United Nations
Farrp	Food Allergy Research and Resource Program
FDA	Food and Drug Administration
FSANZ	Food Standards Australia New Zealand (formerly ANZFA)
GenBank	Genetic sequence databank
GM	Genetically Modified
GMAC	Genetic Manipulation Advisory Committee
GMO	Genetically Modified Organism
GTTAC	Gene Technology Technical Advisory Committee
kDa	1000 Dalton, unit of relative molecular mass
kg	Kilogram
LD <sub>50</sub>	Amount of a substance given in a single dose that causes death in 50% of a test population of an organism
m	Metre
mg	Milligram
mL	Millilitre
µg	Microgram
µM	Microliter
NADH	Nicotinamide adenine dinucleotide
NHMRC	National Health and Medical Research Council
NICNAS	National Industrial Chemicals Notification and Assessment Scheme
NO	Nitric oxide
<i>nos</i>	Gene encoding nopaline synthase
<i>nptII</i>	Gene encoding neomycin phosphotransferase type II
NPTII	Product of the <i>nptII</i> gene
nsHb	Non-symbiotic haemoglobin
NSW	New South Wales
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator

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PC2	Physical containment level 2
PCR	Polymerase Chain Reaction
<i>pdv</i>	pyruvate (2-oxo-acid) carboxylase
QLD	Queensland
RARMP	Risk Analysis and Risk Management Plan
SDAP	Structural Database of Allergenic Proteins
ser	serine
SCSV	Subterranean clover stunt virus
S4S4	Promoter from Subterranean clover stunt virus
T-DNA	Transfer deoxyribonucleic acid
TGA	Therapeutic Goods Administration
thr	threonine
UK	United Kingdom
US FDA	United States Food and Drug Administration
USA	The United States of America
WHO	World Health Organisation

# Technical Summary

## Introduction

The Gene Technology Regulator (the Regulator) has decided to issue a licence (DIR 067/2006) to the Commonwealth Scientific and Industrial Research Organisation (CSIRO) for dealings involving the intentional release of genetically modified (GM) cotton lines into the Australian environment, on a limited scale and under controlled conditions.

The DIR 067/2006 licence permits the limited and controlled release of up to 30 GM cotton lines with tolerance to waterlogging. The release will occur on one site in the shire of Narrabri on a maximum total area of 0.1 ha during each of the three summer growing seasons of 2006/07, 2007/08 and 2008/09.

The *Gene Technology Act 2000* (the Act), the *Gene Technology Regulations 2001* (the Regulations) and corresponding State and Territory law govern the comprehensive and highly consultative process undertaken by the Regulator before making a decision whether to issue a licence to deal with a GMO.

The Regulator's *Risk Analysis Framework* explains the approach used to evaluate licence applications and to develop the Risk Assessment and Risk Management Plans (RARMPs) that form the basis of her decisions<sup>2</sup>.

This RARMP for DIR 067/2006 has been finalised in accordance with the gene technology legislation. Matters raised in the consultation process regarding risks to the health and safety of people and the environment from the proposed dealings were taken into account by the Regulator in deciding to issue a licence and the licence conditions that have been imposed.

## Application

Title:	Limited and controlled release of waterlogging tolerant cotton*
Applicant:	CSIRO
Common name of the parent organism:	Cotton
Scientific name of the parent organism:	<i>Gossypium hirsutum</i> L.
Modified traits:	Waterlogging tolerance, antibiotic resistance
Identity of the genes responsible for the modified traits:	<ul style="list-style-type: none"> <li>• <i>AHb1</i> (non symbiotic haemoglobin), from <i>Arabidopsis thaliana</i> (waterlogging tolerance gene);</li> <li>• <i>npII</i> (neomycin phosphotransferase type II) from the bacterium <i>Escherichia coli</i> (antibiotic resistance selectable marker)</li> </ul>
Proposed location:	One site per season in the shire of Narrabri (NSW)
Proposed release size:	Up to 0.1 hectare per season over 3 seasons
Proposed time of release:	Summer seasons 2006/07, 2007/08 and 2008/09

\* The title of the licence application submitted by the applicant is *Evaluation under field conditions of cotton plants expressing a phytohaemoglobin protein*.

<sup>2</sup> More information on the assessment of licence applications and copies of the *Risk Analysis Framework* are available from the Office of the Gene Technology Regulator (OGTR). Free call 1800 181 030 or at <<http://www.ogtr.gov.au/ir/process.htm>> and <<http://www.ogtr.gov.au/pdf/public/raffinal2.2.pdf>>, respectively.

CSIRO applied for a licence to release up to 30 GM cotton lines with tolerance to waterlogging into the environment on a limited scale and under controlled conditions. The trial is intended to take place at one site in the shire of Narrabri, New South Wales (NSW) on a maximum total area of 0.1 ha during each of the three summer growing seasons of 2006/07, 2007/08 and 2008/09.

The GM cotton lines contain between one and eight copies of the non-symbiotic phytohaemoglobin gene (*AHb1*), derived from thale cress (*Arabidopsis thaliana*) in different positions in the genome. This gene encodes a protein (AHB1) which is induced under conditions of oxygen deficit (hypoxia) that is expected to provide tolerance to waterlogging. Non-symbiotic haemoglobins are common in many food plants such as barley, maize, wheat, rice, soybean and tomato.

The GM cotton lines also contain an antibiotic resistance gene, *nptII* (derived from *Escherichia coli*) conferring resistance to the antibiotics kanamycin and neomycin, that was used to select successfully modified plants during initial research and development work in the laboratory.

The GM cotton lines were derived from the cotton cultivar Coker 315 which is not grown commercially in Australia. The purpose of the release is to conduct early stage ('proof of concept') research to measure the expression levels of the *AHb1* gene; to evaluate the tolerance of the GM cotton plants to waterlogging stress under simulated conditions; and to conduct a preliminary assessment of their agronomic performance in the field. Cotton seed will also be collected for further studies and possible future releases (subject to additional approvals).

The applicant proposed measures to limit the spread and persistence of the GM cotton lines in the environment. These were taken into account in establishing the risk assessment context for the release, and their suitability for limiting the release to the size, duration and location proposed by the applicant was considered as part of the risk assessment process. No products from the GM cotton plants will be used for human food, animal feed or for the production of fabrics and/or other cotton products.

### **Risk assessment**

The risk assessment considered information contained in the application, previous GM cotton assessments, current scientific knowledge, and issues relating to risks to human health and safety and the environment raised in submissions received from consultation with a wide range of prescribed experts, agencies and authorities on the application (summarised in Appendix B of the RARMP). No issues were raised in the comments received on the consultation version of the RARMP that required further analysis or consideration (Appendix D of the RARMP).

The consideration of advice received from a member of the public on the application is summarised in Appendix C.

A reference document, *The Biology and Ecology of Cotton (Gossypium hirsutum) in Australia*, was produced to inform the risk assessment process for licence applications involving GM cotton plants. The document is available from the OGTR or from the website <<http://www.ogtr.gov.au>>.

The hazard identification process considered the circumstances or events by which people or the environment may be adversely affected by exposure to the GMOs, GM plant materials, GM plant by-products, the introduced genes, or products of the introduced genes.

A hazard (source of potential harm) may be an event, substance or organism. A risk is identified when a hazard is considered to have some chance of causing harm. Those events that do not lead to an adverse outcome, or could not reasonably occur, do not advance in the risk assessment process.

Eighteen events were identified and assessed whereby the release of the GM cotton lines might give rise to harm to people or the environment.

These 18 events included consideration of whether expression of the introduced genes could result in products that are toxic or allergenic to people or other organisms, alter characteristics that may impact on the spread and persistence of the GM plants, or produce unintended changes in their biochemistry or physiology. In addition, consideration was given to the opportunity for gene flow to other organisms and its effects.

None of the 18 events are considered to give rise to an identified risk that requires further assessment. The principle reasons comprise:

- small scale of the trial that is limited in both area and duration
- containment and disposal measures proposed by the applicant to limit the spread and persistence of the GM plants
- none of the GM plant materials will be used for human food, animal feed or for the production of fabrics and/or other cotton products
- widespread presence of the same or similar proteins encoded by the introduced genes in the environment and lack of known toxicity or allergenicity from these proteins
- limited capacity of the GM cotton lines to spread and persist in the area for release
- limited ability and opportunity for the GM cotton lines to transfer the introduced genes to other sexually related species.

Therefore, as no risks to the health and safety of people, or the environment were identified from the limited and controlled release of the GM cotton lines the level of risk is considered to be **negligible**.

### ***Risk management***

A risk management plan builds upon the risk assessment to consider whether any action is required to mitigate the identified risks, and what can be done to protect the health and safety of people and the environment.

As none of the 18 events that were characterised in the risk assessment process are considered to give rise to an identified risk that requires further assessment, the level of risk to human health and safety and the environment from the release of GM cotton lines is considered to be **negligible** (ie insubstantial with no present need to invoke actions for their mitigation).

However, containment measures have been imposed to restrict the release to the size, duration and location requested by the applicant, as these were important parameters in establishing the context for assessing the risks.

### **Licence conditions to manage this limited and controlled release**

A number of licence conditions have been imposed to limit and control the release, including requirements to:

- surround the release site with a pollen trap
- locate the release site at least 50 m away from natural waterways

- harvest and gin seed cotton from the release separately from any other cotton crop
- not permit cotton seed or other materials from the release to be used in human food, animal feed or for the production of fabrics and/or other cotton products
- destroy all plant materials remaining at the site after harvest
- clean the site and any equipment used on the site
- conduct regular inspections of the release site following harvest for at least 12 months (and until six consecutive months have passed without any volunteer cotton plants) and destroy any volunteers prior to flowering.

### **Other regulatory considerations**

Australia's gene technology regulatory system operates as part of an integrated legislative framework. The Regulator sought input on the preparation of the RARMP from other agencies that also regulate GMOs or GM products including Food Standard Australia New Zealand (FSANZ), Australian Pesticides and Veterinary Medicines Authority (APVMA), Therapeutic Goods Administration (TGA), National Industrial Chemicals Notification and Assessment Scheme (NICNAS), National Health and Medical Research Council (NHMRC) and Australian Quarantine Inspection Service (AQIS). Dealings conducted under a licence issued by the Regulator may also be subject to regulation by one or more of these agencies<sup>3</sup>.

FSANZ is responsible for human food safety assessment, including GM food. As the trial involves early stage research the applicant does not intend any material from the GM cotton lines to be used in human food. Accordingly the applicant has not applied to FSANZ to evaluate any of the GM cotton lines. FSANZ approval would need to be obtained before they could be used in human food.

### **Identification of issues to be addressed for future releases**

In recognition of the very early stage of research, the risk assessment identified additional information that may be required to assess an application for a larger scale trial, reduced containment conditions or a commercial release of any of these GM cotton lines. This would include:

- molecular characterisation of the introduced genetic materials in the GM cotton lines, genotypic stability, and expression levels of the introduced gene in the GM cotton lines
- data on the potential toxicity of plant material from the GM cotton lines including levels of known endogenous toxins
- data on the allergenicity of the protein encoded by the introduced gene for waterlogging tolerance, and allergenicity of plant material from the GM cotton lines
- details of the survival of the waterlogging tolerant GM cotton lines compared with non-GM cotton in the environment, particularly in habitats such as natural waterways, wetland areas and areas affected by high rainfall or flooding where the GM cotton may have a selective advantage
- biochemical, physiological and agronomic characteristics of the GM cotton lines indicative of weediness including measurement of tolerance to environmental stresses

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<sup>3</sup> More information on Australia's integrated regulatory framework for gene technology is contained in the *Risk Analysis Framework* available from the Office of the Gene Technology Regulator (OGTR). Free call 1800 181 030 or at <<http://www.ogtr.gov.au/pdf/public/raffinal2.2.pdf>>.

(eg drought or pathogen infection) and reproductive capacity (eg growth rate and window of flowering).

### ***Conclusions of the RARMP***

The risk assessment concludes that this limited and controlled release of GM cotton lines with tolerance to waterlogging stress on a maximum of 0.1 ha per annum for 3 years in the shire of Narrabri, NSW poses **negligible** risks to the health and safety of people and the environment posed by or as a result of gene technology.

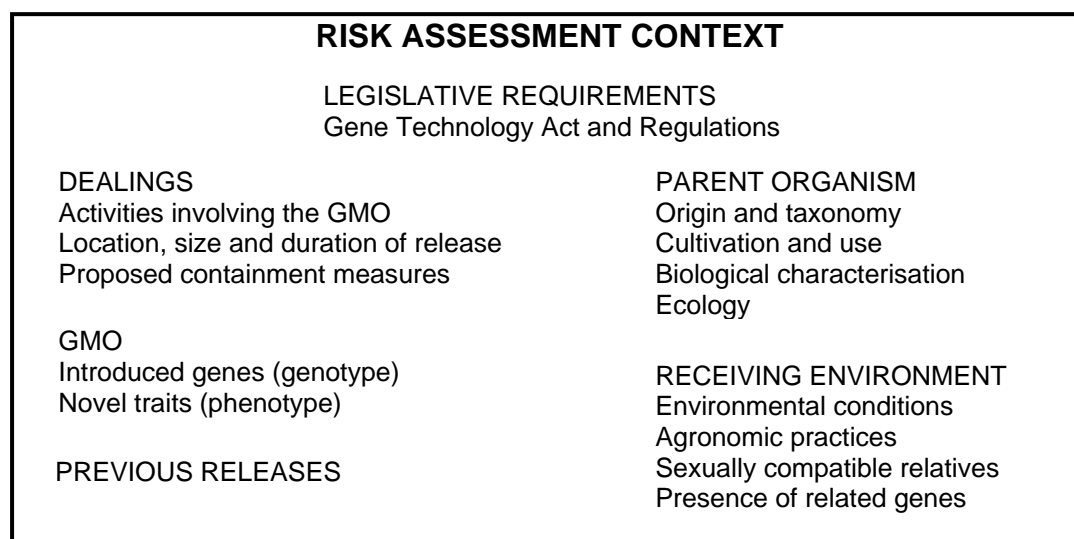
The risk management plan concludes that these **negligible** risks do not require specific risk treatment measures. However, licence conditions have been imposed to limit the release to the size, duration and location requested by the applicant, as these were important parameters in establishing the context for assessing the risks.

# Chapter 1 Risk assessment context

## Section 1 Background

1. This Chapter describes the parameters within which risks that may be posed to the health and safety of people and the environment by the release are assessed. These include the scope and boundaries for the evaluation process required by the gene technology legislation<sup>4</sup>, details of the intended dealings, the GMO(s) and parent organism(s), previous approvals and releases of the same or similar GMOs in Australia or overseas, environmental considerations and relevant agricultural practices. The parameters for the risk assessment context are summarised in Figure 1.

**Figure 1 Components of the risk context considered during the preparation of the Risk Assessment**



2. Sections 49 to 51 of the *Gene Technology Act 2000* (the Act) outlines the matters, which the Regulator must take into account, and who she must consult with, in preparing the RARMPs that form the basis of her decision on licence applications.

3. For this application, establishing the risk assessment context includes consideration of:

- the size, duration and location of the trial proposed by the applicant
- containment measures for the GMOs proposed by the applicant
- characteristics of the parent organism
- the nature and effect of the genetic modification
- the environmental conditions in the location where the release would occur
- relevant agricultural practices

<sup>4</sup> The legislative requirements and the approach taken in assessing licence applications are outlined in more detail at <http://www.ogtr.gov.au/ir/process.htm> and in the *Risk Analysis Framework* (OGTR 2005) <http://www.gov.au/pdf/raffinal2.2.pdf>.

- presence of related GM and non-GM cotton in the environment
- presence of the introduced or similar genes and their encoded proteins in the environment
- any previous releases of these or other GMOs relevant to this application.

4. Initial consideration of the application under section 49 of the Act determined that public consultation was not required for the preparation of the consultation version of the RARMP. In accordance with section 50 of the Act, the Gene Technology Technical Advisory Committee (GTTAC), State and Territory governments, prescribed Australian Government agencies, the Minister for Environment and Heritage and the local council where the release would take place were consulted on matters relevant to the preparation of the RARMP. This advice, and where it was taken into account in the RARMP, is summarised in Appendix B. The consideration of a submission from the public is summarised in Appendix C.

5. In accordance with section 52 of the Act, the Regulator notified the public when the consultation version of the RARMP had been prepared and invited written submissions. No submissions were received from the public on the consultation RARMP. Advice on the RARMP was also sought from the same experts, agencies and authorities as before. None of the latter raised any issues relating to risks to human health and safety and the environment that required further consideration (Appendix D).

## **Section 2 The application**

6. CSIRO proposes to release up to 30 GM cotton lines into the environment under limited and controlled conditions. The cotton lines have been modified to provide tolerance to waterlogging stress.

### **2.1 The proposed size, duration and location**

7. The release is proposed to take place at one site in the shire of Narrabri, New South Wales (NSW) on a maximum total area of 0.1 ha during each of the three summer growing seasons of 2006/07, 2007/08 and 2008/09.

### **2.2 The proposed dealings**

8. The purpose of the proposed trial is to conduct early stage ('proof of concept') research to enable a preliminary assessment of the agronomic performance of the GM cotton lines, both with and without waterlogging.

9. The applicant proposes to examine the capacity of the GM cotton lines expressing a non-symbiotic phytohaemoglobin gene (*AHb1*) from *Arabidopsis thaliana* to tolerate waterlogging stress. The plants will be sampled periodically for biochemical and molecular assays of the *AHb1* gene. The applicant proposes to harvest the cotton bolls by hand and to collect the seed for further studies and possible future releases (subject to additional approvals). No products from the release would be used for human food, animal feed or for the production of fabrics and/or other cotton products.

### **2.3 The proposed measures to limit the spread and persistence of the GMOs**

10. The applicant proposes a number of measures to limit the spread and persistence of the GM cotton lines into the environment. These are taken into account in the risk assessment context (this Chapter) and their suitability is evaluated in Chapter 2 for limiting the release to the size, duration and location proposed by the applicant.

11. The applicant has proposed a number of containment measures including:
- locating the proposed trial site 50 m away from natural waterways or other cotton breeding areas
  - surrounding the trial site by a 20 m pollen trap of non-GM or commercially released GM cotton (Bollgard II®) and treating all plants in this area in the same way as the GM cotton plants proposed for release
  - destruction of all plant materials other than some materials collected for future research or planting
  - post harvest monitoring of trial site for 12 months and destroying any volunteer cotton plants
  - transportation of GM cotton seed and plant materials to and from the proposed trial sites in accordance with OGTR transportation guidelines
  - storage of GM plant materials (required for further study or future release) in certified PC2 facilities.

### **Section 3 The parent organism**

12. The parent organism is cultivated cotton (*Gossypium hirsutum* L.), which is exotic to Australia and is grown as an agricultural crop in NSW, and southern and central QLD. However, the cultivar which has been transformed, Coker 315, is not grown here commercially. More detailed information on cotton can be found in the document, *The Biology and Ecology of Cotton (Gossypium hirsutum) in Australia*, which was produced to inform the risk assessment process for licence applications involving GM cotton plants (OGTR 2002). This document is available at <<http://www.ogtr.gov.au>>.

### **Section 4 The GMOs, nature and effect of the genetic modification**

#### **4.1 Introduction to the GMOs**

13. The GM cotton lines proposed for release contain the non-symbiotic phytohaemoglobin gene, *AHb1*, derived from *Arabidopsis thaliana*. The lines differ in the number of copies of the gene (between one and eight copies) that have been introduced and their positions in the genome. The *AHb1* gene encodes a non-symbiotic phytohaemoglobin protein (AHB1). Expression of this protein is induced during hypoxia (oxygen deficit) and over-expression of the *AHb1* gene in *Arabidopsis* improves tolerance to hypoxia (Hunt et al. 2002; Hebelstrup et al. 2006). The AHB1 protein from *Arabidopsis* is 88% similar to the protein naturally found in cotton (information provided by applicant). Non-symbiotic phytohaemoglobins (nsHb) are common in many food plants such as barley, maize, wheat, rye, rice, soybean, and tomato (Taylor et al. 1994; Andersson et al. 1996; Hunt et al. 2001).

14. The GM cotton lines also contain an antibiotic resistance selectable marker gene, neomycin phosphotransferase type II (*nptII*). The *nptII* gene encoding for the enzyme neomycin phosphotransferase was originally derived from the common gut bacterium *Escherichia coli*, and confers kanamycin and neomycin resistance on the GM plant. The *nptII* gene was used as a selective marker during early stages of development of GM plants in the laboratory.

15. Short regulatory sequences (promoters and transcription terminator sequences) that control expression of the genes are also present in all the GM cotton lines. These are derived

from a plant *Flavaria bidentis*, the bacterium *Agrobacterium tumefaciens* and two plant viruses, Subterranean clover stunt virus (SCSV) and Cauliflower mosaic virus (CaMV). Although *A. tumefaciens*, SCSV and CaMV are plant pathogens, the regulatory sequences comprise only a small part of their total respective genomes, and are not capable of causing disease.

## 4.2 Introduction to non-symbiotic phytohaemoglobins (nsHb)

16. Haemoglobins are most commonly thought of as a constituent of blood and yet they are ubiquitous proteins found in eukaryotes and some bacteria that reversibly bind oxygen (Hardison 1996). In plants at least three distinct types of haemoglobins have been characterised. These are symbiotic, non-symbiotic and truncated haemoglobins.

17. Symbiotic haemoglobins, also called leghaemoglobins after their initial identification in the root nodules of legumes, act as oxygen carriers to regulate oxygen supply to symbiotic nitrogen-fixing bacteria (Appleby 1992).

18. Truncated haemoglobins, as the name suggests consist of shorter protein sequences. They have been identified in bacteria and plants (Watts et al. 2001; Larsen 2003; Vieweg et al. 2005) but their function is presently unknown (Watts et al. 2001).

19. Non-symbiotic haemoglobins (nsHbs) are widely distributed in the plant kingdom, having been identified from at least 20 plant species (Dordas et al. 2003b). These range from liverworts (*Marchantia polymorpha*) (Hunt et al. 2001), mosses (*Physcomitrella patens* and *Ceratodon purpureus*) (Arredondo-Peter et al. 2000; Hunt et al. 2001), monocots such as barley, wheat, rye (Taylor et al. 1994), rice (Sasaki et al. 1994; Arredondo-Peter et al. 1997), maize and teosinte (Aréchaga-Ocampo et al. 2001) as well as dicot plants such as cotton, canola, citrus, tomato (Hunt et al. 2001), chicory (Hendriks et al. 1998), apple and pear (GenBank accession number AY224132 and AY224133), *Parasponia andersonii* (Appleby et al. 1983) *Casuarina glauca* (Landsmann et al. 1986; Christensen et al. 1991) and *Trema tomentose* (Bogusz et al. 1988). They have also been isolated from legumes such as soybean (Andersson et al. 1996), lotus (Uchiumi et al. 2002) and alfalfa (Seregélyes et al. 2000) where they are present in addition to the symbiotic haemoglobin genes. They are expressed at low concentrations, in the order of 1-20  $\mu\text{mol}$  per kg fresh weight tissue (Duff et al. 1997).

20. The nsHbs can be divided into two classes based on sequence homology, biochemical properties and expression patterns. Class 1 nsHbs are present in both monocotyledonous and dicotyledonous plants, but, to date, class 2 nsHbs have only been identified from dicotyledonous plants (Hunt et al. 2001).

### 4.2.1 Expression of nsHbs

21. The two classes of nsHbs are generally differentially expressed in the plants. The *AHb1* gene introduced into the GM cotton lines is a class 1 nsHb so only this class will be discussed further.

22. In *Arabidopsis*, the *AHb1* gene is naturally expressed predominately in plant roots (Trevaskis et al. 1997; Hunt et al. 2001; Gonzali et al. 2005), with some leaf expression (Trevaskis et al. 1997). Root expression of class 1 nsHb has also been seen in other plants such as alfalfa, barley, wheat, *Parasponia*, *Trema* and *Casuarina* (Bogusz et al. 1988; Bogusz et al. 1990; Taylor et al. 1994; Jacobsen-Lyon et al. 1995; Andersson et al. 1997; Franche et al. 1998; Seregélyes et al. 2000; Larsen 2003). In legumes, the class 1 nsHb is also detected in the root nodules (Andersson et al. 1996; Uchiumi et al. 2002).

23. In *Arabidopsis*, *AHb1* showed the highest developmental expression during germination (Trevaskis et al. 1997; Gonzali et al. 2005), in a manner similar to nsHbs from barley (Duff et al. 1998), rice (Ross et al. 2001) and maize (Aréchaga-Ocampo et al. 2001).

24. The localisation of class 1 nsHbs to the roots, especially the tips, and to germinating seeds has led to the conclusion that expression is mostly detected in metabolically active organs and tissues (Larsen 2003), which may be due to hypoxic stress (Guy et al. 2002; Geigenberger 2003).

25. The intracellular localisation of nsHbs has been determined by immunolocalisation. In rice, nsHbs are localised to the cell cytoplasm (Ross et al. 2001). In alfalfa, nsHbs have been localised to the nucleus and cytosol (Seregélyes et al. 2000). Nuclear localisation has also been shown for a cotton *GhHb1*:GFP fusion in onion epidermal cells (Qu et al. 2005), although these results are surprising as none of the sequenced genes have any nuclear targeting sequence. However, it has been suggested that under particular environmental conditions nsHbs may be able to translocate from the cytoplasm to the nucleus (Ross et al. 2002).

26. Although they are seen to be constitutively expressed, class 1 nsHbs have also been referred to as “stress-induced haemoglobins” (Dordas et al. 2003b). A wide range of stresses have been shown to lead to their up-regulation (Table 1). However, they are not just induced by stress *per se*. Generally no class 1 nsHbs have been induced by common stress treatments such as wounding (Trevaskis et al. 1997; Hendriks et al. 1998), heat shock, dehydration, (Trevaskis et al. 1997), oxidative stress (Trevaskis et al. 1997; Lira-Ruan et al. 2001), abscisic acid or gibberellin (Hunt et al. 2001), or heavy metals (except nickel) (Krizek et al. 2003).

**Table 1 Induction of nsHbs by various treatments**

Plant species	Inducer	Ref
<i>Arabidopsis</i> , barley aleurone, maize, wheat, alfalfa, lotus, cotton, rice	hypoxia/anoxia	(Trevaskis et al. 1997; Nie & Hill 1997; Sowa et al. 1998; Seregélyes et al. 2000; Hunt et al. 2001; Lira-Ruan et al. 2001; Hunt et al. 2002; Klok et al. 2002; Larsen 2003; Qu et al. 2005; Shimoda et al. 2005; Liu et al. 2005; Yang et al. 2005; Loreti et al. 2005)
<i>Arabidopsis</i> , lotus	sucrose	(Trevaskis et al. 1997; Hunt et al. 2001; Shimoda et al. 2005)
<i>Arabidopsis</i> , tomato, rice	nitrate	(Wang et al. 2000; Wang et al. 2001; Sakamoto et al. 2004; Ohwaki et al. 2005)
<i>Arabidopsis</i>	nickel	(Krizek et al. 2003)
barley	compounds affecting ATP concentration	(Nie & Hill 1997)
barley	components of signal transduction	(Nie et al. 2006)
cotton	pathogens	(Dowd et al. 2004; Qu et al. 2005)
cotton	H <sub>2</sub> O <sub>2</sub>	(Qu et al. 2005; Qu et al. 2006)
chicory	initiation of somatic embryogenesis	(Hendriks et al. 1998)
tomato	phosphorus, potassium, iron	(Wang et al. 2001)
rice	etiolation	(Lira-Ruan et al. 2001)
alfalfa	G2/M transition in cell cycle	(Seregélyes et al. 2000)
lotus	infection by <i>Mesorhizobium loti</i>	(Shimoda et al. 2005)

#### 4.2.2 Structure of the nsHbs

27. All haemoglobins are characterised by a 3-on-3 sandwich of  $\alpha$ -helices called the globin fold, although the amino acid sequences are highly variable with some showing less than 20% amino acid identity (Watts et al. 2001).

28. NsHbs generally consist of an 18 kDa subunit, which is present in plants as a 36kDa homodimer (Duff et al. 1997; Seregélyes et al. 2000; Hargrove et al. 2000; Sáenz-Rivera et al. 2004).

#### **4.2.3 Biological function of plant class 1 nsHbs**

29. The exact biological function of nsHbs in plants is not known, however the use of GM plants with enhanced or reduced nsHb expression has identified a number of possible roles.

30. The observation that nsHb expression in *Arabidopsis* is induced following hypoxia (Trevaskis et al. 1997) has been followed up with studies showing that over-expression of *AHb1* in GM *Arabidopsis* leads to higher survival rates and increased shoot and root weight after hypoxia than in non-GM plants (Hunt et al. 2002; Perazzolli et al. 2004; Hebelstrup et al. 2006). Conversely, GM *Arabidopsis* lines in which *AHb1* was silenced showed slowed growth and decreased survival following hypoxia (Hebelstrup et al. 2006). In GM alfalfa plants in which nsHb was silenced, this slowed root growth was associated with cellular disintegration characteristic of cell death (Dordas et al. 2003a). GM *Arabidopsis AHb1* silenced lines also showed phenotypic changes, with enlarged hydathodes and stunted inflorescences indicating a role for nsHbs in normal development (Hebelstrup et al. 2006).

31. The over-expression of *AHb1* in GM *Arabidopsis* also leads to enhanced early growth rates under normal growth conditions due to increased size of roots and shoots (Hunt et al. 2002). However, GM tobacco plants expressing alfalfa nsHb did not show any obvious altered phenotype as seedlings or any enhanced growth rates (Seregélyes et al. 2003).

32. Induction of nsHbs during fungal infection has been observed in cotton plants that are susceptible to *Fusarium* or *Verticillium* wilts (Dowd et al. 2004; Qu et al. 2005), however no increased expression was seen in *Arabidopsis* infected with *Alternaria brassicicola* (Schenk et al. 2000). Similar inconsistent reactions to pathogens have been observed in GM plants over-expressing nsHbs. In GM *Arabidopsis*, *AHb1* over-expression has little effect on cell death in plants infected with *Pseudomonas syringae* (Perazzolli et al. 2004), and yet other plant species showed increased tolerance to infection (Seregélyes et al. 2003; Qu et al. 2006) and increased levels of defensive genes and compounds (Seregélyes et al. 2003; Seregélyes et al. 2004).

33. The expression of nsHbs is also altered during symbiotic associations, although both up- and down-regulation has been observed (Uchiumi et al. 2002; Shimoda et al. 2005).

#### **4.2.4 Mechanism of action of plant non-symbiotic haemoglobins**

34. So far, the exact mechanism of action of plant nsHbs is unknown. Initially it was assumed that they would have a similar function to other haemoglobins which are involved in oxygen transport, as NADH oxidases or as oxygen sensing proteins (Appleby et al. 1988; Bogusz et al. 1988; Hill 1998). However, their very high affinity for oxygen and slow dissociation rate make these roles unlikely (Trevaskis et al. 1997; Hill 1998; Perazzolli et al. 2004).

35. Alternatively, it has been suggested that plant nsHbs could act as nitric oxide (NO) scavengers (Arredondo-Peter et al. 1998; Hill 1998). Haemoglobins are well known regulators of NO homeostasis (Perazzolli et al. 2004) and NO has been shown to induce nsHbs (Wang et al. 2001; Ross et al. 2002; Sakamoto et al. 2004). The formation of NO occurs during many of the stresses which upregulate nsHb expression. In GM plants in which the expression of nsHbs have been altered, the level of NO produced during hypoxia is inversely related to the amount of nsHbs (Dordas et al. 2003a; Perazzolli et al. 2004; Dordas

et al. 2004; Igamberdiev et al. 2004). The presence of NO/haem complexes have also been detected in plant cells (Dordas et al. 2003b; Perazzolli et al. 2004).

36. NO is known to cause toxic effects at high concentrations and has been implicated in programmed cell death. In GM *Arabidopsis* lines in which *AHb1* is silenced, NO accumulates in the leaf hydathodes and the inflorescences causing cell death, leading to enlarged leaf hydathodes and stunted inflorescences (Hebelstrup et al. 2006).

37. It has been established that oxygen is not directly involved in the regulation of nsHb gene expression, since nsHbs can be up-regulated in the presence of oxygen. Interference with mitochondrial ATP synthesis strongly up-regulates nsHb gene expression thus suggesting that ATP is a critical component in this process (Nie & Hill 1997). Reduced levels of ATP under hypoxia are seen in GM plant cells in which the nsHb levels have been reduced compared to either non-GM lines or lines engineered to over produce nsHb (Sowa et al. 1998; Dordas et al. 2003a).

38. Correlations have also been seen between the expression of nsHbs and the accumulation of other signalling molecules such as ethylene, hydrogen peroxide and calcium (Sakamoto et al. 2004; Manac'h-Little et al. 2005; Yang et al. 2005; Nie et al. 2006).

39. A possible sequence of events for involvement of nsHbs during tolerance to waterlogging has been proposed (Figure 2) (Igamberdiev et al. 2005). Oxygen deficiency leads to a decline in mitochondrial respiration, triggering an increase in NADH and a drop in ATP levels. This results in nsHb gene expression and activation of nitrate reductase, leading to production of NO. This NO, in combination with the oxygenated form of newly synthesized class 1 nsHb, leads to the oxidation and oxygenation of NO, resulting in restoration of cell redox and energy status. Concomitantly, NO may trigger events leading to cell death and aerenchyma formation, individually or in a combination with ethylene, MAP kinase or guanylate cyclase. The net result would be avoidance of hypoxia through delivery of oxygen from the shoot to the roots, culminating in maintenance of redox and energy status.

### 4.3 The introduced genes and regulatory sequences, and the gene products

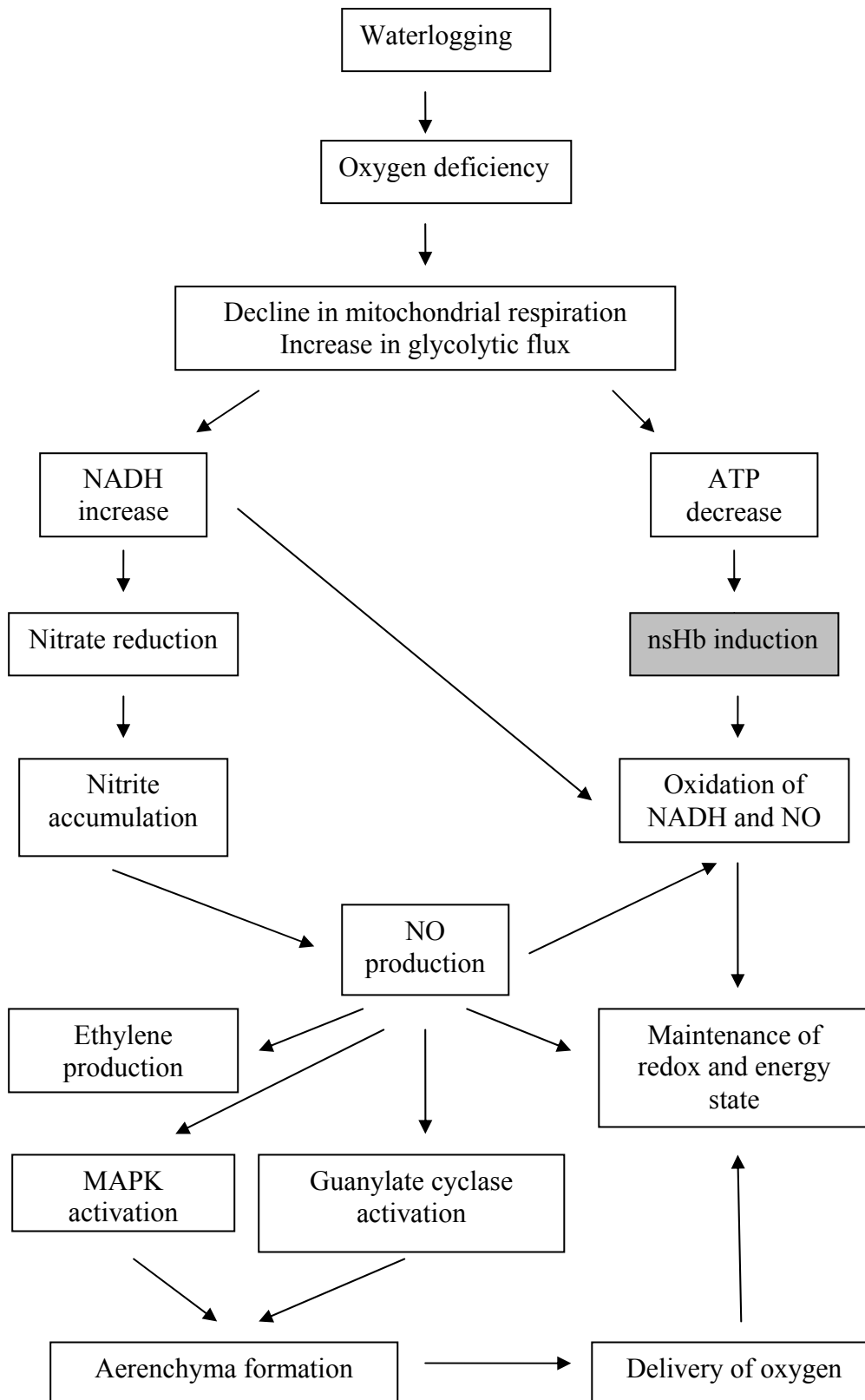
#### 4.3.1 Source organisms for the introduced genes and regulatory sequences

40. Genetic materials from various sources were introduced into cotton (Table 2).

**Table 2 Source organisms of the introduced genes and regulatory sequences**

Genetic element	Source organism	Function in the source organism	Hazardous characteristics of the source organism	Comments
S4S4 promoter	Subterranean clover stunt virus	Transcription initiation	pathogen of legumes	This regulatory sequence does not cause disease
<i>AHb1</i> gene	<i>Arabidopsis thaliana</i>	Non-symbiotic phytohaemoglobin	-	This is a common plant
NADP malic enzyme terminator	<i>Flavaria bidentis</i>	Transcription termination	-	This is a common plant
35S promoter	Cauliflower mosaic virus	Transcription initiation	pathogen of Brassicaceae	This regulatory sequence does not cause disease.
<i>nptII</i> gene	<i>Escherichia coli</i>	Antibiotic resistance	Facultative human pathogen	NPTII does not cause ill effects.
<i>nos</i> terminator	<i>Agrobacterium tumefaciens</i>	Transcription termination	pathogen causing crown gall disease	This regulatory sequence does not cause disease.

41. The genetic elements and their sources are discussed below.



**Figure 2. Proposed sequence of events for tolerance to waterlogging in plants** (redrawn from Igamberdiev A.U.; Baron K.; Manac'h-Little N; Stoimenova M; Hill R.D. (2005) The Haemoglobin/Nitric Oxide Cycle: Involvement in Flooding Stress and Effects on Hormone Signalling. *Annals of Botany* **96** pp557-564 with permission from RD Hill and Oxford University Press).

### **4.3.2 The non-symbiotic haemoglobin gene AHb1 from *Arabidopsis* and the encoded protein**

42. The gene construct used to genetically modify cotton contains the *AHb1* gene. The *AHb1* gene (GenBank accession no.U94998) is derived from a plant, *Arabidopsis thaliana*. Thirty GM cotton lines (derived from different genetic modification events) containing this construct have been selected which differ in the number of copies (1 – 8) and position of the insert in the cotton genome.

43. In *A. thaliana*, *AHb1* is expressed in roots, leaf hydathodes and inflorescences and is upregulated during germination (Trevaskis et al. 1997; Hunt et al. 2001; Gonzali et al. 2005; Hebelstrup et al. 2006). It is strongly induced in both roots and rosette leaves of plants subjected to hypoxia (Trevaskis et al. 1997). It is also induced by sucrose and nitrate, but not by cold, dehydration, heat shock, oxidative stress or wounding.

44. AHB1 shows a high oxygen affinity, with a P<sub>50</sub> value of 1.6 nM at pH7. The P<sub>50</sub> value is the dissolved oxygen concentration at which the haemoglobin is half saturated. *AHb1* has a low oxygen dissociation rate constant (Trevaskis et al. 1997).

45. The mode of action of AHB1 is currently unknown, although it affects a number of cellular processes (for details see Section 4.2.4 of this Chapter).

#### **Regulatory sequences for *AHb1* gene expression**

46. Expression of the introduced *AHb1* gene in GM cotton lines is controlled by the S4S4 promoter from Subterranean clover stunt virus (SCSV). SCSV is a plant virus which infects pasture legumes. Although SCSV is a plant pathogen, the regulatory sequence does not cause disease. The S4S4 promoter leads to constitutive expression of inserted genes in plants (Schunmann et al. 2003).

47. Also required for gene expression in plants is an mRNA termination region, including a polyadenylation signal. The mRNA termination region for the *AHb1* gene in the GM cotton lines is the 3' untranslated region (*MeI*) derived from the NADP malic enzyme gene from the plant *Flavaria bidentis* (Ali & Taylor 2001).

#### **Toxicity and allergenicity of AHB1**

48. No tests have been performed to assess the toxicity or allergenicity of purified AHB1 protein as the trial is still in the early research stage.

49. nsHbs have been identified from more than 20 plant species. (Dordas et al. 2003b), most of which are food plants. These include barley, maize, wheat, citrus, rice, soybean and tomato (Taylor et al. 1994; Andersson et al. 1996; Hunt et al. 2001; Shimoda et al. 2005). These plants are not toxic and they have no allergens in common. The *AHb1* gene shows high homology to these other nsHbs genes from food plants and during phylogenetic analysis of plant haemoglobins the nsHbs form a distinct cluster (Shimoda et al. 2005). The AHB1 protein shows 88% similarity to the nsHb from cotton itself (information supplied by applicant).

50. The introduced *AHb1* gene is from *Arabidopsis thaliana*, a member of the Brassicaceae family which also includes many food plants such as broccoli, cabbage and radish. *Arabidopsis* is widely used throughout the world for scientific research and there have been no reports of toxicity or allergenicity.

51. AHB1 is in the molecular weight range identified as being typical of allergens (15-70 kDa). A motif search (<http://www.expasy.org/prosite/>) with the AHB1 amino acid sequence identified a putative *N*-glycosylation site (Asp-X-Ser/Thr). Many protein allergens are

*N*-glycosylated (Herouet et al 2005 9065), leading to the concern that glycosylation may contribute to the allergenicity (Jenkins et al. 1996; Buchanan 2001). However, it is unknown if the protein is glycosylated in the GM cotton lines.

52. Comparisons of the AHB1 amino acid sequence to amino acid sequences in publicly available databases [SDAP ([http://fermi.utmb.edu/SDAP/sdap\\_who.html](http://fermi.utmb.edu/SDAP/sdap_who.html)), Agmobiol (<http://ambl.lsc.pku.edu.cn/english/index.phpallergen>), Farrp (<http://www.allergenonline.com>) The Allergen Database (<http://allergen.csl.gov.uk/>) and Allermatch (<http://allermatch.org/>)] were carried out. FAO/WHO (2001) guidelines propose that cross-reactivity between an unknown protein and a known allergen has to be considered when there is more than 35% identity in the amino acid sequence over a sliding window of 80 amino acids or a match of six identical contiguous amino acids. Two matches of AHB1 of 80 amino acids with greater than 35% sequence identity to known allergens were observed. These were to the pollen allergens Cry j II from Japanese cedar (*Cryptomeria japonica*) and Zea m1 from maize (*Zea mays*). A match of six contiguous identical amino acids (VAAIKA) was identified between AHB1 and the dust mites *Euroglyphus maynei* (an apolipoprotein-like protein (Eur m 14) and *Dermatophagoides pteronyssinus* (Der p 14). However, the significance of a six amino acid match is unclear. An optimal length of between eight and twelve amino acids is required for binding to T-cells (International Food Biotechnology Council/International Life Sciences Institute) and an immunological significant sequence identity requires a match of at least eight contiguous amino acids (Metcalf et al. 1996). There were no matches of the AHB1 protein greater than six identical contiguous amino acids with any known allergens. For a detailed discussion on this refer to Events 3 and 4 in Chapter 2. In addition data on the allergenicity of the AHB1 protein has been identified as a future research requirement.

53. Although no nsHbs have been identified as allergens, haemoglobins from the insect family Chironomidae (non-biting midges) have been identified as highly potent human allergens (Liebers & Baur 1994). These haemoglobins have been divided into a different subgroup from the nsHbs as although they are both single chain, single domain proteins, the *Chironomus* haemoglobin is extracellular compared to the cytoplasmic location of the plant nsHbs (Vinogradov et al. 1993). These insect haemoglobins show low (<25%) sequence identity with the AHB1 sequence with matches to only three identical contiguous amino acids.

#### **4.3.3 The antibiotic resistance marker gene *nptII* and the encoded protein**

54. The GM cotton lines also contain the *nptII* gene, which was isolated from the bacterial Tn5 transposon from *E. coli* (Beck et al. 1982). It encodes an enzyme, neomycin phosphotransferase type II (NPTII) that confers resistance to aminoglycoside antibiotics, eg kanamycin and neomycin. NPTII uses ATP to phosphorylate those antibiotics, thereby inactivating and preventing them from killing the NPTII-producing cells. *NptII* was used as a selectable marker gene during the laboratory stages of cotton plant tissue selection following genetic modification, allowing GM cells to grow in the presence of the antibiotic while inhibiting the growth of non-GM cells. The *nptII* gene is commonly used as a selectable marker in the production of GM plants (Miki & McHugh 2004).

55. Other regulatory agencies, in Australia and in other countries, have previously assessed the *nptII* gene as safe for use in human food (US FDA 1998; ANZFA 2001a; ANZFA 2001b; ANZFA 2001c; ANZFA 2001d; FSANZ 2003). In addition, a number of genetically modified food crops containing the *nptII* gene have been approved for commercial release both in Australia (DIRs 012/2002, 021/2002, 022/2002, 059/2005 and 066/2006) and overseas. No adverse effects on humans, animals or the environment have been reported from these releases (US FDA 1998; Flavell et al. 1992; EFB 2001).

### ***Regulatory sequences for the expression of the nptII gene***

56. The bacterial *nptII* gene was modified by the addition of Cauliflower mosaic virus and *A. tumefaciens* regulatory sequences (CaMV 35S promoter and *A. tumefaciens nos* termination regions respectively) to allow efficient expression in plant cells.

57. *A. tumefaciens* is a common gram-negative soil bacterium that causes crown gall disease in a wide variety of plants. CaMV is a virus which infects brassicas. Although *A. tumefaciens* and CaMV are plant pathogens, the regulatory sequences comprise only a small part of their total respective genomes, and are not capable of causing disease.

### ***Toxicity of NPTII***

58. Protein and DNA sequence comparisons using sequences from four separate databases (Genbank, EMBL, PIR29, Swiss-Prot) indicated that NPTII does not have significant homology to any proteins listed as food toxins in these databases (FDA 1994).

59. Humans (and, by implication, other animals) continually ingest kanamycin-resistant micro-organisms, some containing the NPTII enzyme. The diet, especially raw salad, is the major source: estimated conservatively, each human ingests  $1.2 \times 10^6$  kanamycin-resistant micro-organisms daily (Flavell et al. 1992). Large numbers of kanamycin- or neomycin-resistant bacteria already inhabit the human digestive system (Levy et al. 1998) with Flavell et al. (1992) estimating about  $10^{12}$  per person. Kanamycin-resistant bacteria have been isolated from soil, river water and sewage (Smalla et al. 1993).

60. The insertion of the *nptII* gene into a wide range of GMOs has not resulted in any adverse effects (Flavell et al. 1992). The *nptII* gene was introduced into mammalian cell lines with no effects on viability or growth. During gene therapy experiments, mammalian cells expressing the NPTII protein have been infused into cancer patients. Again, no adverse effects have been observed (Flavell et al. 1992).

61. The NPTII protein produced in GM tomatoes has been fed to rodents and reported to be rapidly inactivated and degraded (Calgene Inc. 1990). An acute oral toxicity study in mice, in which the purified NPTII protein was fed at doses of up to 5000 mg/kg of body weight (2500 mg/kg administered twice, four hours apart), did not show any adverse effects (Berberich et al. 1993). A similar study in mice also reported no adverse effects of NPTII at 5000 mg/kg of body weight (Fuchs et al. 1993b).

### ***Allergenicity of NPTII***

62. The NPTII protein is approximately 29 kDa in size, which is within the typical size range of allergenic proteins. However, it does not possess glycosylation sites, is not stable in the mammalian digestive system and is heat labile, decreasing the probability that it is allergenic (US FDA 1998; Fuchs et al. 1993a; FDA 1994; ANZFA 2001e; ANZFA 2001f). Fuchs et al. reported that no NPTII was detected 10 seconds after addition of simulated gastric fluid as measured by both western blot and enzymatic activity (Fuchs et al. 1993b). Protein sequence comparisons using sequences from four separate protein databases (EMBL, GenBank, PIR29 and Swiss-Prot) indicated that NPTII does not have significant sequence identity to any known protein food allergens (Fuchs & Astwood 1996).

63. The FDA has evaluated data submitted for deliberate releases of GMOs expressing the NPTII protein and concluded that NPTII does not have any of the characteristics associated with allergenic proteins (US FDA 1998). The UK Royal Society have concluded that there is at present no evidence that available GM foods cause allergic reactions, and that the risks posed by GM plants are in principle no greater than those posed by conventional breeding or by plants introduced from other areas of the world (The Royal Society 2002).

#### 4.4 Method of genetic modification

64. The GM cotton lines were generated by *Agrobacterium*-mediated transformation of the readily transformed cotton cultivar Coker 315 using standard protocols (Firoozabady et al. 1987; Cousins et al. 1991; Umbeck 1991).

65. *A. tumefaciens* is a common gram-negative soil bacterium that causes crown gall disease in a wide variety of plants (Van Larebeke et al. 1974). Plants can be genetically modified by the transfer of DNA (transfer-DNA or T-DNA, located between specific border sequences on a resident plasmid) from *A. tumefaciens* through the mediation of genes from the virulence region of Ti plasmids. *Agrobacterium*-mediated transformation has been widely used in Australia and overseas for introducing genes and regulatory sequences for their expression into plants. Unintended changes in phenotype as well as mutations can occur upon transformation and are similar to those in conventional breeding and mutation breeding (Bradford et al. 2005; Cellini et al. 2004).

66. Disarmed *Agrobacterium* strains have been constructed specifically for plant transformation. The disarmed strains do not contain the genes responsible for the overproduction of auxin and cytokinin (*iaaM*, *iaaH* and *ipt*), which are required for tumour induction and rapid callus growth (Klee & Rogers 1989). *Agrobacterium* plasmid vectors used to transfer T-DNAs contain well characterised DNA segments required for their replication and selection in bacteria, and for transfer of T-DNA from *Agrobacterium* and its integration into the plant cell genome (Bevan 1984; Wang et al. 1984).

67. The gene construct was generated using the disarmed binary vector, pPLEX-3002 (pPLEX T-DNA: GenBank accession number AY159023) (Bevan 1984). The *AHb1* gene, the antibiotic selective marker gene, *nptII*, and regulatory sequences for the expression of the genes are present in the gene construct.

68. *A. tumefaciens* (strain AGL1) was transformed by electroporation and vector-containing *A. tumefaciens* was used to infect cotyledons of the parent cotton cultivar Coker 315. Infected cotyledons were washed and cultured in the presence of the antibiotic kanamycin. Those that were transformed showed resistance to kanamycin and developed embryogenic calli from which embryos germinated. Plants were regenerated and transferred onto soil in contained facilities. Plants were tested by Southern blotting to identify low copy number insertions and screened for the presence of the *AHb1* gene by western blotting or PCR.

69. Up to 30 lines (derived from different transformation events) have been selected, these are from the T1, T2 or T3 generations and contain from one to eight copies of the *AHb1* gene inserted into different positions in the genome.

70. The genetic modification has not been introduced into any of the Australian elite cotton cultivars; the GM cotton lines for release are GM Coker 315 plants.

#### 4.5 Characterisation of the GM cotton lines proposed for release

##### 4.5.1 Stability and molecular characterisation

71. The GM cotton lines have been propagated by self pollination in contained facilities in the course of their development.

72. The applicant has used Southern blot analysis to estimate that GM cotton lines contain between one to eight copies of the inserted *AHb1* gene. Western blotting of leaves with an antibody raised to AHB1 has determined that the lines have different expression levels. PCR has been used in the T2 generation to identify homozygous lines. The GM cotton lines to be

field tested may be T1, T2 or T3 generation depending on the timing of the trial and the stage of evaluation of the material.

73. The applicant states that the GM cotton lines are in early development stage and have not been tested for genotypic stability or site of insertion as they form part of a 'proof of concept' field trial. Detailed molecular characterisation using standard molecular biology techniques would take place once the best performing GM cotton lines have been identified under field conditions. Those GM cotton lines would then be selected for molecular characterisation. Such data would be required for possible future applications involving large scale or commercial releases of these GM cotton lines. However, this information is not required for assessing the risks of this release because of the containment measures proposed by the applicant to limit the spread and persistence of the GM cotton lines, and the trial is limited in size, duration and location.

#### **4.5.2 Characterisation of the phenotype of the GMOs**

74. The applicant stated that under glasshouse conditions there is no obvious altered phenotype resulting from the expression of the *AHb1* gene. However, the lines to be tested are primarily for experimental evaluation of waterlogging tolerance so the applicant only intends to do a preliminary assessment of agronomic performance. The applicant proposes to monitor the performance of the GM lines relative to non-GM cotton lines under field conditions.

75. Expression of *AHb1* was determined by immunoblot analysis using AHB1-specific antisera. The applicant has not provided data on expression levels in different plant tissues. The applicant states that the introduced *AHb1* gene in the GM cotton lines is stably expressed. Data on expression levels and stability of *AHb1* in the GM cotton lines would be required for possible future applications involving large scale or commercial releases of these GM cotton lines. However, this information is not required for assessing the risks of this release because of the containment measures proposed by the applicant to limit the spread and persistence of the GM cotton lines, and the trial is limited in size, duration and location.

#### **Toxicity and allergenicity of GM cotton plant materials**

76. Data on the toxicity and allergenicity of the GM cotton lines would be required for possible future applications involving large scale or commercial releases of these GM cotton lines. However, this information is not required for assessing the risks of this release because of the containment measures proposed by the applicant to limit the spread and persistence of the GM cotton lines, and the trial is limited in size, duration and location.

### **Section 5 The receiving environment**

77. The receiving environment forms part of the context in which the risks associated with dealings involving the GMOs are assessed. This includes the size, duration and location of the dealings, any relevant biotic/abiotic properties of the areas where the release would occur; intended agronomic practices, including those that may be altered in relation to normal practices; other relevant GMOs already released; and any particularly vulnerable or susceptible entities that may be specifically affected by the release (OGTR 2005).

#### **5.1 Relevant abiotic factors**

78. The size and duration of the release is outlined in Section 2 of this Chapter. The release is to occur in the shire of Narrabri, NSW. This is a cotton growing region and has a typical climate for summer cotton growing regions in Australia, with warm summers and higher summer than winter rainfall (see Table 3).

**Table 3** Climatic data for Narrabri, NSW

	Narrabri Post Office
Average daily max/min temperature (summer)	33.3 °C /18.7 °C
Average daily max/min temperature (winter)	18.9 °C /4.5 °C
Average monthly rainfall (summer)	73.2 mm
Average monthly rainfall (winter)	45.7 mm

Source: <<http://www.bom.gov.au>>.

## 5.2 Relevant agricultural practices

### 5.2.1 General information

79. The applicant intends to treat the GM cotton lines following standard agricultural protocols used at the Australian Cotton Research Institute. The cotton plants in the release sites would therefore receive applications of water, fertilizers, herbicides, insecticides and other agronomic management practices similar to non-GM cotton.

### 5.2.2 Waterlogging

80. In Australia, waterlogging in cotton is estimated to cause annual yield losses of approximately 1 bale/ha or 11% (Dennis et al. 2000). Waterlogging occurs mainly when heavy rain follows a scheduled irrigation, especially when combined with poorly draining soils and inadequate field slope. In the 2005/06 season in Australia, 84% of cotton was grown as a furrow irrigated crop (Cotton Australia 2006) and fields are commonly irrigated five or six times during the growing season between flowering and peak boll development (McLeod et al. 1998). In NSW, cotton production occurs mainly on cracking grey clay soils (vertisols) of the Namoi and Gwydir River Valleys which have inherently low drainage rates (Hodgson & Chan 1982).

81. Research in the early 1980's showed that a 32 hour waterlogging treatment of cotton could lead to yield losses of 42% (Hodgson & Chan 1982; Hodgson 1982), although another study showed a recovery of plants following waterlogging stresses leading to no reduction in yield (Hocking et al. 1987). A more recent experiment following a similar protocol to the Hodgson study recorded approx. 40% yield loss but only when more severe waterlogging conditions were imposed (Bange et al. 2004b). The reduced yield loss due to waterlogging seems to be partly related to improvements in field design and soil structure. An increased awareness of soil management programs by cotton farmers has led to a reduction in soil compaction and there have been improvements in the furrow irrigation fields with more even water flow due to the use of laser guided levelling systems. The more even slope and hill heights have meant that water does not collect in low areas.

82. Waterlogging damages plants due to low oxygen concentrations (hypoxia) around the roots. This is caused because water displaces the oxygen in the soil, and cannot be replaced by diffusion of atmospheric oxygen. The low oxygen conditions inhibit energy production in the plant roots and other oxygen-dependent pathways, including those involving cytochromes, oxidases and desaturases.

83. The visual symptoms of waterlogging are initially wilting (Hocking et al. 1985; Reicosky et al. 1985) then leaf chlorosis, premature senescence and reduced boll number, leading to lint yield loss (Hodgson & Chan 1982). Damage to crop yields has already

occurred once leaf yellowing is observed (Constable 1995). The impact of waterlogging early in crop growth has a far greater influence on yield than waterlogging at mid-flowering or later (Bange et al. 2004a), although yield loss due to waterlogging can be sustained at all stages of crop growth (Hodgson & Chan 1982).

84. Uptake of potassium, phosphorus (Hocking et al. 1987) and nitrogen (Hocking et al. 1985; Constable 1995) is impaired in waterlogged cotton, especially in young plants just before flowering and can result in the plants becoming temporarily deficient in these nutrients. During the first three to four days of waterlogging most of the yield loss is due to less nitrogen being absorbed from the soil (Constable 1995).

### ***Strategies to reduce waterlogging***

85. Strategies have been recommended to reduce waterlogging (Bange et al. 2004a). These include measures to ensure that irrigation water or rainfall can quickly drain away from the crop, minimising the irrigation period by attempting to ensure that the cotton is not irrigated just prior to anticipated heavy rainfall and the addition of nitrogen prior to a possible waterlogging event.

86. Other crop species such as maize, rice and soybean tolerate waterlogging due to the formation of aerenchyma (Bacanamwo & Purcell 1999; Ray et al. 1999; Colmer 2003). Aerenchyma tissue comprises a high proportion of airspaces extending from the roots up into the aerial parts of the plant, providing a system for transfer of oxygen to the roots during waterlogging. The formation of aerenchyma can be induced by waterlogging (Drew et al. 2000).

87. Previous attempts to genetically engineer cotton plants with tolerance to waterlogging have focussed on over-expression of pyruvate (2-oxo-acid) carboxylase (*pdh*) and alcohol dehydrogenase (*adh*) genes. These enzymes occur at the start and end of the ethanolic fermentation pathway, respectively (Agarwal & Grover 2006) and constitutive expression of *pdh* in GM *Arabidopsis* led to improved survival under flooding (Ismond et al. 2003). Constitutive expression of the endogenous cotton genes did not lead to improved tolerance to low oxygen conditions in cotton plants (Ellis et al. 2000).

## **5.3 Presence of related plants in the receiving environment**

88. Cotton (*G. hirsutum* and *G. barbadense*) is grown in the shire of Narrabri including herbicide tolerant and/or insect resistant GM cotton plants that have previously been approved for commercial release (DIR 012/2002, DIR 022/2002, DIR 023/2002, DIR 059/2005 and DIR 062/2005, see Event 11 in Chapter 2 for details). As a result of these commercial releases in southern Australia (south of latitude 22°S), GM cotton plants are widespread in the agricultural environment, comprising about 90% of commercially grown cotton crops in the 2005/2006 growing season (Cotton Australia 2006).

## **5.4 Presence of the introduced or similar genes and their products in the environment**

89. The *AHb1* gene introduced into the GM cotton lines is from *A. thaliana*, a plant commonly known as thale cress. *Arabidopsis* is native to Europe and central Asia but is naturalised worldwide. Many plant species, and possibly all plants, contain nsHb genes, including the native Australian plants *Casuarina glauca* (swamp oak) and *Trema tomentosa* (native peach) (Bogusz et al. 1988) and food plants such as such as barley, maize, wheat, rye, rice, soybean, and tomato (Taylor et al. 1994; Andersson et al. 1996; Hunt et al. 2001). Cotton itself contains a nsHb gene which is 88% similar to the introduced *AHb1* gene. The

haemoglobin family, to which the nsHbs belong, are present in all organisms from soil bacteria to humans (Hardison 1996).

90. The kanamycin resistance protein NPTII is widespread in the environment since it is naturally produced by a common gut bacterium and is widespread in human and animal digestive systems (Blattner et al. 1997). A number of GM cotton plants have previously been approved for commercial release which also produce the NPTII protein (for details see Section 4.3.3 of this Chapter).

## **Section 6 Australian and international approvals**

### **6.1 Australian approvals of waterlogging tolerant GM cotton lines**

#### **6.1.1 Previous releases approved by GMAC or the Regulator**

91. There have been no previous releases of these *AHb1* containing GM cotton lines in Australia. CSIRO has performed four field trials of cotton, using genes other than *AHb1*, for tolerance to waterlogging under the former voluntary system that was overseen by the Genetic Manipulation Advisory Committee (GMAC): PR99 (1998), PR99X (1999), PR99X2 (2000) and PR99X3 (2001).

#### **6.1.2 Approvals by other Australian government agencies**

92. The *Gene Technology Act 2000* is designed to operate in a cooperative legislative framework with other regulatory authorities that have complementary responsibilities and specialist expertise. As well as enhancing coordinated decision making, this arrangement avoids duplication.

93. While the Regulator is responsible for identifying, assessing and managing risks to the health and safety of people and the environment associated with the use of gene technology, other government regulatory requirements may also have to be met in respect of release of GMOs.

94. FSANZ is responsible for human food safety assessment and food labelling, including GM food. The applicant does not intend to use materials from the GM cotton lines in human food, accordingly an application to FSANZ has not been submitted. FSANZ approval would need to be obtained before materials from cotton lines containing the AHB1 protein could be used in food.

### **6.2 International approvals**

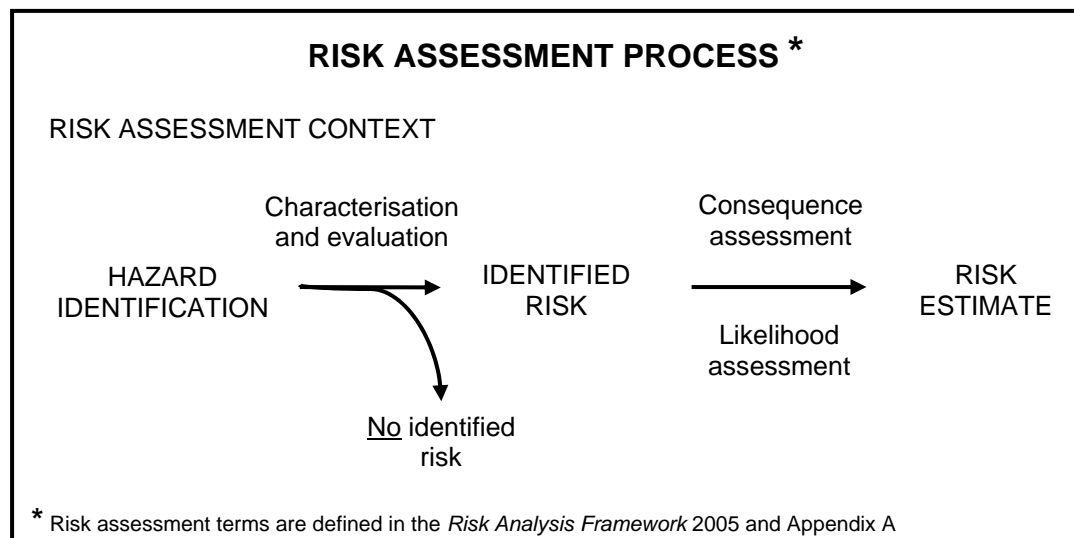
95. There has been no release of these GM cotton lines or other nsHb containing GM plants world wide. However, there are introduced symbiotic haemoglobin genes being tested for their effects on carbohydrate metabolism in potato in Germany ([http://gmoinfo.jrc.it/gmp\\_report\\_onepag.asp?CurNot=B/DE/03/150](http://gmoinfo.jrc.it/gmp_report_onepag.asp?CurNot=B/DE/03/150)).

## Chapter 2 Risk assessment

### Section 1 Introduction

96. Risk assessment is the overall process of identifying the sources of potential harm (hazards) and determining both the seriousness and the likelihood of any adverse outcome that may arise. The risk assessment (summarised in Figure 3) considers risks from the proposed dealings with the GMOs that could result in harm to the health and safety of people or the environment posed by or as a result of gene technology.

Figure 3 The risk assessment process



97. Once the risk assessment context has been established (see Chapter 1) the next step is hazard identification to examine what harm could arise and how it could happen during a release of these GMOs into the environment.

98. It is important to note that the word ‘hazard’ is used in a technical rather than a colloquial sense in this document. The hazard is a source of *potential* harm. There is no implication that the hazard will *necessarily* lead to harm. A hazard can be an event, a substance or an organism (OGTR 2005).

99. Hazard identification involves consideration of events (including causal pathways) that may lead to harm. These events are particular sets of circumstances that might occur through interactions between the GMOs and the receiving environment as a result of the proposed dealings.

100. A number of hazard identification techniques are used by the Regulator and staff of the OGTR, including the use of checklists, brainstorming, commonsense, reported international experience and consultation (OGTR 2005). In conjunction with these techniques, hazards identified from previous RARMPs prepared for licence applications of the same and similar GMOs are also considered.

101. The hazard identification process results in the compilation of a list of events. Some of these events lead to more than one adverse outcome and each adverse outcome can result from more than one event.

## Section 2 Hazard characterisation

102. The list of events compiled during hazard identification are characterised and evaluated to determine which events represent a risk to the health and safety of people or the environment posed by or as a result of gene technology.

103. A risk is identified only when there is some chance that harm will occur. Those events that do not lead to an adverse outcome or could not reasonably occur do not represent an identified risk and will not advance in the risk assessment process. Risks associated with the remaining events are assessed further to determine the seriousness of harm (consequence) and chance of harm (likelihood). The identified risks must be posed by or result from gene technology.

104. The criteria used by the Regulator to determine harm are described in Chapter 3 of the *Risk Analysis Framework* (OGTR 2005). Harm is assessed in comparison to the parent organism and in the context of the proposed dealings and the receiving environment. The risk assessment process focuses on measurable criteria for determining harm.

105. The following factors are taken into account during the analysis of events that may give rise to harm:

- the proposed dealings, which may include experimentation, development, production, breeding, propagation, and possession, use, supply, transport or disposal of the GMOs during the course of these dealings
- the size, duration and location of the release
- containment measures proposed by the applicant
- comparisons with the non-GM parent
- routes of exposure to the GMOs, the introduced gene(s) and its product(s)
- potential effects of the introduced gene(s) and its product(s) expressed in the GMOs
- potential exposure to the introduced gene(s) and its product(s) from other sources in the environment
- properties of the biotic and abiotic environment at the site of release
- agronomic management practices for the GMOs.

106. Events that are discussed in detail later in this Section are summarised below in Table 4. Events that share a number of common features are grouped together in broader hazard categories as indicated in the table. Eighteen events were characterised, none of which were considered to lead to an identified risk that required further assessment.

107. The prevalence of the *nptII* gene in the environment and the lack of evidence of toxicity or allergenicity of the NPTII protein are discussed in Section 4.3.3 of Chapter 1. The potential effects of the *nptII* gene and its product were considered in detail in previous DIR applications including some for commercial releases. RARMPs for those DIR applications are available from the OGTR or from the website <<http://www.ogtr.gov.au>>. In those cases, no risks were identified when NPTII was expressed in GM cotton. The proposed release would pose negligible risks, highly similar to those identified in the previous releases and the potential effects of NPTII will not be assessed further.

**Table 4 Summary of events that may give rise to adverse outcomes**

Hazard category	Event that may give rise to an adverse outcome	Potential adverse outcome	Identified risk?	Reason
Section 2.1 Production of a substance toxic to people	1. Ingestion of GM plant materials containing the protein encoded by the introduced <i>AHb1</i> gene.	Toxicity for people	No	<ul style="list-style-type: none"> <li>• People are exposed to the same protein from the plant <i>Arabidopsis thaliana</i> or similar proteins via ingestion of other food plants with no reports of evidence of toxicity.</li> <li>• None of the GM plant materials from the proposed release would be used as human food.</li> </ul>
	2. Contact with, or inhalation of, GM plant materials containing the protein encoded by the introduced <i>AHb1</i> gene via: <ul style="list-style-type: none"> <li>• Occupational exposure</li> <li>• Exposure to the wider community.</li> </ul>	Toxicity for people	No	<ul style="list-style-type: none"> <li>• People are exposed to the same protein from the plant <i>Arabidopsis thaliana</i> or similar proteins via contact with other plants with no reports of evidence of toxicity.</li> <li>• Contact with, or inhalation of GM plant materials would be limited to people working with the GM plants. Contact would be further limited by the small size and short duration of the proposed release.</li> </ul>
Section 2.2 Production of a substance allergenic to people	3. Use of GM plant materials, containing protein encoded by the introduced <i>AHb1</i> gene, in food.	Allergic reactions in people	No	<ul style="list-style-type: none"> <li>• People are exposed to the same protein from the plant <i>Arabidopsis thaliana</i> or similar proteins via ingestion of other food plants.</li> <li>• No nSHb proteins are known to cause allergic reactions.</li> <li>• None of the GM plant materials from the proposed release would be used as human food.</li> </ul>
	4. Contact with, or inhalation of, GM plant materials (including pollen), containing the protein encoded by the introduced <i>AHb1</i> gene via: <ul style="list-style-type: none"> <li>• Occupational exposure</li> <li>• Exposure to the wider community.</li> </ul>	Allergic reactions in people	No	<ul style="list-style-type: none"> <li>• None of the GM plant materials from the proposed release would be used for the production of fabrics and/or other cotton products.</li> <li>• People are exposed to the same protein from the plant <i>Arabidopsis thaliana</i> or similar proteins via contact with other plants.</li> <li>• No nSHb proteins are known to cause allergic reactions.</li> <li>• Contact with, or inhalation of, GM plant materials would be limited to people working with the GMOs.</li> <li>• Contact would be further limited by the small size and short duration of the proposed release.</li> </ul>
Section 2.3 Production of a substance toxic to organisms other than people	5. Direct or indirect ingestion of GM plant materials containing AHB1 by vertebrates, invertebrates or micro-organisms.	Toxicity for vertebrates, invertebrates or micro-organisms	No	<ul style="list-style-type: none"> <li>• Vertebrates, invertebrates and micro-organisms are exposed to the same protein from the plant <i>Arabidopsis thaliana</i> or similar proteins via contact with other plants.</li> <li>• Exposure of organisms to the AHB1 protein is expected to be restricted due to the small size and short duration of the proposed release.</li> <li>• None of the GM plant materials from the proposed release would be used as animal feed.</li> </ul>

Hazard category	Event that may give rise to an adverse outcome	Potential adverse outcome	Identified risk?	Reason
Section 2.4 Spread and persistence of the GM cotton in the environment	6. Expression of the <i>AHb1</i> gene improving the survival of cotton volunteers through tolerance to waterlogging.	Weediness	No	<ul style="list-style-type: none"> <li>Waterlogging is not a factor limiting the spread and persistence of cotton in the area proposed for release. The main factors are water and nutrient availability, temperature and soil type.</li> <li>The release would be of small size and short duration and the applicant proposes measures to limit the spread and persistence of the GM cotton lines including destroying GM cotton volunteers.</li> </ul>
	7. Expression of the <i>AHb1</i> gene improving the survival of cotton volunteers through enhancement of : <ul style="list-style-type: none"> <li>Abiotic stress tolerances (other than waterlogging)</li> <li>Biotic stress tolerance.</li> </ul>	Weediness	No	<ul style="list-style-type: none"> <li>The main factors limiting the spread and persistence of cotton in the area proposed for release are water and nutrient availability, temperature and soil type.</li> <li>The release would be of small size and short duration and the applicant proposed measures to limit the spread and persistence of the GM cotton lines.</li> </ul>
	8. Expression of the <i>AHb1</i> gene improving the survival of cotton volunteers through altered dormancy, seed viability, germination rates and/or seedling viability.			
	9. Dispersal of GM seed or other GM plant materials during transport, research, storage, equipment use, flooding or via animals.	Weediness	No	<ul style="list-style-type: none"> <li>The proposed trial site is liable to flooding. However, it is located at least 50 m away from natural waterways which would limit dispersal in the event of flooding.</li> <li>Once established, plants within the pollen trap would act as a physical barrier to GM plant materials being dispersed. Cotton does not propagate vegetatively.</li> <li>The applicant proposes to transport and store all GM plant materials including seeds according to OGTR guidelines, to clean equipment on site and to burn all harvested plant material not required for further research at the end of each growing season.</li> <li>Research is to be carried out in certified PC2 facilities and any GM plant waste materials will be autoclaved.</li> <li>Mammals and birds are unlikely to transport GM cotton seeds because cotton bolls are unattractive to them.</li> </ul>
	10. Exposure of vertebrates (including people), invertebrates and micro-organisms to GM cotton volunteers expressing the <i>AHb1</i> gene.	Toxicity for, or allergic reactions in, people. Toxicity for other vertebrates, invertebrates and/or micro-organisms	No	<ul style="list-style-type: none"> <li>The potential effects on people are discussed in Events 1-4.</li> <li>The potential effects on organisms other than people are discussed in Event 5.</li> <li>The release would be of small size and short duration and the applicant proposed measures to limit the spread and persistence of the GM cotton lines including destroying GM cotton volunteers.</li> </ul>

Hazard category	Event that may give rise to an adverse outcome	Potential adverse outcome	Identified risk?	Reason
Section 2.5 Vertical transfer of genes or genetic elements to sexually compatible plants	11. Expression of the introduced gene in other <i>G. hirsutum</i> (including commercially released GM cotton lines) or <i>G. barbadense</i> cotton plants.	Weediness	No	<ul style="list-style-type: none"> <li>Cotton is primarily a self-pollinating plant so gene transfer to other cotton plants is expected to occur at low frequencies.</li> <li>Outcrossing to other <i>G. hirsutum</i> and <i>G. barbadense</i> plants would be limited due to the small size, short duration and containment measures proposed by the applicant.</li> <li>Agricultural practices proposed by the applicant include the use of insecticides which would further limit pollen dispersal.</li> <li>The factors limiting the spread and persistence of cotton are discussed in Events 6-9.</li> </ul>
	12. Exposure of vertebrates (including people), invertebrates and micro-organisms to other <i>G. hirsutum</i> (including commercially released GM cotton lines) or <i>G. barbadense</i> cotton plants expressing the introduced gene.	Toxicity for, or allergic reactions in, people. Toxicity for other vertebrates, invertebrates and/or micro-organisms	No	<ul style="list-style-type: none"> <li>The limited potential for gene flow is discussed in Event 11.</li> <li>The potential effects on people are discussed in Events 1-4.</li> <li>The potential effects on organisms other than people are discussed in Event 5.</li> </ul>
	13. Presence of the introduced regulatory sequences in other <i>G. hirsutum</i> or <i>G. barbadense</i> plants as a result of gene transfer.	Toxicity for, or allergic reactions in, people. Toxicity for other vertebrates, invertebrates and/or micro-organisms. Weediness	No	<ul style="list-style-type: none"> <li>Outcrossing to other <i>G. hirsutum</i> and <i>G. barbadense</i> plants would be limited due to the small size, short duration and containment measures proposed by the applicant.</li> <li>The introduced regulatory sequences are not known to behave any differently than endogenous regulatory sequences in plants.</li> </ul>
	14. Gene transfer to native <i>Gossypium</i> species.	Toxicity for, or allergic reactions in, people. Toxicity for other vertebrates, invertebrates and/or micro-organisms. Weediness	No	<ul style="list-style-type: none"> <li>Well established genetic incompatibility prevents vertical gene transfer to native <i>Gossypium</i> species.</li> </ul>
Section 2.6 Horizontal transfer of genes or genetic elements to sexually incompatible organisms	15. Presence of the <i>AHb1</i> gene, or the introduced regulatory sequences, in other organisms as a result of gene transfer.	Toxicity for, or allergic reactions in, people. Toxicity for other vertebrates, invertebrates and/or micro-organisms. Weediness Increased pathogenicity	No	<ul style="list-style-type: none"> <li>The introduced gene or similar genes and the introduced regulatory sequences are already present in the environment and are available for transfer via demonstrated natural mechanisms.</li> <li>Gene transfer from plants to bacteria has not been demonstrated under natural conditions, and the likelihood of such transfer is greatly exceeded by the likelihood of transfer from other natural sources of this gene.</li> </ul>

Hazard category	Event that may give rise to an adverse outcome	Potential adverse outcome	Identified risk?	Reason
Section 2.7 Unintended changes in toxicity and/or allergenicity	16. Altered levels of innate toxic or allergenic compounds as a result of the expression, or random insertion of the gene construct into the cotton genome during genetic modification.	Toxicity for, or allergic reactions in, people. Toxicity for other vertebrates, invertebrates and/or micro-organisms. Weediness	No	<ul style="list-style-type: none"> <li>Compositional analysis has not been done on the GM cotton lines expressing AHB1 protein, as the proposed trial represents early stage research. Information on toxicity and allergenicity of the GM cotton plants would be a prerequisite for a large scale release.</li> <li>The small scale and short duration of the proposed release, along with containment measures proposed by the applicant, will limit any possible adverse outcomes.</li> </ul>
Section 2.8 Unintended changes in biochemistry, physiology or ecology	17. Altered biochemistry, physiology or ecology of the GM cotton lines resulting from expression of the introduced <i>Ahb1</i> gene.	Toxicity for, or allergic reactions in, people. Toxicity for other vertebrates, invertebrates and/or micro-organisms. Weediness	No	<ul style="list-style-type: none"> <li>Unintended adverse effects, if any, would be limited by the small scale and short duration of the proposed release.</li> <li>Results from glasshouse trials show that there is no evidence of altered growth rates or fertility of the GM cotton lines compared to the parent cultivar.</li> </ul>
Section 2.9 Unauthorised activities	18. Use of GMOs outside the licence conditions.	Potential adverse outcomes mentioned in Events 1-17	No	<ul style="list-style-type: none"> <li>The Act provides for substantial penalties for non-compliance and unauthorised dealings with GMOs and also requires consideration of the suitability of the applicant to hold a licence prior to the issuing of a licence by the Regulator.</li> </ul>

## 2.1 Production of a substance toxic to people

108. Toxicity is the cascade of reactions resulting from exposure to a dose of a chemical that is sufficient to cause direct cellular or tissue injury, or otherwise inhibit normal physiological processes (Felsot 2000). Toxic proteins are known to act via acute mechanisms rather than through chronic exposure (Sjoblad et al. 1992). Toxicity may occur through ingestion, contact or inhalation. The level of toxicity is often expressed as the LD<sub>50</sub>. This is the amount of a substance given in a single dose that causes death in 50% of a test population of an organism.

109. Toxicity assays generally use the purified toxin of interest rather than the product that expresses the protein (eg GM plant material). This is necessary because the aim of the assays is to determine the concentration of toxin at which an adverse effect is seen. The level of expression in the product is used to determine the level of exposure to the toxin and comparison to the results of the toxicity assay indicate whether or not this is a safe level of exposure (OECD 1998; Konig et al. 2004). The use of purified toxin also increases the reproducibility of the assays.

### **Event 1: Ingestion of GM plant materials containing the protein encoded by the introduced *AHb1* gene**

110. The GM cotton lines differ from non-GM cotton in the expression of two additional proteins, the AHB1 and NPTII proteins. The absence of toxicity of NPTII is discussed in Section 4.3.3 of Chapter 1 and will not be considered further. As the proposal is a 'proof of concept' field trial, no toxicity testing has been carried out of the protein encoded by the introduced *AHb1* gene, or of the GM cotton lines.

111. An extensive search of the literature did not reveal any reports that imply AHB1 or any other plant nsHb are toxins. Plant nsHb genes similar to *AHb1* are expressed in a wide range of plants. The food plants barley, maize, wheat, rye, soybean, and tomato all contain nsHbs

(Taylor et al. 1994; Andersson et al. 1996; Hunt et al. 2001) (for details see Sections 4.1 and 4.2 in Chapter 1). People are exposed to AHB1 or similar proteins through normal diet or the environment.

112. Food Standards Australia New Zealand (FSANZ) is responsible for human food safety assessment and FSANZ approval would be required before products from the GM cotton lines could be used in human food.

113. The applicant does not intend to use GM plant materials (including cotton seed oil or linters) from the release in human food or as animal feed, but to destroy all plant materials other than some materials collected for future research or planting. Ingestion of materials from the GM cotton lines containing the AHB1 protein is not expected to occur from the release.

114. Therefore, **no risk is identified**. The potential for toxicity for people as a result of ingestion of the AHB1 protein will not be assessed further.

**Event 2: Contact with, or inhalation of, GM plant materials containing the protein encoded by the introduced AHb1 gene**

115. Exposure to the AHB1 protein could occur as a result of contact with, or inhalation of, material from the GM cotton lines. This may occur via occupational exposure or general exposure to the wider community from living near the proposed release site.

116. Dermal and inhalation toxicity studies have not been conducted with the AHB1 protein. However, on the basis of the presence of this or similar proteins in many plant species, it is also expected to be of very low acute dermal and inhalation toxicity. In *A. thaliana*, the AHB1 protein is retained within the plant cells and the same is expected in the GM cotton cells. Therefore, dermal and inhalation contact may occur when the plant cells have been damaged or broken or via pollen. People working with damaged GM cotton plants may come into contact with the AHB1 protein during handling and/or processing the GM cotton lines or its products that contain the protein. Contact with pollen is likely to be limited because cotton pollen is relatively large, sticky and heavy, and not easily dispersed by wind (OGTR 2002). Furthermore, nsHb proteins are widespread in the environment, through the presence of other plants (see Sections 4.1 and 4.2 in Chapter 1 for details) and therefore people are already widely exposed to nsHb proteins, including AHB1.

117. Since the proposed release is of small size and short duration (for details see Section 5 in Chapter 1), the frequency and duration of contact with the AHB1 protein is expected to be very limited. In addition, the applicant does not intend to use any cotton plant materials from the release in human food, animal feed or for the production of fabrics and/or other cotton products.

118. Therefore, **no risk is identified** and the potential for toxicity for people as a result of contact with, or inhalation of, GM plant materials containing the proteins encoded by the introduced genes for waterlogging tolerance will not be assessed further.

## **2.2 Production of a substance allergenic to people**

119. The possibility that exposure of people to the AHB1 protein expressed by the GM cotton plants may result in an allergic reaction is considered. Routes of exposure to the AHB1 protein could include consumption of food containing cotton products or dermal contact with material from cotton plants such as fibres or pollen as a result of occupational exposure.

**Event 3: Use of GM plant material, containing protein encoded by the introduced AHb1 gene, in food**

120. No nsHb proteins are known to cause allergic reactions. However, the AHB1 protein has some of the characteristics of an allergen. It is in the molecular weight range identified as being typical of allergens (15-70 kDa) and possesses an *N*-glycosylation site (Asp-X-Ser/Thr) typical of allergens. AHB1 also shows some amino acid identity with known allergens. FAO/WHO (2001) guidelines propose that cross-reactivity between an unknown protein and a known allergen has to be considered when there is more than 35% identity in the amino acid sequence over a sliding window of 80 amino acids or a match of six identical contiguous amino acids. AHB1 has two matches of 80 amino acids with 36% and 37% sequence identity to the pollen allergens Cry j II from Japanese cedar (*Cryptomeria japonica*) and Zea m1 from maize (*Zea mays*) respectively, and a match of six contiguous identical amino acids with a protein found in two species of dust mite (for details see Section 4.3.2 in Chapter 1). There is no significant homology with any proteins known to cause allergenicity or toxicity when ingested.

121. The introduced *AHb1* gene is from *Arabidopsis thaliana*, a member of the Brassicaceae family which also includes many food plants such as broccoli, cabbage and radish. *Arabidopsis* is widely used throughout the world for scientific research and there have been no reports of allergenicity. nsHbs have been identified from more than 20 plant species (Dordas et al. 2003b), most of which are food plants. The AHB1 protein shows 88% similarity to the nsHb from cotton itself (information supplied by applicant).

122. None of the GM cotton materials from the proposed release would be used in human food or animal feed. Thus, the potential for allergic reactions in people resulting from exposure to food is unlikely. Food Standards Australia New Zealand (FSANZ) is responsible for human food safety assessment and FSANZ approval would be required before products from the GM cotton lines could be used in human food.

123. Therefore, **no risk is identified** and the potential for production of a substance allergenic to people will not be assessed further.

**Event 4: Contact with, or inhalation of, GM plant materials (including pollen), containing the protein encoded by the introduced AHb1 gene**

124. There are a number of different routes by which people could be exposed to the AHB1 protein expressed by the introduced gene in the GM cotton lines proposed for release, including:

- contact with items containing GM cotton fibre
- contact with damaged GM cotton plant materials during handling
- inhalation of pollen
- inhalation of cotton dust.

125. There would be limited contact between some people and GM cotton materials during the proposed release. There are no reports of AHB1 causing allergic reactions in people, however AHB1 does have some characteristics associated with known allergens (for details see Event 3, and Section 4.3 in Chapter 1).

126. AHB1 shows 36 and 37% sequence identity over 80 amino acids with allergens from maize and Japanese cedar respectively. These are both pollen allergens. The allergen from Japanese cedar, Cry j II, has been implicated in causing Japanese cedar pollinosis, one of the most important allergic diseases in Japan. The protein is found in the amyloplast and has

homology to polygalacturonases (Namba et al. 1994). The maize allergen Zea m1 is found only in pollen and is homologous to the major allergen of rye grass pollen (Broadwater et al. 1993).

127. The six amino acid sequence VAAIKA from AHB1 matches a protein from the European dust mite *Dermatophagoides pteronyssinus* and the house dust mite *Euroglyphus maynei*. This protein is an inhalant allergen. It shows similarities to apolipoproteins (lipid transport proteins) and peptides from this protein bind IgE in serum of mite-allergic people (Epton et al. 1999). However, the sequence similarity with AHB1 is confined to the six amino acid match with no additional homology in the remaining protein sequence.

128. Matches of six amino acids with any allergen have been shown to occur frequently by chance which limits the utility of this criteria for predicting allergenicity (Hileman et al. 2002; Stadler & Stadler 2003; Thomas et al. 2005; Silvanovich et al. 2006). The International Food Biotechnology Council/International Life Sciences Institute have suggested that an optimal length of between eight and twelve amino acids is required for binding to T-cells and that an immunological significant sequence identity requires a match of at least eight contiguous amino acids (Metcalf et al. 1996). A search for homology of the AHB1 protein with known allergens was conducted based on detecting identities of eight or more contiguous amino acids and no sequence homologies were found.

129. A more refined method for detecting possible allergenic epitopes has been suggested (Kleter & Peijnenburg 2002; Kleter & Peijnenburg 2003) based on the type of amino acid present (Hopp & Woods 1981). The method was applied to the amino acid sequence of the AHB1 protein and the results suggest that the region with the six amino acid match to the dust mite allergen is not hydrophilic and will therefore not be exposed on the outside of the protein to act as an epitope.

130. However, all these approaches are limited as they cannot identify discontinuous or conformational epitopes that depend on the tertiary structure of the protein (Metcalf et al. 1996).

131. Although no nsHbs have been identified as allergens, haemoglobins from non-biting midges are known to be potent inhalant allergens (see Section 4.3.2 in Chapter 1). Human polyclonal antibodies from patients sensitised to one midge recognise antigenic sites present in all 33 species of the Chironomid insect family (Baur et al. 1991) yet there is no cross-reactivity to other animal haemoglobins (Tichy et al. 1981) suggesting the epitope is not common to all haemoglobins.

132. People working with cotton plants would be exposed primarily to the outer waxy cuticle layer at the plant surface, to the seed coat or to the cotton lint, all of which are essentially free of protein. However, dermal exposure to proteins (including the AHB1 protein) or to other cellular components of the cotton plants may occur if damage to the plants during handling results in rupture of plant cells.

133. Inhalation of pollen by workers could potentially result in allergic reactions if this protein was allergenic and was expressed in the pollen. However, due to the physical characteristics of cotton pollen and flowers (OGTR 2002), it is expected that people would be exposed to very small quantities of pollen.

134. More data on the allergenicity of the AHB1 protein itself and plant material from the GM cotton lines would be required before any future application for a large scale or commercial release of these GMOs could be assessed. However, for this limited and controlled release human contact with the GM plant materials containing the AHB1 protein would be limited because cotton pollen is sticky, heavy and not easily dispersed by wind; the

proposed release is of small scale and short duration; and none of the materials would be used in human food, animal feed or for the production of fabrics and/or other cotton products.

135. Therefore, **no risk is identified** and the potential for allergic reactions in people resulting from contact with GM plant materials (including pollen) containing the AHB1 protein will not be assessed further.

### **2.3 Production of a substance toxic to organisms other than people**

136. A range of organisms may be exposed directly or indirectly to AHB1. Organisms may be exposed directly to AHB1 through biotic interactions with GM cotton plants (vertebrates, insects, symbiotic micro-organisms and/or pathogenic fungi) or through contact with root exudates or dead plant material (soil biota). Indirect exposure would include organisms that feed on organisms that feed on GM-cotton or degrade it (vertebrates, insects, fungi, Oomycetes and/or bacteria).

#### **Event 5: Direct or indirect ingestion of GM plant materials containing AHB1 by vertebrates, invertebrates or micro-organisms**

137. Vertebrates such as livestock and wildlife (including mammals) may be exposed to the GM cotton lines containing the protein encoded by the introduced *AHb1* gene through direct feeding or indirectly through consumption of other organisms which have fed on GM cotton plants.

138. Cotton tissue (from either GM or non-GM plants), particularly the seeds, can be toxic to mammals if ingested in large quantities because of the presence of toxic and anti-nutritional factors including gossypol and cyclopropenoid fatty acids (eg dihydrosterculic, sterculic and malvalic acids).

139. Vertebrates generally avoid feeding on cotton plants because they find them unpalatable. The presence of gossypol and cyclopropenoid fatty acids in cotton seed limits the use of whole cotton seed as a protein supplement in animal feed, except for cattle which are less affected by these components. Inactivation or removal of these components during processing enables the use of some cotton seed meal for farmed fish, poultry and swine. The meal and hulls of cotton seed can also be used for cattle feed. Its use as stockfeed is limited, nonetheless, to a relatively small proportion of the diet and it must be introduced gradually, to avoid potential toxic effects. In the field, seed cotton is present as large lint covered bolls that are unattractive to avian species (OGTR 2002), so birds are unlikely to be exposed to the AHB1 protein in the seeds of GM cotton lines.

140. Invertebrates, including beneficial insects, may be directly exposed to the GM cotton lines containing the AHB1 protein through feeding on the GM cotton plants. Exposure may also occur indirectly through eating other organisms which have previously fed on the GM cotton plants. Exposure in the soil may occur when cotton tissues decompose or as a result of root exudation. Relative exposure will be greatest for herbivorous species feeding on the cotton plants. Pollinator species and various adult insects that feed on pollen will also be exposed to the protein. Sap feeders, such as aphids, will have minimal exposure, as the sap is composed mainly of sugars and mineral salts dissolved in water.

141. Micro-organisms, particularly soil micro-organisms, will be exposed to the GM cotton plants and the expressed AHB1 protein during the growth and decomposition of plant material. After harvest of cotton seed the remaining cotton residues will be tilled into the soil so soil micro-organisms are likely to be exposed to the AHB1 protein as the residues are broken down.

142. Cotton seed and pollen from the release are not expected to enter aquatic habitats in any significant quantities (OGTR 2002). Therefore the level of exposure of aquatic organisms to the GM cotton will be low. Irrigation practices (Good Management Practice of Cotton Industry) used by cotton growers in Australia retain irrigation water run-off as well as the first 15 mm of storm water run-off, on-farm to minimise the entry of pesticide residues into natural waterways.

143. AHB1 and other nsHbs are present naturally in plants (OGTR 2002; Dordas et al. 2003b), including cotton (Hunt et al. 2001)(see Section 5.4 in Chapter 1). Since the same or similar proteins are widespread in the environment, the AHB1 protein is not expected to be a novel source of harm for vertebrates, invertebrates or micro-organisms.

144. Contact with, or ingestion by vertebrates, invertebrates or micro-organisms of the GM plant materials containing AHB1 would be limited because of the small scale and short duration of the proposed release, and none of the materials would be used as animal feed.

145. Therefore, **no risk is identified** and the potential for toxicity for organisms (other than people) as a result of direct or indirect indigestion of the AHB1 protein will not be assessed further.

## 2.4 Spread and persistence of the GM cotton lines in the environment

146. Information on non-GM cotton is included here to establish a baseline for comparison with the GM cotton being considered in this risk assessment. Attributes of non-GM cotton associated with potential weediness are discussed in the document *The Biology and Ecology of Cotton (Gossypium hirsutum) in Australia* (OGTR 2002). This document concludes that non-GM cotton is not a serious weed in Australia. Firstly, cotton does not have certain characteristics typically associated with problematic weeds such as prolonged seed dormancy, persistence in soil seed banks, germination under adverse environmental conditions, rapid vegetative growth, a short life cycle, very high seed output, high seed dispersal and long-distance dispersal of seeds. Secondly, abiotic and biotic factors including temperature (particularly frost), soil moisture, nutrient levels and roadside management practices limit the establishment and/or persistence of cotton outside of agricultural and other disturbed environments.

147. Additional limiting abiotic and biotic factors that determine whether cotton will persist in the environment include short summer seasons, soil type, fire, competition from other plants, herbivory (insects and other animals), and physical destruction such as trampling (Farrell & Roberts 2002; OGTR 2002; Eastick & Hearnden 2006). The relative impact of each of these factors is dependent on whether the cotton plants are in coastal or inland areas, as well as whether they are in northern or southern areas of Australia. For example, frost is a major limiting factor in southern areas of Australia, whereas the reliable availability of water is a limiting factor in most areas of Australia.

148. An important indicator of potential weediness of a particular plant is its history of weediness in any part of the world and its taxonomic relationship to declared weeds (Bergelson et al. 1998; Panetta 1993; Pheloung 1995). Cotton has been grown for centuries throughout the world without any reports that it is a serious weed. Likewise, cotton is not considered to be a serious weed in Australia (Groves et al. 2000; Groves et al. 2002; Groves et al. 2003). Worldwide, there are about 50 species of *Gossypium* (Fryxell 1992; Craven et al. 1994), none of which is listed as a serious weed (Holm et al. 1979; Holm et al. 1997; Randall 2002; Groves et al. 2003).

149. The weed status of cotton has also been considered previously in many of the RARMPs produced during the assessment of a variety of GM cotton lines (eg DIRs 012/2002,

022/2002, 023/2002, 055/2004, 056/2006, 059/2005, 062/2005 063/2005, 064/2006, 065/2006 and 066/2006). In addition to the information in the Biology and Ecology document (OGTR 2002), these RARMPs have considered new data that has been collected during the assessment of application for previous releases of GM cotton lines in Australia.

**Event 6: Expression of the *AHb1* gene improving the survival of cotton volunteers through tolerance to waterlogging**

150. The GM cotton lines produce the AHB1 protein, which gives tolerance to waterlogging stress. In an environment in which susceptibility to waterlogging was the main factor limiting the spread and persistence of cotton, expression of AHB1 could result in weediness of the GM cotton lines.

151. The applicant states that this is a proof of concept field trial. Therefore the ability of the GM cotton lines to withstand waterlogging throughout different stages of their lifecycle as compared to commercially available cotton cultivars is unknown. Data on the waterlogging tolerance of the GM cotton lines would be required for possible future applications involving large scale or commercial releases of these GM cotton lines. However, the data are not required for the proposed release because the trial is limited in size, duration and location.

152. In Australia, waterlogging of cotton is a problem due to the furrow irrigation system. In the areas in which cotton is grown, waterlogging of soils outside the irrigated cropping areas is less likely.

153. Some GM cotton seed may to be dispersed during flooding. As a result, cotton volunteers may establish along waterways (eg drains, creeks and rivers) or in flood prone areas where the *AHb1* gene may confer some advantage. If the waterlogging tolerant cotton were to spread outside of a cultivated area there are numerous factors which would limit its persistence, as for non-GM cotton (as discussed above). Waterlogging is not a main limiting factor for cotton in the natural environment. The applicant proposed to locate the release site 50 m away from natural waterways and the irrigation practices (Good Management Practice of Cotton Industry) used by cotton growers in Australia to retain irrigation water run-off, as well as the first 15 mm of storm water run-off, on-farm to minimise the entry of pesticide residues into natural waterways would also reduce the dispersal of seed.

154. The limited area and duration of this proposed release would result in the introduction of a relatively small number of GM cotton plants into the environment. The chance of volunteer GM plants establishing as weeds by finding suitable ecological niches, would be no greater than for the non-GM parent organism. Furthermore, containment measures proposed by the applicant will further limit the spread and persistence of the GM cotton lines (for details see Section 2.3 in Chapter 1).

155. Therefore, **no risk is identified** and the potential for the spread and persistence (weediness) of cotton as a result of the production of the AHB1 protein will not be assessed further.

**Event 7: Expression of the *AHb1* gene improving the survival of cotton volunteers through enhancement of tolerances to abiotic stress (other than waterlogging) or biotic stress**

156. The GM cotton lines produce the AHB1 protein, which is intended to give tolerance to waterlogging. It is possible that the AHB1 protein confers cross tolerance to other environmental stresses, which could lead to increased spread and persistence of the GM cotton plants.

***Cross tolerance to abiotic environmental stresses other than waterlogging***

157. If AHB1 conferred cross tolerance to abiotic stresses such as cold, drought or nutrient uptake, an increase in the spread and persistence of the GM cotton lines could occur.

158. The expression of *AHb1* is not induced by cold or dehydration stress (Trevaskis et al. 1997) suggesting that it is not involved in tolerance to cold or drought. However, in DNA microarray experiments *AHb1* expression in seedlings is increased by addition of nitrate (Wang et al. 2000; Sakamoto et al. 2004) and reduced by nitrate removal (Zimmermann et al. 2004). This suggests that *AHb1* expression may increase in response to nitrogenous fertiliser.

159. *AHb1* expression has also been shown in lab experiments to be increased by exposure to nickel (but not the heavy metals cadmium, copper or zinc) (Krizek et al. 2003), high CO<sub>2</sub>, ethylene and heat stress (Zimmermann et al. 2004).

160. *AHb1* belongs to the class 1 nsHbs (see Section 4.2 in Chapter 1 for details). A class 1 nsHb in tomato has been shown in DNA microarray experiments to be up-regulated by the addition of nitrate, iron, potassium and phosphorus (Wang et al. 2001).

161. These results are all from lab experiments and the expression and effects of AHB1 to abiotic stresses under field conditions has not been assessed previously. The increased expression of *AHb1* in response to these abiotic stresses could initiate a cascade of events leading to increased tolerance to the stress, which could lead to an increase in the spread and persistence of the GM cotton lines.

162. Root architecture is an important factor in water and nutrient uptake (Lynch 1995). Root growth has been seen to be altered in GM *Arabidopsis* lines over-expressing *AHb1* (Hunt et al. 2002). The roots from the 35S-*AHb1* plants were longer with fewer root hairs, more lateral roots and a longer root elongation zone than the non-GM plants. This could impact on the ability of these plants to respond to soil factors such as water and nutrient availability as has been seen in other plants (Misra et al. 1988; Brouder & Cassman 1994; Gahoonia & Nielsen 1997; Gahoonia et al. 2001).

163. Despite these possible effects the applicant has reported no differences in growth of the GM cotton lines in the glasshouse compared to non-GM cotton plants.

***Cross tolerance to biotic environmental stresses***

164. If AHB1 conferred cross tolerance to biotic stresses such as insect pests, diseases or micro-organisms, an increase in the spread and persistence of the GM cotton lines could occur.

165. There have been no reports of the effect of *AHb1* on the palatability or toxicity of plants to insects. Microarray experiments comparing gene expression in healthy *Arabidopsis* plants with those following aphid (*Myzus persicae*), thrip (*Frankliniella occidentalis*) or caterpillar (*Pieris rapae*) infestation showed no altered expression of *AHb1* (Zimmermann et al. 2004).

166. Numerous microarray studies have been performed using *Arabidopsis* following various biotic stresses and analysed on the GENEVESTIGATOR database. The biotic stresses which showed no effect on *AHb1* expression include fungal infection (*Alternaria brassicicola*, *Botrytis cinerea*, *Erysiphe cichoracearum* and *Erysiphe orontii*), oomycete infection (*Phytophthora infestans*), pseudomonad infection (*Pseudomonas syringae*) and mycorrhizal colonisation (*Gigaspora rosea*). Increased expression of *AHb1* was observed following infestation with the nematode *Heterodera schachtii* or infection with the bacterium *Agrobacterium tumefaciens*.

167. No alterations to the resistance reaction following challenge with an incompatible fungus or bacterium have been attributed to *AHb1*. No changes in *AHb1* expression were seen in *Arabidopsis* infected with the incompatible fungus *Alternaria brassicicola* (Schenk et al. 2000). GM *Arabidopsis* plants over-expressing *AHb1* showed a similar hypersensitive cell death reaction to non-GM plants when infected with *Pseudomonas syringae* (Perazzolli et al. 2004).

168. Effects have been observed on other class 1 nsHbs following infection with a pathogen. In cotton plants, *GhHb1* expression increased following infection with *Fusarium* or *Verticillium* wilts (Dowd et al. 2004; Qu et al. 2005). Similarly, GM *Arabidopsis* over-expressing cotton *GhHb1* had increased tolerance to the pathogens *Verticillium dahliae* and *Pseudomonas syringae* pv *tomato* (Qu et al. 2006). In GM tobacco, over-expression of alfalfa nsHb results in fewer necrotic lesions from the induced non-host hypersensitive response when challenged with the incompatible bacteria *P. syringae* pv *phaseolicola*. These GM plants also showed reduced lesion numbers when infected with tobacco necrosis virus (Seregélyes et al. 2003). After challenge with the pathogen *P. syringae* pv *maculicola* the GM tobacco plants showed an enhanced stress response with more reactive oxygen species (ROS), *PR-1a* and salicylic acid produced during infection than the non-GM plants (Seregélyes et al. 2003; Seregélyes et al. 2004).

169. Alterations to the expression levels of nsHbs have been observed during symbiotic interactions. Symbiotic rhizobium infection causes the up regulation of a class-1 nsHb in *Lotus japonicus* (Shimoda et al. 2005), but repression of the nsHb was seen following colonisation by mycorrhizal fungi (*Glomus* sp.). This has been confirmed in alfalfa, where the nsHb, *Mhb1*, was repressed in mycorrhizal colonisation (Uchiumi et al. 2002).

### **Conclusion**

170. The main factors limiting the spread and persistence in the areas proposed for release are water and nutrient availability, temperature and soil type (Farrell & Roberts 2002; OGTR 2002; Eastick & Hearnden 2006). Therefore, an increase in spread and persistence of the GM cotton lines could only occur in an environment where enhanced resistance to insect pests and micro-organisms was limiting the spread and persistence of cotton.

171. In addition, the release would be of limited size and short duration and the applicant proposed a number of measures to limit the spread and persistence of the GM cotton lines proposed for release.

172. Therefore, **no risk is identified** and the potential for the spread and persistence of cotton through cross tolerance to environmental stresses other than waterlogging as a result of the expression of the *AHb1* protein will not be assessed further.

### **Uncertainty**

173. It is possible that the GM cotton lines may have enhanced tolerances to a number of biotic stresses as compared to their non-GM parent. However, in the unlikely instance where the plants may have enhanced tolerances to several environmental stresses, the GM plants will most likely be less fit as compared to commercially available cotton varieties because of the potential metabolic/physiological burdens. For example, the cotton may have stunted growth, produce less seeds, or have a decreased ability to tolerate competition from other plants.

174. Data on abiotic and biotic stress tolerances, additional to waterlogging tolerance, of the GM cotton lines would be required for future applications involving large scale or commercial releases of these GM cotton lines. However, the data are not required for the proposed release because the trial is limited in size, duration and location.

**Event 8: Expression of the *AHb1* gene improving the survival of cotton volunteers through altered dormancy, seed viability, germination rates and/or seedling viability**

175. Expression of the *AHb1* gene in the GM cotton lines could lead to altered dormancy, seed viability, germination rate and/or seedling viability resulting in increased spread and persistence of these GM cotton lines.

176. There have been no reports of *AHb1* affecting seed dormancy, although *AHb1* is expressed during germination (Hunt et al. 2001; Gonzali et al. 2005). In barley, the viability of stored seed was found to be correlated with nsHb levels. The correlation was found to be so robust that nsHb has been used as a marker for seed germination, and thus viability, of barley seeds (Guy et al. 2002).

177. The over-expression of *AHb1* in GM *Arabidopsis* leads to enhanced early growth rates (Hunt et al. 2002). GM *Arabidopsis* plants constitutively expressing *AHb1* had 1.5-fold greater wet weight than non-GM plants at 14 days after germination. This is not due to faster development as the leaf numbers were not altered, but due to increased size of roots and shoots (Hunt et al. 2002). The plants were measured for a further 18 days on both sucrose-supplemented and non-supplemented media and did not show any significant growth differences between the non-GM and *AHb1* over-expressing GM plants.

178. However, this altered growth rate has not been seen for all class 1 nsHbs. GM tobacco plants expressing alfalfa nsHb did not show any obvious phenotype as seedlings or any enhanced growth rates (Seregélyes et al. 2003).

179. Increased early growth rates have also been observed in GM plants over-expressing a haemoglobin gene from the gram negative bacterium *Vitreoscilla*. Expression of the haemoglobin gene from *Vitreoscilla* in GM petunia or cabbage has shown enhanced tolerance to flooding and waterlogging (Mao et al. 2003; Li et al. 2005). The GM cabbage also showed faster seed germination times (Li et al. 2005). GM tobacco over-expressing *Vitreoscilla* haemoglobin showed enhanced dry matter and chlorophyll, and faster germination and flowering times than non-GM control plants (Holmberg et al. 1997). Tobacco cell suspension cultures over-expressing *Vitreoscilla* haemoglobin showed no lag phase and exhibited improved cell growth (Farrés & Kallio 2002). Conversely, other experiments with GM tobacco and hybrid aspen (*Populus tremula x tremuloides*) over-expressing *Vitreoscilla* haemoglobin showed no phenotypic differences between the GM and non-GM plants (Häggman et al. 2003; Frey et al. 2004).

180. Sequence comparisons indicate that *Vitreoscilla* haemoglobin exists as a dimer (Wakabayashi et al. 1986) and is more homologous to plant than to animal haemoglobins (Arredondo-Peter & Escamilla 1991). However, plant and *Vitreoscilla* haemoglobins may not function in the same way as the *Vitreoscilla* haemoglobin possesses a 60x lower oxygen affinity than the plant nsHbs (Bülow et al. 1999).

181. In an environment where the speed of germination and early growth of cotton seedlings gives a selective advantage, the GM cotton lines could become established. However, the main factors limiting the spread and persistence of cotton in the areas proposed for release are water and nutrient availability, temperature and soil type (Farrell & Roberts 2002; OGTR 2002; Eastick & Hearnden 2006).

182. In addition, the release would be of limited size and short duration and the applicant proposed a number of measures to limit the spread and persistence of the GM cotton lines proposed for release (for details see Section 2.3 in Chapter 1).

183. Therefore, **no risk is identified** and the potential for the spread and persistence of cotton through altered dormancy, seed viability, germination rates and/or seedling viability as a result of the expression of the AHB1 protein will not be assessed further.

**Event 9: *Dispersal of GM seed or other GM plant materials during transport, research, storage, equipment use, flooding or via animals***

184. In the course of the proposed dealings the applicant proposed to transport seed to and from the release sites, cultivate GM cotton plants, store the GM cotton seed hand-harvested from the crop and collect GM plant materials for research purposes, laboratory research or possible future release (subject to further applications and approvals). Accidental spillage or dispersal of GM plant materials, especially seed, in the course of these dealings could allow the GM cotton plants to spread and persist in the environment.

185. Cotton does not propagate vegetatively (Serdy et al. 1995; OGTR 2002), so dispersal of GM cotton materials other than seed would be highly unlikely to result in the dissemination of the GM cotton lines proposed for release.

***Dispersal by spillage during transport, storage or from equipment***

186. The Regulator has issued guidelines and policies for the transport, supply and storage of GMOs (*Guidelines for the transport of GMOs, June 2001* and *Policy on transport and supply of GMOs, July 2005*). The applicant proposed to transport and store seed according to OGTR guidelines. Any GM cotton seed not required for future research or planting will be destroyed. Therefore, any spillage of seed during transport to and from the release sites or while in storage would be rare. Any incident involving spillage of GM seed is expected to be readily controlled through cleaning and monitoring of the site of the spill. In addition, the opportunity for an adverse outcome from any such rare occurrence is further diminished by the need for appropriate environmental conditions for germination, survival and persistence of any few escaped seeds.

187. Furthermore, the applicant proposed to thoroughly clean pickers and module builders after the GM cotton material has been harvested to prevent dispersal of seed to other locations. The applicant proposed to destroy all plant materials other than some materials collected for future research or planting. The sites would be monitored for volunteers and any volunteer cotton plants will be destroyed.

***Dispersal by flooding of the release site***

188. Severe weather conditions (eg flooding) could lead to the dispersal of GM plant materials, including seed. As a result, cotton volunteers may establish along waterways (eg drains, creeks and rivers) or in flood prone areas where the AHB1 protein may confer some advantage. However, the flooding is likely to be a transient event and once the ground is no longer waterlogged the GM cotton will have no advantage compared to non-GM cotton. The other factors which prevent cotton from establishing such as reliable water availability and low winter temperatures would likely limit its spread and persistence.

189. The proposed trial site is prone to flooding, but even in the event of flooding the field is surrounded by levy banks and laser levelled to control the movement of irrigation water and floods rise and fall gently, normally without disturbing the plants growing in the field (information supplied by the applicant). In case of a flood, any seed or other GM plant material on the ground is therefore not expected to be dispersed beyond the area of the proposed release (including the pollen trap, which would act as a physical barrier once the plants were established). Cotton does not generally propagate vegetatively, so dispersal of GM cotton plant material other than seed would be highly unlikely to help establish the GM

cotton lines (OGTR 2002). However, the applicant proposed to monitor all plots and drains and actively control cotton volunteers.

190. In addition, the applicant proposes to locate the site 50 m away from natural waterways (see Section 2.3 in Chapter 1). Irrigation practices (Good Management Practice of Cotton Industry) used by cotton growers in Australia retain irrigation water run-off, as well as the first 15 mm of storm water run-off, on-farm to minimise the entry of pesticide residues into natural waterways. This practice would also reduce the dispersal of seed.

#### ***Dispersal by animals***

191. In the field, seed cotton is present as large lint-covered bolls. Mammals, including rodents, generally avoid feeding on cotton plants and therefore are unlikely to carry bolls any great distance from the cotton fields. The cotton bolls are also unattractive to avian species, so birds are unlikely to transport cotton seeds (OGTR 2002).

#### ***Conclusion***

192. Therefore, **no risk is identified** and the potential for an adverse outcome as a result of dispersal of GM cotton seed or other GM plant materials during transport, research, storage, equipment use, flooding or via animals will not be assessed further.

#### ***Event 10: Exposure of vertebrates (including people), invertebrates and micro-organisms to GM cotton volunteers containing AHB1***

##### ***Increased spread and persistence of the GM cotton lines proposed for release***

193. The potential of increased spread and persistence of the GM cotton lines in the environment was assessed in Events 6, 7 and 8 and no risk was identified. However, in the highly unlikely instance of this occurring, spread and persistence of the GM cotton plants in the environment could lead to increased exposure of vertebrates (including people), invertebrates and micro-organisms to GM cotton volunteers containing the expressed AHB1 protein.

194. An adverse outcome could occur, if the AHB1 protein were toxic or allergenic for people. The potential for AHB1 causing toxic or allergic reactions in people was assessed (Events 1-4 of this Chapter) and no risk was identified.

195. Organisms other than people may be exposed directly, through feeding on the GM cotton plants or indirectly through eating organisms that have fed on or degrade the GM cotton plants as a result of spread and persistence of the GM cotton in the environment. These organisms include vertebrates, invertebrates and micro-organisms. The potential for toxicity of AHB1 to organisms other than people was considered (Event 5 of this Chapter) and no risk was identified.

196. The release would be of limited size and short duration and the applicant proposed a number of measures to limit the spread and persistence of the GM cotton lines in the environment (for details see Section 2.3 of Chapter 1).

197. Therefore, **no risk is identified** and the potential for toxicity or allergic reactions in people or other organisms as a result of spread and persistence of the GM cotton lines in the environment will not be assessed further.

## **2.5 Vertical transfer of genes or genetic elements to sexually compatible plants**

198. Transfer of genetic material to offspring by sexual reproduction (vertical gene transfer) could result in the transfer of the *AHb1* gene or its associated regulatory elements to other

plants. The only sexually compatible species present in Australia that could receive genes from the GM cotton lines are *G. hirsutum* (including both cultivated–GM and non-GM, and naturalised cotton) and *G. barbadense*.

199. Weediness resulting from an increase in the spread and persistence of other cotton plants is contingent on both of the following steps:

- transfer of the introduced gene construct to other cotton plants
- weediness of the recipient plants as a result of expression of the introduced gene.

**Event 11: Expression of the introduced gene in other *G. hirsutum* (including commercially released GM cotton lines) or *G. barbadense* cotton plants**

***Outcrossing to sexually compatible species***

200. In the area proposed for release, both cotton species (*G. hirsutum* and *G. barbadense*) are grown. The gene for waterlogging tolerance could be transferred to these sexually compatible cotton plants resulting in an increased potential for weediness. The following GM cotton lines are currently approved for commercial release in Australia south of latitude 22°South:

- insect resistant INGARD® cotton (DIR 022/2002; withdrawn from the market in 2004 in favour of Bollgard II® cotton)
- glyphosate tolerant Roundup Ready® cotton (DIR 023/2002)
- glyphosate tolerant/insect resistant (Roundup Ready®/INGARD®) cotton (DIR 023/2002; withdrawn from the market in favour of Bollgard II®/Roundup Ready® cotton)
- insect resistant Bollgard II® cotton (DIR 012/2002)
- insect resistant/glyphosate tolerant (Bollgard II®/Roundup Ready®) cotton (DIR 012/2002)
- glyphosate tolerant Roundup Ready Flex® cotton (DIR 059/2005)
- glyphosate tolerant/insect resistant (Roundup Ready Flex®/Bollgard II®) cotton (DIR 059/2005)
- glufosinate ammonium tolerant (LibertyLink® Cotton) cotton (DIR 062/2005).

201. In the 2005-2006 season, 90% of cotton grown in Australia was GM, consisting of Bollgard II®, Roundup Ready® and Bollgard II®/Roundup Ready® cotton lines (Cotton Australia 2006).

202. Roundup Ready Flex® and Roundup Ready Flex®/ Bollgard II® cotton were approved in February 2006 (under DIR 059/2005 licence) and are expected to replace Roundup Ready® and Bollgard II®/Roundup Ready® respectively, in future seasons.

203. LibertyLink® Cotton was approved for commercial release in August 2006 and as yet there are no widespread plantings.

204. Cotton is primarily self-pollinating with pollen that is large, sticky and heavy, and not easily dispersed by wind (Jenkins 1992; OGTR 2002). Overseas studies have shown that insect pollinators can transfer pollen to other nearby cotton plants at rates up to 80% (eg Oosterhuis & Jernstedt 1999). However, cotton pollen dispersal studies consistently show that outcrossing is localised around the pollen source and decreases significantly with distance [(OGTR 2002), and references therein]. The separation distance of 4 metres required in

Australia for certified commercial seed production reflects the relatively short distances observed for cotton pollen dispersal in Australian studies (Brubaker et al. 1999). For vertical gene transfer to occur, the GM cotton lines would need to be planted within pollination distance of sexually compatible cotton plants.

205. Although commercial cotton crops may be planted near to the trial sites, outcrossing would be limited due to the containment measure of a 20 m pollen trap proposed by the applicant that would surround the GM cotton lines. All plant materials other than some materials collected for future research or planting would be destroyed at the end of each growing season.

206. In addition, the GM cotton lines proposed for release have no traits conferring insect resistance. This would likely result in the applicant having to adopt the heavy insecticide spraying regime that is necessary for maintaining non-insect-resistant cotton. This in turn would further limit the chance of pollen transfer to plants outside the proposed trial site.

207. *G. barbadense* is the closest relative of *G. hirsutum* occurring in Australia (OGTR 2002). It is grown commercially on a small scale in Australia and will be grown near the trial site (information supplied by applicant). Hybridisation can occur naturally between these two species (Brubaker et al. 1999). Hybrid progeny exhibit characteristics intermediate to the parents but typically with a lower capacity to produce fruit. *G. barbadense* and hybrids are not weedier or more difficult to control than *G. hirsutum* (Warwick Stiller & Greg Constable, CSIRO, pers. comm.). As for *G. hirsutum*, pollen dispersal from the GM cotton lines would be low and insecticides will be used.

208. The limited area and duration of this proposed release would result in the introduction of a relatively small number of GM cotton plants into the environment. The chance of volunteer GM plants establishing as weeds by finding suitable ecological niches, would be no greater than for the non-GM parent organism.

#### ***Potential for weediness in plants resulting from outcrossing***

209. In the highly unlikely case of outcrossing to a commercially grown non-GM cotton plant, the potential for weediness would be the same as for the GM cotton lines itself, which was assessed (Events 6-9, this Chapter) and no risk was identified. The spread and persistence of cotton in the areas proposed for release is limited by multiple factors other than tolerance to waterlogging including water availability, frost, nutrient levels, roadside management practices, soil type, and competition from other plants and diseases (Farrell & Roberts 2002; OGTR 2002; Eastick & Hearnden 2006).

210. If the pollen recipient were a commercially released GM cotton plant, stacking of genes conferring herbicide tolerance and/or insect resistance with *AHb1* conferring tolerance to waterlogging could occur. This may give a fitness advantage in an environment where waterlogging, herbicide tolerance and insect herbivory were the main limiting factors for the spread and persistence of cotton. However, the main factors limiting the spread and persistence of cotton in the areas proposed for release are water and nutrient availability, temperature and soil type, (OGTR 2002). In the rare event of outcrossing to commercially released GM cotton plants, the resulting seed would not be used for subsequent plantings as farmers are required to buy certified GM cotton seed for each growing season. This would further reduce the already limited possibility of spread and persistence of the GM cotton lines proposed for release.

## Conclusion

211. In addition to the proposed measures to limit the spread and persistence of the GM cotton lines, the release is of limited size and short duration. The applicant also proposed to conduct post harvest monitoring of the trial sites for at least 12 months and destroy all volunteers.

212. Therefore, **no risk is identified** and the potential for expression of the introduced genes for waterlogging tolerance in other *G. hirsutum* or *G. barbadense* cotton plants leading to weediness will not be assessed further.

## Uncertainty

213. The *AHb1* gene could potentially affect the growth rate and thereby also alter flowering time of the GM cotton lines as compared to commercially grown cotton. If flowering times of any of the GM cotton lines during the field trials was altered as compared to pollen trap, then the potential for pollen flow to flowering cotton plants outside the trial site would be increased.

214. For example, the over-expression of *AHb1* in GM *Arabidopsis* leads to enhanced early growth rates (Hunt et al. 2002) (see Event 8 in this Chapter) but there is no data presented on alteration of flowering times. GM tobacco over-expressing *Vitreoscilla* haemoglobin showed faster germination and flowering times than non-GM control plants (Holmberg et al. 1997).

215. The applicant is not aware of differences in growth of the GM cotton plants compared to the non-GM parent in glasshouse studies. Data on the growth of the GM cotton lines selected for further development and how this may affect weediness would be required before any future application for a large scale or commercial release of these GMOs could be assessed. However, this information is not required for assessing the risks of this proposed release because of the containment measures proposed by the applicant to limit the spread and persistence of the GM cotton lines, and the trial is limited in size, duration and location.

216. Considering the above points, it is highly unlikely the genes for waterlogging tolerance will be transferred to sexually compatible cotton plants ie *G. hirsutum* or *G. barbadense* cotton plants (including commercially released GM cotton lines).

### **Event 12: Exposure of vertebrates (including people), invertebrates and micro-organisms to other *G. hirsutum* (including commercially released GM cotton lines) or *G. barbadense* cotton plants expressing the introduced gene.**

217. The *AHb1* gene could be transferred to sexually compatible cotton plants, ie *G. hirsutum* (including commercially released GM cotton lines), resulting in increased exposure of vertebrates (including people), invertebrates and micro-organisms to AHB1.

218. The transfer of the introduced genes to other *G. hirsutum* or *G. barbadense* cotton plants was assessed in Event 11 and considered highly unlikely. If gene transfer to other *G. hirsutum* or *G. barbadense* cotton plants occurred the potential adverse outcomes of these cotton lines would be highly similar to the GM cotton lines proposed for release, which was assessed in Events 1-5.

219. In addition to the proposed measures to limit the spread and persistence of the GM cotton lines, the release is of limited size and short duration. The applicant also proposed to conduct post harvest monitoring of the trial site for at least 12 months and destroy all volunteers.

220. Therefore, **no risk is identified** and the potential for toxicity or allergenicity to vertebrates (including people), invertebrates and micro-organisms through increased exposure

as a result of the *AHb1* gene being expressed in other *G. hirsutum* and *G. barbadense* cotton plants will not be assessed further.

**Event 13: Presence of the introduced regulatory sequences in other *G. hirsutum* or *G. barbadense* plants as a result of gene transfer**

221. All of the introduced regulatory sequences operate in the same manner as regulatory elements endogenous to cotton plants. The transfer of either endogenous or introduced regulatory sequences could result in unpredictable effects. The impacts from the introduced regulatory elements are equivalent and no greater than the endogenous regulatory elements.

222. Therefore, **no risk is identified** and the potential for an adverse outcome as a result of vertical gene transfer of introduced regulatory sequences will not be assessed further.

**Event 14: Gene transfer to native *Gossypium* species**

223. As discussed in the *Biology and Ecology of Cotton (Gossypium hirsutum) in Australia* (OGTR 2002), Australian flora contains 17 native *Gossypium* species, all of which are diploid (C, G or K genomes), while the cultivated cotton species are tetraploid (AD genomes). Thus there is well established genetic incompatibility between native *Gossypium* species and cultivated cotton. The native cotton species with highest potential for hybridising with *G. hirsutum* is *G. sturtianum*. Hybrids between these two species have been produced without application of plant hormones, when plants were in close proximity to each other. However, these hybrids were sterile, effectively eliminating any potential for introgression of *G. hirsutum* genes into *G. sturtianum* populations.

224. The centre of native *Gossypium* diversity in Australia is in northern Western Australia and the Northern Territory. Most of the Australian *Gossypium* species have limited distributions and occur at considerable geographic distance from cultivated cotton fields. Thus, gene transfer from the GM cotton lines to native cotton plants is prevented not only by genetic incompatibility but also by geographic constraints on cross pollination (OGTR 2002).

225. Therefore, **no risk is identified** and the potential for weediness in these sexually incompatible species as a result of gene transfer will not be assessed further.

## **2.6 Horizontal transfer of genes or genetic elements to sexually incompatible organisms**

**Event 15: Presence of the *AHb1* gene or the introduced regulatory sequences in other organisms as a result of gene transfer**

226. Transfer of the *AHb1* gene, or the introduced regulatory sequences, from the GM cotton plants to sexually incompatible plants, animals or micro-organisms (horizontal gene transfer) rarely occurs without human intervention.

227. Most gene transfers have been identified through analyses of gene sequences (Worobey & Holmes 1999; Ochman et al. 2000). In general, gene transfers are detected over evolutionary time scales of millions of years (Lawrence 1999). Most gene transfers have been from virus to virus (Lai 1992), or between bacteria (Ochman et al. 2000). In contrast, transfers of plant genetic materials to other micro-organisms such as bacteria, viruses or fungi have been exceedingly rare.

228. Transfer of the regulatory sequences to other organisms including viruses could alter the expression of endogenous genes in unpredictable ways. However, all of the introduced regulatory sequences operate in the same manner as regulatory elements endogenous to cotton plants. The transfer of either endogenous or introduced regulatory sequences could result in

adverse unpredictable effects. As there is no difference between those two events, this does not represent a novel adverse outcome as a result of the genetic modification.

229. It is possible that recombination could occur between the introduced SCSV S4S4 and CaMV35S promoters and a virus invading the GM cotton lines. This could lead to the development of a viral variant with altered properties. However, these promoter sequences are present in viruses which exist naturally in the environment and transfer of sequences from virus to virus is more likely than from plant to virus.

230. Horizontal gene transfer has been examined in detail in a number of other RARMPs (most recently DIR 057/2004), which are available from the OGTR website (<http://www.ogtr.gov.au>) or by contacting the Office. These assessments have concluded that horizontal gene transfer from plants to other sexually incompatible organisms occurs rarely and usually only on evolutionary timescales. Reports of horizontal gene transfer from plants to bacteria occurring during laboratory experiments have not only relied on the use of highly similar sequences to allow homologous recombination to occur, but also on conditions designed to enhance the selective advantage of gene transfer events (Nielsen et al. 2000; Gebhard & Smalla 1998; Mercer et al. 1999; Nielsen 1998; De Vries et al. 2001). Horizontal gene transfer is not expected to produce any adverse outcomes during this proposed limited and controlled release.

231. Therefore, **no risk is identified**. The potential for an adverse outcome as a result of horizontal gene transfer will not be assessed further.

## 2.7 Unintended changes in toxicity and/or allergenicity

232. A single plant gene can have an influence on multiple, sometimes unrelated, plant traits. This phenomenon is known as pleiotropy. Single genes inserted into a plant by genetic modification can also result in pleiotropy. It is therefore necessary to evaluate GM plants for unintended pleiotropic effects, such as changes in agronomic characteristics, which may be a consequence of the gene insertion.

233. Gene technology has the potential to cause unintended effects due to the process used to insert new genetic material or by producing a gene product that affects multiple traits. Such effects may include:

- altered expression of an unrelated gene at the site of insertion
- altered expression of an unrelated gene distant to the site of insertion, for example, due to the encoded protein of the introduced gene changing chromatin structure, affecting methylation patterns or regulating signal transduction and transcription
- increased metabolic burden associated with high level expression of the introduced genes
- novel traits arising from interactions of an introduced gene product with endogenous non-target molecules
- secondary effects arising from altered substrate or product levels in the biochemical pathway incorporating the protein encoded by the introduced gene.

234. Unintended pleiotropic effects might result in adverse outcomes such as toxicity or allergenicity; weediness, pest or disease burden; or reduced nutritional value as compared to the parent organism. However, accumulated experience with genetic modification of plants indicates that the process has little potential for unexpected outcomes that are not detected and eliminated during the early stage of selecting plants with new properties (Bradford et al.

2005). Additionally, unintended changes that occur as a result of gene insertions are rarely advantageous to the plant (Kurland et al. 2003).

235. All methods of plant breeding can induce unanticipated changes in plants, including pleiotropic effects (Haslberger 2003). Most often the important nutrients, toxicants and other components are present in the new plant variety at levels that are within the range expected for commercial varieties.

**Event 16: Altered levels of innate toxic or allergenic compounds as a result of the expression or random insertion of the gene construct into the cotton genome during genetic modification**

236. Cotton tissue (from either GM or non-GM plants), particularly the seeds, can be toxic if ingested in large quantities because of the presence of toxic and anti-nutritional compounds including gossypol and cyclopropenoid fatty acids (eg dihydrosterculic, sterculic and malvalic acids). Further discussion regarding the toxicity and allergenicity of non-GM cotton is provided in *The Biology and Ecology of Cotton (Gossypium hirsutum) in Australia* (OGTR 2002) and the *Consensus document on compositional considerations for new varieties of cotton (Gossypium hirsutum and Gossypium barbadense): Key food and feed nutrients and anti-nutrients* (OECD 2004). There is potential for these GM cotton plants to have increased levels of toxic or allergenic compounds as a result of the expression of the *AHb1* gene for waterlogging tolerance. Additionally, random insertion of the gene construct during the genetic modification process could potentially result in increased toxic or allergenic compounds in the GM cotton lines.

237. Thus far, exposure to plant materials (including pollen) from the GM cotton lines has been limited to a few workers maintaining these plants in the glasshouse. The applicant has reported no adverse outcomes from exposure to the GM cotton plant materials, suggesting that expression of the introduced genes has not altered the toxicity or allergenicity endogenous to non-GM cotton.

238. In addition, the release would be of limited size and short duration and the applicant proposed a number of measures to limit the spread and persistence of the GM cotton lines.

239. Data on toxicity of plant material and/or levels of known endogenous toxins would be required for risk assessments of applications for large scale or commercial release of these GM cotton lines. Further information is not required for assessing the risks of this proposed release because the trial is limited in size, duration and location and none of the GM cotton plant materials are intended for use in human food, animal feed or in the production of fabrics and/or other cotton products

240. Therefore, **no risk is identified** and the potential for changes in levels of innate toxic or anti-nutritional compounds as a result of random insertion of the gene construct into the GM cotton lines will not be assessed further.

## **2.8 Unintended changes in biochemistry, physiology or ecology**

**Event 17: Altered biochemistry, physiology or ecology of the GM cotton lines resulting from expression of the introduced gene**

241. Unintended changes in gene expression could alter the biochemistry, the physiology or the ecology of the GM cotton lines. Biochemical, physiological or ecological changes to the GM cotton lines proposed for release could occur either as a result of the expression of the introduced genes or of the transformation process itself. The GM cotton lines proposed for release were selected from a number of initial individual GM events. Based on the applicant's observations, there was no difference between the parental and GM cotton lines grown under

glasshouse conditions. Further information is not required at this stage as the proposed release is limited in size, duration and location and none of the GM plant materials are intended for use in human food or animal feed or for the production of fabrics and/or other cotton products.

242. The potential adverse outcomes (toxicity, allergenicity and weediness) resulting from altered biochemistry, physiology or ecology of the GM cotton lines proposed for release were assessed (Events 1-15 in this Chapter) and no risks were identified.

243. The expression of the introduced *AHb1* gene could result in unintended pleiotropic effects, and the uncertainty of potential effects is discussed in Event 16. The introduction of a nsHb gene has been shown to affect a number of different pathways in plants such as response to pathogen infection or symbiotic interactions, enhanced seedling growth rates and root architecture (discussed in Events 7 and 8). Introduction of a haemoglobin gene from the gram negative bacterium *Vitreoscilla* into tobacco or *Datura* has also shown altered metabolic pathways. GM tobacco contained a higher level of nicotine and lower levels of anabasine indicating that the metabolic pathways had changed (Holmberg et al. 1997). When *Datura innoxia* was transformed with *Vitreoscilla* haemoglobin, a six-fold increase in the levels of the alkaloid scopolamine could be observed over the controls (Bülow et al. 1999).

244. However, the release would be of limited size and short duration and the applicant proposed a number of measures to limit the spread and persistence of the GM cotton lines proposed for release (for details see Section 2.3 in Chapter 1).

245. More data on the potential pleiotropic effects of the genetic modifications of the GM cotton lines selected for further development and how these may affect potential toxicity, allergenicity and weediness would be required before any future application for a large scale or commercial release of these GMOs could be assessed.

246. Therefore, **no risk is identified** and the potential for weediness, toxicity or allergenicity to people and other organisms, as a result of unintended changes in biochemistry, physiology or ecology will not be assessed further.

## 2.9 Unauthorised activities

### **Event 18: Use of GMOs outside the proposed licence conditions (non-compliance)**

247. If a licence were to be issued, non-compliance with the proposed conditions of the licence could lead to spread and persistence of the GM cotton lines outside of the proposed release areas. The adverse outcomes that this event could cause are discussed in the events above. The Act provides for substantial penalties for non-compliance and unauthorised dealings with GMOs. The Act also requires that the Regulator has regard for the suitability of the applicant to hold a licence prior to the issuing of a licence. These legislative provisions are considered sufficient to minimise risks from unauthorised activities.

248. Therefore, **no risk is identified** and the potential for an adverse outcome as a result of unauthorised activities will not be assessed further.

## **Section 3 Risk estimate process for identified risks**

249. The hazard identification process considered the circumstances by which people or the environment may be exposed to the GMOs, GM plant materials, GM plant by-products, the introduced genes, or products of the introduced genes.

250. Eighteen events were identified and assessed whereby the proposed release of the GM cotton lines might give rise to harm to people or the environment.

251. These 18 events included consideration of whether expression of the introduced genes could result in products that are toxic or allergenic to people or other organisms, produce unintended changes in the biochemistry, physiology or ecology of the GM cotton lines, or alter characteristics that may impact on spread and persistence of the GMOs. In addition, consideration was given to the opportunity for gene flow to other organisms and its effects.

252. None of the 18 events are considered to give rise to an identified risk that required further assessment. The principle reasons include:

- small scale of the trial that is limited in both area and duration
- containment and disposal measures proposed by the applicant to limit the spread and persistence of GM cotton plants
- none of the GM plant materials will be used in human food, animal feed or for the production of fabrics and/or other cotton products
- widespread presence of the same or similar proteins encoded by the introduced genes in the environment and lack of known toxicity or allergenicity from these proteins
- limited capacity of the GM cotton lines to spread and persist in the area proposed for release
- limited ability and opportunity for the GM cotton lines to transfer the introduced genes to other sexually related species or other organisms.

253. Therefore, as no risks to the health and safety of people or the environment were identified from the proposed limited and controlled release of GM cotton lines the level of risk is considered to be **negligible**.

## Chapter 3 Risk management

254. Risk management includes evaluation of risks identified in Chapter 2 to determine whether or not specific treatments are required to mitigate harm to human health and safety, or the environment, from the proposed release. Other risk management considerations required under the Act are also addressed in this Chapter, including conditions that limit and control the release. Together, these risk management measures are used to inform the decision-making process and determine licence conditions that are imposed by the Regulator under the Act. In addition, the roles and responsibilities of other regulators under Australia's integrated regulatory framework for gene technology are explained.

### Section 1 Background

255. Under section 56 of the Act, the Regulator must not issue a licence unless satisfied that any risks posed by the dealings proposed to be authorised by the licence are able to be managed in a way that protects the health and safety of people and the environment. All licences are required to be subject to three conditions prescribed in the Act.

256. Section 63 requires that each licence holder inform relevant people of their obligations under the licence. Other mandatory statutory conditions contemplate the Regulator maintaining oversight of licensed dealings. For example section 64 requires that licence holders provide access to premises to OGTR monitors, and section 65 requires the licence holder to report any information about risks or unintended effects of the dealing to the Regulator on becoming aware of them. Matters related to the ongoing suitability of the licence holder are also required to be reported to the Regulator.

257. It is a further requirement that the licence be subject to any conditions imposed by the Regulator. Examples of the matters to which conditions may relate are listed in section 62 of the Act. Licence conditions can be imposed to limit and control the scope of the dealings and the possession, supply, use, transport or disposal of the GMO for the purposes of, or in the course of, a dealing. In addition, the Regulator has extensive powers to monitor compliance with licence conditions under section 152 of the Act.

### Section 2 Other Australian regulators

258. Australia's gene technology regulatory system operates as part of an integrated legislative framework. Other agencies that also regulate GMOs or GM products include FSANZ, APVMA, Therapeutic Goods Administration (TGA), National Industrial Chemicals Notification and Assessment Scheme (NICNAS), National Health and Medical Research Council (NHMRC) and Australian Quarantine and Inspection Service (AQIS). Dealings conducted under any licence issued by the Regulator may also be subject to regulation by one or more of these agencies<sup>5</sup>.

259. The *Gene Technology Act 2000* requires the Regulator to consult these agencies during the assessment of DIR applications. The *Gene Technology (Consequential Amendments) Act 2000* requires the agencies to consult the Regulator for the purpose of making certain decisions regarding their assessments of products that are, or contain a product from, a GMO.

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<sup>5</sup> More information on Australia's integrated regulatory framework for gene technology is contained in the *Risk Analysis Framework* available from the Office of the Gene Technology Regulator (OGTR). Free call 1800 181 030 or at <<http://www.ogtr.gov.au/pdf/public/raffinal2.2.pdf>>.

260. FSANZ is responsible for human food safety assessment, including GM food. As the trial involves early stage research the applicant does not intend any material from these GM cotton lines to be used in human food. Accordingly the applicant has not applied to FSANZ for evaluation of any of the GM cotton lines for use in human food. However, FSANZ approval would need to be obtained before they could be used in this way.

261. No other approvals are required.

### **Section 3 Risk treatment measures for identified risks**

262. The risk assessment of events listed in Chapter 2 concluded that there are **negligible** risks to people and the environment from the proposed release. *The Risk Analysis Framework* (OGTR 2005), which guides the risk assessment and risk management process, defines negligible risks as insubstantial with no present need to invoke actions for their mitigation.

263. These events were considered in the context of the scale of the release (on one site per season in the shire of Narrabri, NSW on a maximum total area of 0.1 ha during each of the three summer growing seasons of 2006/07, 2007/08 and 2008/09), the containment measures and agricultural practices proposed by the applicant and the receiving environment (see Chapter 1, Section 5).

### **Section 4 General risk management**

264. Containment measures consistent with the risk assessment context have been imposed to limit the spread and persistence of the GM cotton lines in the environment.

#### **4.1 Summary of licence conditions associated with managing the limited and controlled release**

##### **4.1.1 Measures to limit and control the release**

265. A number of licence conditions have been imposed to limit the spread and persistence of the GMOs:

- surround the release site with a pollen trap
- locate the release site at least 50 m away from natural waterways
- harvest and gin seed cotton from the release separately from any other cotton crop
- not permit cotton seed or other materials from the release to be used in human food, animal feed or for the production of fabrics and/or other cotton products
- destroy all plant materials remaining at the site after harvest
- clean the site and any equipment used on the site
- conduct regular inspections of the release site following harvest for at least 12 months (and until six consecutive months have passed without any volunteer cotton plants) and destroy any volunteers prior to flowering.

##### **4.1.2 Measures to control other activities associated with the release**

266. The Regulator has issued guidelines and policies for the transport and supply of GMOs (*Guidelines for the transport of GMOs, June 2001; Policy on transport and supply of GMOs, July 2005*). Licence conditions based on these guidelines and policies have been imposed regarding transportation and storage, and to control possession, use or disposal of the GMOs for the purposes of, or in the course of, the authorised dealings.

## 4.2 Other risk management considerations

267. All DIR licences issued by the Regulator contain a number of general conditions that relate to general risk management. These include, for example:

- applicant suitability
- contingency and compliance plans
- identification of the persons or classes of persons covered by the licence
- reporting structures, including a requirement to inform the Regulator if the applicant becomes aware of any additional information about risks to the health and safety of people or the environment
- monitoring for compliance.

### 4.2.1 Applicant suitability

268. In making a decision whether or not to issue a licence, the Regulator must have regard to the suitability of the applicant to hold a licence. Under section 58 of the Act matters that the Regulator must take into account include:

- any relevant convictions of the applicant (both individuals and the body corporate)
- any revocation or suspension of a relevant licence or permit held by the applicant under a law of the Commonwealth, a State or a foreign country
- the applicant's history of compliance with previous approved dealings
- the capacity of the applicant to meet the conditions of the licence.

269. Before making the decision whether or not to issue a licence for this application (DIR 067/2006), the Regulator determined that CSIRO is suitable to hold a licence.

270. Conditions in the licence include a requirement for the licence holder to inform the Regulator of any circumstances that would affect their suitability or their capacity to meet the conditions of the licence.

271. In addition, any applicant organisation must have access to a properly constituted Institutional Biosafety Committee and be an accredited organisation under the Act.

### 4.2.2 Contingency and compliance plans

272. The licence requires CSIRO to submit a plan detailing how it intends to ensure compliance with the licence conditions and document that compliance. This plan is required before the planting of the GM cotton lines occurs.

273. CSIRO is also required to submit a contingency plan to the Regulator within 30 days of the issue date of the licence. This plan must detail measures to be undertaken in the event of any unintended presence of the GM cotton lines outside of the permitted areas.

274. CSIRO is also required to provide a method to the Regulator for the reliable detection of the presence of the GMO and the introduced genetic materials in a recipient organism. This instrument would be required within 30 days of the issue date of the licence.

### 4.2.3 Identification of the persons or classes of persons covered by the licence

275. The persons covered by this licence are the licence holder and employees, agents or contractors of the licence holder and other persons who are, or have been, engaged or otherwise authorised by the licence holder to undertake any activity in connection with the dealings authorised by this licence.

#### **4.2.4 Reporting structures**

276. The licence obliges the licence holder to immediately report any of the following to the Regulator:

- any additional information regarding risks to the health and safety of people or the environment associated with the release
- any contraventions of the licence by persons covered by the licence
- any unintended effects of the release.

277. The licence holder is also obliged to submit an Annual Report within 90 days of the anniversary of the licence containing any information required by the licence, including the results of inspection activities.

278. A number of written notices are also required under the licence that would assist the OGTR in designing and implementing its risk based monitoring program for all licensed dealings. The notices include:

- expected and actual dates of planting
- expected and actual dates of commencement of flowering
- expected and actual dates of final destroying and cleaning at the end of the trial.

#### **4.2.5 Monitoring for compliance**

279. The Act stipulates, as a condition of every licence, that a person who is authorised by the licence to deal with a GMO, and who is required to comply with a condition of the licence, must allow inspectors and other persons authorised by the Regulator to enter premises where a dealing is being undertaken for the purpose of monitoring or auditing the dealing. Post-release monitoring continues until the Regulator is satisfied that all the GMOs resulting from the authorised dealings have been removed from the release sites.

280. If monitoring activities identify changes in the risks associated with the authorised dealings, the Regulator may also vary licence conditions, or if necessary, suspend or cancel the licence.

281. In cases of non-compliance with licence conditions, the Regulator may also instigate an investigation to determine the nature and extent of non-compliance. These include the provision for criminal sanctions of large fines and/or imprisonment for failing to abide by the legislation, conditions of the licence or directions from the Regulator, especially where significant damage to health and safety of people or the environment could result.

### **Section 5 Issues to be addressed for future releases**

282. In view of the very early stage of research involved in the release, the risk assessment identified additional information that may be required to assess an application for a larger scale trial, reduced containment conditions or a commercial release of any of these GM cotton lines. This would include:

- molecular characterisation of the introduced genetic materials in the GM cotton lines, genotypic stability, and expression levels of the introduced *AHb1* gene in the GM cotton lines
- data on the potential toxicity of plant material from the GM cotton lines including levels of known endogenous toxins

- data on the allergenicity of the protein encoded by the introduced *AHb1* gene for waterlogging tolerance
- details of the survival of the waterlogging tolerant GM cotton lines compared with non-GM cotton in the environment, particularly in habitats such as natural waterways, wetland areas and areas affected by high rainfall or flooding where the GM cotton may have a selective advantage
- biochemical, physiological and agronomic characteristics of the GM cotton lines indicative of weediness including measurement of tolerance to environmental stresses (eg drought or pathogen infection) and reproductive capacity (eg growth rate and window of flowering).

## **Section 6 Conclusions of the RARMP**

283. The risk assessment concludes that this limited and controlled release of up to 30 GM cotton lines on a maximum of 0.1 ha per annum for 3 years in the shire of Narrabri (NSW) poses a **negligible** risk to the health and safety of people and the environment.

284. The risk management plan concludes that this **negligible** risk does not require specific risk treatment measures. However, licence conditions have been imposed to contain the release to the size, duration and location requested by the applicant.

## **Section 8 DIR 067/2006 Licence**

285. The licence DIR 067/2006 is available on the OGTR website <<http://www.ogtr.gov.au/gmorec/ir.htm#table>>, following the path to DIR 067/2006.

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## Appendix A Definitions of risk analysis terms used by the Regulator

(\* terms defined as in Australia New Zealand Risk Management Standard AS/NZS 4360:2004)

### *Consequence*

outcome or impact of an adverse event

Marginal: there is minimal negative impact

Minor: there is some negative impact

Major: the negative impact is severe

### *Event\**

occurrence of a particular set of circumstances

### *Hazard\**

source of potential harm

### *Hazard identification*

the process of analysing hazards and the events that may give rise to harm

### *Intermediate*

the negative impact is substantial

### *Likelihood*

chance of something happening

Highly unlikely: may occur only in very rare circumstances

Unlikely: could occur in some circumstances

Likely: could occur in many circumstances

Highly likely: is expected to occur in most circumstances

### *Quality control*

to check, audit, review and evaluate the progress of an activity, process or system on an ongoing basis to identify change from the performance level required or expected and opportunities for improvement

### *Risk*

the chance of something happening that will have an undesired impact

Negligible: risk is insubstantial and there is no present need to invoke actions for mitigation

Low: risk is minimal but may invoke actions for mitigation beyond normal practices

Moderate: risk is of marked concern requiring mitigation actions demonstrated to be effective

High: risk is unacceptable unless actions for mitigation are highly feasible and effective

***Risk analysis***

the overall process of risk assessment, risk management and risk communication

***Risk analysis framework***

systematic application of legislation, policies, procedures and practices to analyse risks

***Risk assessment***

the overall process of hazard identification and risk estimation

***Risk communication***

the culture, processes and structures to communicate and consult with stakeholders about risks

***Risk Context***

parameters within which risk must be managed, including the scope and boundaries for the risk assessment and risk management process

***Risk estimate***

a measure of risk in terms of a combination of consequence and likelihood assessments

***Risk evaluation***

the process of determining risks that require treatment

***Risk management***

the overall process of risk evaluation, risk treatment and decision making to manage potential adverse impacts

***Risk management plan***

integrates risk evaluation and risk treatment with the decision making process

***Risk treatment\****

the process of selection and implementation of measures to reduce risk

***Stakeholders\****

those people and organisations who may affect, be affected by, or perceive themselves to be affected by a decision, activity or risk

***States***

includes all State governments, the Australian Capital Territory and the Northern Territory governments

***Uncertainty***

imperfect ability to assign a character state to a thing or process; a form or source of doubt

## **Appendix B Summary of issues raised in submissions received from prescribed experts, agencies and authorities<sup>6</sup> on application DIR 067/2006**

All issues relating to risks to the health and safety of people and the environment were considered in the context of the currently available scientific evidence that was used in the preparation of the RARMP.

### ***Issues raised relating to the Risk Assessment and where they have been considered:***

- Risk of enhanced spread and persistence (weediness) of the GM cotton (Events 6, 7 and 8, Chapter 2)
- Risks arising from any gene flow to other commercial cotton crops and naturalised populations of cultivated cotton species (Events 11 and 13, Chapter 2)
- Risks arising from gene flow to native cotton (Event 14, Chapter 2)
- Risks arising from gene flow to other organisms, including microbes (Event 15, Chapter 2)
- Risks arising from the dissemination of the GM cotton material beyond the intended area (Events 9 and 11, Chapter 2)
- Risks resulting from unintended and potential pleiotropic effects of the introduced genes (Events 16 and 17, Chapter 2).
- Risks arising from recombination between viral sequences and compatible viruses (Event 15, Chapter 2)
- Risks arising from presence and accumulation of proteins encoded by introduced genes in the environment (Events 1-5, Chapter 2)

### ***Issues raised and addressed by the Risk Management Plan (considered in Chapter 3 and the licence):***

- Adequate containment measures
- Adequate transport procedures for the GM cotton
- Adequate post-harvest monitoring and practices
- Adequate disposal of GM plant materials not required for further research or approved plantings.

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<sup>6</sup> GTTAC, State and Territory governments, Australian Government agencies, the Minister for Environment and Heritage and Local councils where the release may occur.

## **Appendix C Summary of submissions received from the public on application DIR 067/2006**

One submission from the public was received. All issues relating to risks to the health and safety of people and the environment were considered in the context of the currently available scientific evidence.

### **Issues raised relating to the Risk Assessment and where they have been considered:**

- Environmental effects (see Chapters 1 and 2)
- Use of GM products in animal feed (see Event 5, Chapter 2)
- Effects on microorganisms in soil (see Events 5 and 10, Chapter 2).

### **Issues that were outside the scope of assessments conducted under the *Gene Technology Act 2000*:**

- Expansion of agriculture into new areas
- Impact of agriculture.

## **Appendix D Summary of issues raised in submissions received from prescribed experts, agencies and authorities<sup>7</sup> on the consultation RARMP for DIR 067/2006**

None of the experts, agencies and authorities prescribed for consultation under the Gene Technology Act 2000, raised any issues on the RARMP relating to risks to human health and safety and the environment that required further consideration.

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<sup>7</sup> GTTAC, State and Territory governments, Australian Government agencies, the Minister for Environment and Heritage and Local councils where the release may occur