SAFETY ASSESSMENT OF NOVEL FOODS

REPORT OF
G02 RESEARCH PROGRAMME
REVIEW

G02 RESEARCH PROGRAMME REVIEW

CONTENTS **Page**

1. EXECUTIVE SUMMARY

All genetically modified crops for food and feed use are evaluated for safety before they can be grown or marketed in the European Union. Information on the overall composition of the plant is considered as part of the assessment, in addition to the nature of the genetic modification, data on toxicological studies, the potential for any new proteins to be allergenic and the intended use of the food. To keep abreast of developments in genetic modification technology and to improve the ability of detecting unintended effects, new techniques were considered for development and validation.

This three-year research programme was launched in September 2001, with funding of £5.5M provided by the Treasury department, focusing on the applicability and practicality of a variety of existing and emerging techniques for the safety assessment procedures for the next generation of GM foods. The programme examined the use of transcriptomic, proteomic and metabolomic techniques in a number of different plant species.

The independent scientific review panel, which met on 18 March 2005, was impressed with the achievements of the programme, which they considered contributed greatly to advancing the application of proteomic and metabolomic techniques to the analysis of plants. The techniques explored, methods evaluated and baseline data generated with further development will be invaluable to the future safety assessment of GM food, in addition to their wider application throughout the research community.

2. BACKGROUND TO THE REVIEW

2.1 The Review Panel

Chairman

Dr. Peter Lund	ACNFP member. School of Biosciences, University of Birmingham

External Reviewers

Prof. John Lindon	Division of Biomedical Sciences, Imperial College, London
Prof. Mike Burrell	Department of Animal and Plant Sciences, University of Sheffield
Prof. Malcolm Bennett	School of Biosciences, University of Nottingham
Dr. Jaap Keijer	Rikilt Institute of Food Safety, Wageningen, Netherlands
Dr. Rainer Craimer	The Biocentre, University of Reading

Programme Advisor

Dr Sandy Primrose	Independent Consultant

FSA Officials

Novel Foods Additives and Supplements Division
- Dr. Clair Baynton
- Dr. Trudy Netherwood
- Dr. James Ridsdale
- Dr. Chris Jones
- Dr. David Jefferies
- Annie Laurie Robin
- Kate Halliwell
- Michelle Gardner

Research Co-ordination Unit
- Dr. Sîan Thomas

2.2 Rationale of the programme

All genetically modified crops for food and feed use are evaluated for safety before they can be grown or marketed in the European Union. In order to provide the highest possible level of consumer protection, there is a requirement for a detailed risk assessment to be carried out for each Genetically Modified Organism (GMO). Since 2003 this has been carried out by an independent body of scientific experts on behalf of the European Food Safety Authority (EFSA). The assessment involves a comparative approach based on the concept of substantial equivalence. This means that a new food is compared with an equivalent existing food, for example, its equivalent GM food might be compared with a non-GM variety. This is a starting point for the assessment and each application is reviewed on a case-by-case basis.

Information on the overall composition of the plant is considered as part of the assessment, in addition to the nature of the genetic modification, data on toxicological studies, the potential for any new proteins to be allergenic and the intended use of the food.

In May 2000, a FAO/WHO expert consultation on foods derived from biotechnology recognised that there were no alternative strategies, at that time, that would provide a better assurance of safety for genetically modified foods than the appropriate use of the concept of substantial equivalence. It was agreed that some aspect of the steps in the safety assessment process could be refined to keep abreast of developments in genetic modification technology and to improve the ability of detecting unintended effects, profiling techniques were considered as useful alternatives which should be further developed and validated.

This three-year research programme was launched in September 2001, with funding of £5.5M provided by the Treasury department, focusing on the applicability and practicality of a variety of existing and emerging techniques for the safety assessment procedures for the next generation of GM foods. The programme examined the use of transcriptomic, proteomic and metabolomic techniques in a number of different plant species.

2.3 Objectives of the Programme

The key objective of this programme was to explore new and emerging techniques for the evaluation of the next generation of genetically modified plants and how they might be used to refine the current safety assessment of novel (including GM) foods. This was undertaken as a result of the following objectives:

- The development and validation of genomic, proteomic and metabolomic techniques for identifying unintended changes which may occur as a result of genetic modification of plants.
- To establish systems for interpreting the data and applying it to the safety assessment.

The two objectives were directly dependent on each other for achieving the overall programme aim. Profiling techniques generate a large amount of complex data that sets new challenges for interpretation and collation into a manageable and comparable format. In particular, a format needed to be developed so that it could be used in the safety assessment of new GM foods.

2.4 Review of the Research Programme

The aim of the review was to:

1. Assess whether the projects under review had met the programme's scientific and policy objectives.
2. Assess the scientific quality of completed research projects
3. Make recommendations on the future direction of the programme

All 6 projects (Annex A) were reviewed based on the final report submitted by the contractors and a presentation from the project leader. Reviewers were provided with the final reports for each project, a detailed programme review paper written by the Programme Advisor, appraisal forms and guidance for appraisal.

Each of the external reviewers was asked to provide a detailed written appraisal of two final reports and to be familiar with all six reports for the review panel meeting held on 18 March 2005. The panel of independent experts heard presentations from the project leaders and questioned the researchers about their work, before commenting on the outcome of each project and of the programme as a whole.

2.5 Outputs of the Programme

In reviewing the achievements of the G02 programme, it was important to recognise that when the programme began (April 2001) many techniques were still in their infancy and others, although well established for other uses, had not been applied to the analysis of plants. For example, only a very limited number of plant species microarrays were available and there were none for any of the major crop species. Plant metabolomics was in its infancy, with the first papers on plant metabolomics only being published in 1999 and 2000. Methods for data collection and analysis for the vast amounts of data, particularly that generated by metabolomics studies, were yet to be fully developed.

An executive summary of each project is presented below and full details are presented in the technical report at Annex E.

2.5.1 G02001 – Transcriptome, Proteome and Metabolome Analysis to Detect Unintended Effects in Genetically Modified Potato

This study applied transcriptomics, proteomics and metabolomics to the analysis of genetically modified potatoes. The objective was to ensure that

the technologies were robust and reproducible in analysing a range of GM and non-GM potato varieties.

The procedures used were effective and have potential to be used in the risk assessment of GM foods. The project developed operating practices, which could be applied across the scientific community to ensure consistency when analysing the same plant lines.

Transcriptomic and metabolomic analyses successfully detected both intended and unintended effects in transgenic potatoes. However, in most cases the magnitude of the changes was small, even in a line with a defect in a key biosynthetic step (polyamine biosynthesis), and seldom exceeded twofold. By contrast, the range of natural variation in metabolite levels seen in common cultivars was much greater and frequently exceeded fivefold. This natural variation was measured in plants grown in one location in one season.

Analysis with NMR and GC/MS detected no new compounds but four novel polyamines were detected in the various cultivars of potato by HPLC/MS.

Data suggested that changes induced by plant tissue culture rather than genetic modification per se were responsible for some of the cluster separations observed between the wild type Desiree, GM and control lines (vector only and tissue culture derived lines). Glycoalkaloid levels were generally lower in these lines compared with the wild types. The latter observation also was made in the EU GMOCARE project using targeted analysis and using plants grown under containment in different years.

2.5.2 G02002 – Methods for the Analysis of GM Wheat and Barley Seed for Unexpected Consequences of the Transgenic Insertion

This project assessed the suitability of several analytical methods, such as Poly-Acrylamide Gel Electrophoresis (PAGE), metabolite profiling and novel techniques such as Serial Analysis of Gene Expression technology (SAGE), and junction analysis, which may be used in the safety assessment of genetically modified wheat and barley. The techniques were applied to the same GM wheat and barley material, enabling researchers to compare the techniques in terms of sensitivity, reliability and suitability in different plant species.

All methods used in the study demonstrated the potential to be used in the safety assessment of genetically modified foods. Nuclear Magnetic Resonance (NMR) was particularly suitable for high throughput screening, providing detailed information about the chemical composition of the extracts from wheat and barley seed.

Analysis of junction sequences showed that in most of the transgenic lines that were selected, insertion had occurred in gene rich regions and had led to gene disruption. None of the barley lines had any DNA from outside of the T-DNA at the right border whereas five out of the eight wheat lines did have extraneous DNA.

Transcript analysis was undertaken on only a limited sub-set of the transgenic lines. SAGE showed that in one line there were 58 genes whose expression was up- or down-regulated more than 5-fold. Use of Affymetrix arrays with two other lines did not identify any significant changes in gene expression except for identifying the expression of the inserted gene. Analysis of seed storage proteins showed changes in the levels of all detected proteins in some lines and changes in individual proteins in other lines. These changes are not considered significant in the context of food safety.

Metabolomic analysis using NMR showed that, there are far greater differences between the unmodifed lines compared to wild type controls, than when the unmodified lines are compared with the transgenic lines. Thus changes induced by plant tissue culture accounts for most of the metabolomic changes rather than the transgenes themselves. The ploidy of the host genome could influence the extent of the changes seen in transgenic plants since the changes observed in hexaploid wheat were far less than those seen in diploid barley.

2.5.3 G02003 – Comparison of the Metabolome and Proteome of GM and Non-GM Wheat: Defining Substantial Equivalence

In this project, the researchers developed several techniques based on proteomics and metabolomics and examined their future potential in the safety assessment of genetically modified wheat.

A protocol for collection of NMR metabolite fingerprints was developed for white flour and applied to samples producing a large data set of fingerprints. These were analysed and compared with results of two mass spectrometry-based fingerprinting techniques, demonstrating the compatibility of NMR in the assessment of wheat flour.

The production of 2-Dimentional protein separation images of the wheat samples that could be compared objectively was a major challenge that was overcome in this project. The development of a standard operating procedure for the method was a major output of the study and will be of value to the wider scientific community.

Proteomic and metabolomic analysis has revealed unintended effects in white flour in one out of four transgenic wheat lines, genetically engineered to have a different glutenin content, compared with their non-GM controls.

Metabolomic analysis showed that the GM line had higher sugar levels than the comparative parental line. However, the differences observed between this GM line and its parental control generally were smaller than that seen between different cultivation sites or years for any particular line whether it was GM or non-GM.

2.5.4 G02004 – Development and Comparison of Molecular Profiling Methods for Improved Safety Evaluation Using GM Brassicas

This project investigated the application of transcriptomics, proteomics and metabolomics techniques, such as DNA microarrays and 2D gel electrophoresis, to analysing Brassicas, using Arabidopsis as a model plant system.

Project G02004 differed from the other projects in that a principle objective was to use the 'omics techniques to analyse a number of independent lines carrying the same transgenes but with vastly differing levels of expression. This work was undertaken in the model plant *Arabidopsis thaliana* because its genome has been sequenced thereby providing detailed molecular data. A second objective was to determine the transferability of the methods to another Brassica species (broccoli) that is grown as a food crop.

It was possible to demonstrate that data produced by each of transcriptomics, proteomics and metabolomics techniques could be integrated providing a complete biological picture.

During the course of the project a new approach, Multi-dimentional Protein Identification Technology (MudPIT), became available. MudPIT was demonstrated to be highly sensitive with more than 300 proteins, from crude extracts of conventional Arabidposis leaves, being identified by amino acid sequence.

No unintended proteomic effects were seen in transgenic Arabidopsis plants expressing the 'neutral' gene GUS. In plants expressing a gene for a novel enzyme (HCHL) that is metabolically active, the extent of the changes in RNA and protein species and metabolites is directly proportional to the level of expression of the transgene. The activity of the HCHL led to the synthesis of new metabolites in Arabidopsis. It was also demonstrated that changing light levels lead to very significant differences in the numbers of genes expressed.

In all the crops analysed, environmental stress had a much greater impact on the transcriptome, proteome and metablome than transgene introduction.

2.5.5 G02005 – The Application of Metabolic Profiling to the Safety Assessment of GM Foods

Most of the effort in this project was spent on method development rather than analysis of transgenic crops *per se*. The overall aim of this project was to develop mass spectrometry methods to identify metabolites in GM and non-GM crops, with a focus on tomatoes. A reproducible protocol for preparing samples and extracting metabolites was developed and used to evaluate different techniques.

The objective of the potato and soybean analysis was to determine if it would be possible to apply the GC/MS profiling method developed previously for tomatoes. The outcome was that while it was possible to successfully apply

the techniques with potatoes, the differences between individual tubers are as big as the differences between different potato lines and with soybeans, the method of sample preparation greatly affected the outcome of the analysis.

The most robust and reliable technique in this project was found to be Gas Chromatography Mass Spectrometry (GC-MS) which demonstrated its potential for being adapted to the analysis of other GM crops or foods. The GC/MS methods developed for profiling transgenic tomatoes were capable of detecting unintended effects but the changes observed were small and due principally to alterations in the levels of amino acids. Between-plant variation was found to be much greater than within plant variation.

2.5.6 G02006 – Metabolome Technology for the Profiling of GM and Conventionally Bred Plant Materials

The aim was to determine which combination of metabolomic procedures, coupled with a purpose built data storage and analysis programme (ArMET), was the most appropriate for routine use to reproducibly analyse potato crops.

A successive metabolomics approach was used successfully to separate GM potatoes (carrying genes for fructan and inulin synthesis) from non-GM potatoes although single transgenic lines (fructan synthesis only) were very similar to the parental Desiree cultivar. The transgenic potato lines were found to be no more different from the parental Desiree cultivar than the different cultivars were from each other.

Different methods of generating potato metabolomics data (electrospray MS fingerprinting; GC/MS profiling) and different methods of data analysis (Principal Components Analysis, Discriminant Function Analysis, etc) gave essentially the same results.

The ArMET programme has a core section, covering all phases of the metabolomic process that can be tailored by research groups to their individual needs. In this project, a particular achievement was the refinement of a module to store Gas Chromatography Mass Spectrometry (GC-MS) metabolite profile data for comparison with data from alternative sources. A major publication describing ArMET has been endorsed by key laboratories in the international science community and is expected to form a future focus for development of standards in metabolomics.

Further information on G02 Research Projects

The Technical Report (Annex E) contains a detailed discussion of experimental methodologies and an analysis of results. A list of publications can be found at Annex C. The contractors' reports are available from Dr John Dixon at the Food Standards Agency Information Centre.

3. The review meeting

3.1 Chairman's introduction

Dr. Lund opened the meeting, outlining its purpose. He emphasised that the reason for the review was to consider the nature and the value of the research programme, ensuring that the FSA was funding good science within the Agency's aims and objectives, and covered areas relevant to the stated interests and possible future concerns of the Agency. He noted that the reviewers would be asked to provide recommendations on the future direction of work in this area and identify strengths and weaknesses of the programme under review.

The lead contractor for each project then made a presentation of their work and was questioned by the panel.

3.2 Reviewers' Comments.

In addition to providing written comments for each project, the review panel discussed their views at the meeting, following presentations by each of the project leaders. Their overall views are summarised here.

3.2.1 G02001 – Transcriptome, Proteome and Metabolome Analysis to Detect Unintended Effects in Genetically Modified Potato.

It is important to be able to compare genetically modified (GM) crops and their non-GM counterparts for unintended differences, in order to assess whether they pose an unacceptable risk to human health (for example through the introduction of allergens or alteration of chemical composition).

Current analysis uses targeted approaches, which risk missing unexpected differences. This study, however, applied modern technologies (transcriptomics, metabolomics and proteomics) to assess potatoes for many more possible differences, thereby allowing safety assessments to be made with greater certainty.

Results suggested that unintended effects were not common but indicated a wide range of within-crop variation for both GM and non-GM potato varieties, highlighting the need to place any unintended changes into a wider context.

Reviewers agreed that this had been a very ambitious project, which met all of its objectives. The quality of work was good and it was viewed to have provided value for money. Reviewers agreed that the report was well presented over all. Reviewers suggested that to maximise outputs of the research, more detail would have been beneficial to explain the rationale for the choice of data presentation method, as well as the limitations of the technologies, and more emphasis should have been placed on highlighting the implications of the data. It was also thought that interpretation of the report would rely heavily on the reader's perspective and knowledge of plants and their metabolism.

The contractors were considered to have performed well in addressing a number of issues including food safety and within-crop natural variation. They had also linked the metabolomics results to biochemical pathways and the microarray results had been subjected to thorough data analysis.

The conclusions drawn by the contractors, were felt by the reviewers to be justified, however it was suggested that as only a small proportion of known metabolites and proteins can be measured, quantification of the measurements would allow determination of significant differences of the crops.

3.2.2 G02002 – Methods for the Analysis of GM Wheat and Barley Seed for Unexpected Consequences of the Transgenic Insertion. Project

Information on the location of inserted genes in GM foods would provide knowledge to help predict unintended effects of gene insertion. This combined with approaches looking at the production of proteins, other compounds and levels of gene activity would make a comprehensive safety assessment.

The main objective of this project was to examine a range of methods for the analysis of GM wheat and barley seed to assess their suitability for the detection of unintended effects following genetic modification.

Each of the methods examined has been shown to be suitable for the detection of certain types of unexpected effects, with each having some advantages such as cost, specificity or speed. Nuclear Mass Resonance (NMR) was particularly suitable for high throughput screening, which cannot be achieved using more targeted approaches.

There was agreement that this project had largely achieved and perhaps even exceeded its original technical aims and objectives in certain areas and had given value for money with work being performed to a high standard. However, reviewers questioned whether the original rationale had been fully addressed. Two areas of work were felt to be left incomplete; development of arrays and characterisation of junction sequencing, however problems with both had only been finally resolved near the end of the project and so this was considered to be justified.

The report was well presented and the work of high quality with conclusions being largely justified by the robustness of the data.

3.2.3 G02003 – Comparison of the Metabolome and Proteome of GM and Non-GM Wheat: Defining Substantial Equivalence.

Substantial equivalence is a concept used to identify similarities and differences between the genetically modified food and a comparator with a history of safe food use, which subsequently guides the safety assessment process and contributes to a robust safety assessment framework. A

consideration of compositional changes should not be the sole basis for determining safety. Safety can only be determined when the results of all aspects under comparison are integrated.

In this project, the researchers developed technologies based on transcriptomics, proteomics and metabolomics.

The work has shown that metabolomics can be applied to the safety assessment of GM foods. Application to wheat in this study also showed that variability due to the environment is generally greater than that due to genetic manipulation.

Reviewers considered the rationale to have been met in that similarities and differences had been established between genotypes grown at the same and different locations. However it was felt that the report could have explained the original rationale more fully.

The work was viewed to be timely and that it had produced some useful techniques, but the general output could have been better focussed to the original programme goals.

It was felt that this project had advanced the ability to assess novel foods of any type as well as the obvious exclusion of some other techniques. Some of the protocols could be a starting point to develop a 2D gel-based analysis strategy for the proteome of wheat.

However, it was also noted that a more robust multivariate statistical analysis of the data (by extending it to a discrimination analysis) was still underway.

The technical quality of the work was viewed to be good. Some concern was expressed over whether or not all of the conclusions could be justified based on the data obtained. In particular:

- The first conclusion from the EST study, because the replicates did not cluster well and many adjustments had been made by the data processing.
- There was a question about the legitimacy of eliminating data points and changing data mining tools in order to see significant differences between samples.
- While it was clear that more differences were observed with the metabolite analysis, which might be expected, reviewers questioned whether assessments should be based on this one aspect.

Reviewers felt that value for money had been achieved overall, but noted that a more cost-effective approach could have been taken.

3.2.4 G02004 – Development and Comparison of Molecular Profiling Methods for Improved Safety Evaluation Using GM Brassicas.

In this project, the techniques studied (transcriptomics, proteomics and metabolomics) were shown to be capable of detecting, resolving and quantifying a wide range of compounds.

The project demonstrated that the methods investigated could generate data usable in a food safety assessment. It was also shown that environment has a much more significant impact on overall gene expression than the deliberately inserted gene. The observations made provide reassurance that the GM process does not contribute in any significant way, to unintended effects.

The project was felt to have addressed its rationale for funding, however the panel was disappointed than the contractor did not pursue the original objective of studying broccoli, but had focussed more on Arabidopsis while cultivating the GM broccoli and adapting the methods for application to broccoli. They noted that only T0 and T1 generations of broccoli could be grown during the length of this project and that better results would only have been achieved if a T2 generation of broccoli had been available.

The reviewers agreed that contractors performed well in what was a complex and ambitious study but felt that investigations could been developed further. They were content that the project had been presented clearly in the report.

Reviewers agreed that the conclusions drawn from the work were justified, but felt that the work would have benefited from the application of the methods to food crops, rather than using the model plant Arabidopsis, and further data mining of the results to investigate links between the outputs of different profiling methods.

Overall, it was agreed that the project had provided good value for money.

3.2.5 G02005 – The Application of Metabolomic Profiling to the Safety Assessment of GM Foods.

The overall aim of this project was to develop mass spectrometry methods to profile and identify metabolites in GM crops, with a focus on tomatoes. A reproducible protocol for preparing samples and extracting metabolites was developed and used to develop different techniques. The most robust and reliable technique was found to be Liquid Chromatography Mass Spectrometry (LCMS).

Data showed that the extent of unintended effects caused by genetic modification was small compared to the natural variation seen between varieties of tomato.

This project was felt to have met its objectives and addressed the programme aims of feasibility and practicability. The reviewers agreed that scientific quality was high and the report clearly written.

The conclusions were considered to be justified, but reviewers thought that more work was needed to develop a robust operating procedure for the metabolite profiling method. The achievements did represent good value for money.

3.2.6 G02006 – Metabolome Technology for the Profiling of GM and Conventionally Bred Plant Materials.

It has been suggested that genetic modification may lead to unforeseen traits being introduced in food crops, such as changes in metabolite content (low molecular weight chemicals naturally produced by an organism). This project addressed technology developed to determine whether different metabolite profiles exist between GM crops and their non-GM counterparts.

The aim was to determine which if any combination of metabolomics analytical routines, coupled with a purpose built data storage programme (ArMET) and advanced data analysis, was the most appropriate for routine use to determine whether different metabolite profiles exist for GM and non-GM potato raw materials.

Researchers concluded that metabolomics would allow a robust analysis of individual metabolites to be made in any assessment of food composition.

It was possible to standardise metabolomics procedures to allow data to be compared when generated on similar instruments in different laboratories. This study also paves the way for robust, internationally accepted tests for raw food material composition that would have multiple applications for assessment of food quality, safety and provenance.

Reviewers agreed that this project had successfully addressed the original rationale for funding. The reviewers felt that the quality of the technical and bioinformantic scientific work was high and that the linkage to the biological understanding, using multivariate statistics was thorough.

It was clear that serious consideration has been given to how best to determine whether different metabolite profiles exist between GM crops and their non-GM counterparts. The conclusions were considered to be justified by the evidence presented.

4. Review Panel conclusions

The panel was impressed with the achievements of the programme, which they considered contributed greatly to advancing the application of proteomic and metabolomic techniques to the analysis of plants.

They agreed that the data analysis techniques produced by the research contractors was essential to the success of the programme, given the potential for the techniques to produce vast amounts of data. The techniques explored, methods evaluated and baseline data generated with further development will be invaluable to the future safety assessment of GM food, in addition to their wider application throughout the research community.

5. The scientific quality of the programme

Metabolomics, proteomics and transcriptomics were in their infancy when the G02 programme was conceived. G02 contractors focussed on quality issues at an early stage in the programme and they collectively were able to identify the key sources of variability and to manage them. The principal sources of variability are:

- Efficient extraction of metabolites.
- The derivatisation step (GC-MS only) needs to be optimised and the stability of derivatised metabolites determined.
- The appropriate analytical instrument needs to be selected with careful thought being given to sensitivity and selectivity.
- The performance of the instrument needs to be assessed constantly and appropriate internal and external.
- Where large numbers of samples are being run it is important to ensure that 'new' peaks are shown to be genuine and are not the result of instrument drift. That is, data pre-processing is essential before data analysis is undertaken.

In addition, three of the G02 contractors participated in the preparation of a checklist of the information necessary to provide context for metabolomics data that is to be published, known as MIAMET (minimum information on a metabolomics experiment). MIAMET has been developed into a database (called ArMet) and has received wide endorsement from the plant metabolomics community.

High quality peer reviewed publications have been produced from the work funded in the G02 programme with more publications planned. The quality of the work has increased the recognition of UK research in the top international level in this field.

6. Suggestions for future work

Reviewers were asked if they considered that the G02 programme provided scope for future development.

Suggested future work fell into the following areas:

- Apply technologies developed under G02 to safety assessment of GM and novel foods (following a prioritisation exercise of which technologies to be investigated first).

- Mining of G02 data, in particular, to explore natural variation within and between cultivars as well as unintended effects in GM and novel foods.
- Further develop the work of G02002, regarding NMR identification of transgene biomarkers, pursuing commercial exploitation. This was identified as being useful for high throughput testing.

These suggestions will be considered by the Agency in future discussions on research priorities and potential areas of work to be funded from 2005 onwards.

7. Overall conclusions

- At the conception of the programme, knowledge of how to apply 'omics technologies to food safety assessment was very limited, but it was recognised that it could provide a much more detailed comparison when establishing substantial equivalence.
- Project costs varied widely and did not always reflect the level or importance of outputs, however all work provided value for money.
- Effective and thorough analysis and interpretation of data is critical to success.
- The technical quality of work produced under this programme was high.
- There is now a better understanding of which techniques could allow us to assess the safety of GM foods.

8. The programme's impact on Agency policy

The genomics methods evaluated in the GO2 programme could now be applied to the safety assessment of novel GM foods and also explore the application to novel non-GM foods.

The lessons learned in the GO2 programme, especially the value of different experimental approaches and the utility of the quality approach, could be applied very usefully in other Agency-funded projects involving metabolomics and proteomics.

The major outstanding issue is the lack of comprehensive compound libraries and spectral databases, especially for the secondary metabolites that normally are found in plants. Creation of a baseline database accessible by scientists working in the field is essential for future application of these methods.

9. Action plan

A new G03 research programme has been developed to build on, and continue to support the mandatory safety assessment of novel foods, applying existing information. The programme will build upon the results that have been obtained in two previous programmes on the safety, and safety assessment, of novel foods (G01 and G02), as well as recommendations from relevant experts and the Agency's Advisory Committee on Novel Foods and Processes (ACNFP).

The programme will aim to develop further, the profiling technologies for GM plants, established under the G02 programme, for use in the safety assessment of both novel and GM foods particularly focussing on metabolomics and their potential in toxicological assessment. It will also investigate the use of MudPIT (Multidimensional protein identification technology) as a quantitative method for protein analysis in GM and non-GM safety assessments.

The programme will build upon the success of the G02 programme by collating the data it generated, into easily accessible internet databases. This will ensure that the knowledge gained in G02 can be added to and can be specifically applied to research being carried out in the international arena (e.g. setting baselines for metabolite variation in GM crop plants in order to inform safety assessment under the GM Food and Feed Regulation). Such information can easily be utilised by others in the Agency e.g. for nutritional profiling and authenticity work, and the wider research community.

Annex A List of G02 projects assessed by the review

PROJECT TITLE	CONTRACTOR	VALUE
GO2001 Transcriptome, proteome and metabolome analysis to detect unintended effects in genetically modified potato.	Prof. Howard Davies Scottish Crop Research Institute Invergowrie, Dundee	£988,319
GO2002 Methods for the analysis of GM wheat and barley seed for unexpected consequences of the transgene insertion.	Prof. John Snape John Innes Centre Norwich	£386,862
GO2003 Comparison of the metabolome and proteome of GM and non-GM wheat: defining substantial equivalence.	Prof. Mike Beale Long Ashton Research Station Bristol (later transferred to Rothamsted following closure of Long Ashton)	£556,419
GO2004 Development and comparison of molecular profiling methods for improved safety evaluation using GM brassicas.	Prof. Mike Gasson Institute of Food Research Norwich	£911,123
GO2005 The application of metabolic profiling to the safety assessment of GM foods.	Prof. Peter Bramley Royal Holloway College University of London Egham	£338,116
GO2006 Metabolome technology for the profiling of GM and conventionally bred materials.	Prof. John Draper University of Wales Aberystwyth	£933,362

Annex B Review agenda

Agenda for the Food Standards Agency's Review of the G02 Programme.
Friday 18th March 2005, Conference Room 5, Aviation House

9:30- 10:00	**Tea and Coffee**
10:00-10:20	G02005 - Prof. Peter Bramley, from Royal Holloway will give a presentation and answer questions on the G02005 project
10:20-10:30	**Brief discussion of the G02005 project**
10:30- 11:00	**G02003 - Dr. Mike Beal, From Rothamsted will give a presentation and answer questions on the G02003 project**
11:00-11:10	**Brief discussion of the G02003 project**
11:10- 11:50	**G02006 - Prof. John Draper, from the Aberystwyth will give a presentation and answer questions on the G02006 project**
11:50 – 12:00	**Brief discussion of the G02006 project**
12:00 – 12:45	**Lunch**
12:45-13:25	**G02001- Prof. Howard Davis, from the Scottish Crop Research Institute will give a presentation and answer questions on the G02001 project**
13:25-13:35	**Brief discussion of the G02001 project**
13:35-14:15	**G02002- Prof. John Snape, From the John Innes Centre will give a presentation and answer questions on the G02002 project.**
14:15-14:25	**Brief discussion on the G02002 project**
14:25-15:10	**G02004- Prof. Mike Gasson from the Institute of Food Research will give a presentation and answer question on the G02004 project**
15:10-15:20	**Brief Discussion of the G02004 project**
15:20-15:30	**Tea and Coffee**
15:30-16:30	**Wash Up**
16:30-17:00	**Meeting Closed**

Annex C Publications List

Chassy, B., Hlywka, G.A., Kleter, G.A., Kok, E.J., Kuiper, H.A., McGloughlin, M., Munro, I.C., Phipps, R.H. & Reid, J.E. (2004). Nutritional and safety assessment of foods and feeds nutritionally improved through biotechnology. Prepared by a task force of the ILSI International Food Biotechnology Committee. *Comprehensive Reviews in Food Science and Food Safety* **3**, 35-104.

Defernez, M. & Colquhoun, I.J. (2003). Factors affecting the robustness of metabolite fingerprinting using ^{1}H NMR spectra. *Phytochemistry* **62**, 1009-1017.

Defernez, M., Gunning, Y.M., Parr, A.J., Shepherd, L.V.T., Davies, H.V. & Colquhoun, I.J. (2004). NMR and HPLC/UV profiling of potatoes with genetic modifications to metabolic pathways. *Journal of Agriculture and Food Chemistry* **52**, 6075-6085.

Jenkins, H., Hardy, N., Beckmann, M., Draper, J., Smith, A.R., Taylor, J., Fiehn, O., Goodacre, R., Bino, R.J., Hall, R., Kopka, J., Lane, G.A., Lange, B.M., Liu, J.R., Mendes, P., Nikolau, B.J., Oliver, S.G., Paton, N.W., Rhee, S., Roessner-Tunali, U., Saito, K., Smedsgaard, J., Sumner, L.W., Wang, T., Walsh, S., Wurtele, E.S. and Kell,D.B. A proposed framework for the description of plant metabolomics experiments and their results. (2004) *Nature Biotechnology* **22**, 1601-1606.

Kok, E.J. & Kuiper, H.A. (2003). Comparative safety assessment for biotech crops. *Trends in Biotechnology* **21**, 439-444.

Kuiper, H.A., Kok, E.J. & Engel, K.-H. (2003). Exploitation of molecular profiling techniques for GM food safety assessment. *Current Opinion in Biotechnology* **14**, 238-243.

Lewis, J., Baker J.M., Beale, M.H. and Ward, J.L. Metabolite profiling of GM plants: the importance of robust experimental design and execution**.** In: Genomics for Biosafety in Plant Biotechnology (*Proceedings of NATO Advanced Research Workshop on Biosafety, Bulgaria, 2003)*, Eds. J-P. Nap, A. Atanassov and W.J. Stiekema. IOS Press, Amsterdam, 2004, pp 47-57

Salvo G H, Travella S, Bilham L J, Harwood W A, Snape J W (2004) The distribution of transgene insertion sites in barley determined by physical and genetic mapping. Genetics, **167**: 1371-1379.

Taylor, J., King, R. D., Altmann, T. & Fiehn, O. Application of metabolomics to plant genotype discrimination using statistics and machine learning. *Bioinformatics* **18**, S241-S248 (2002).

Annex D List of acronyms

FAO: Food and Agriculture Organisation

WHO: World Health Organisation

PCR: Polymerase Chain Reaction

RT-PCR: Real Time Polymerase Chain Reaction

EST: Expressed Sequence Tag

DapA: Gene involved in leucine biosynthesis

BETv1: *Betula verrucosa* (birch) pollen allergen gene 1

PAT: phosphinothricin acetyltransferase gene/protein from *Streptomyces viridochromogenes*

Bar: Bialaphos resistance gene (confers resistance to the herbicide PPT)

PPT: Herbicide phosphinothricin

HILIC-MS: Hydrophilic Interaction Chromatography-Mass Spectrometry

CAMV: Cauliflower Mosaic Virus

Annex E Technical Report

REVIEW OF G02 PROGRAMME: SAFETY ASSESSMENT OF NOVEL FOODS

S B Primrose PhD
Programme Advisor

June 2005

TABLE OF CONTENTS

SECTION A: OVERVIEW

1 EXECUTIVE SUMMARY

Overview

The Food Standards Agency G02 programme comprised 6 projects devoted to an assessment of the utility of genomics technologies in the safety assessment of novel foods, particularly those resulting from plant gene manipulation. The technologies assessed were analysis of junction sequences, transcriptomics (analysis of gene expression), proteomics and metabolomics. Each project involved a direct comparison of Genetically Modified (GM) plants with their non-GM counterparts and the plant materials analysed were derived from potatoes, tomatoes, barley, wheat and the model plant *Arabidopsis*.

At the outset of the programme it was realised that the numbers of experimental variables in each project were very high and that these would need to be controlled if meaningful results were to be generated. To ensure this, a quality initiative was instituted and the use of quality procedures by contractors was assessed continually.

Method development

Analysis of junction sequences is considered to be an essential requirement for the safety assessment of GM foods. A number of different methods for determining junction sequences were assessed and a suitable generic method was identified.

A number of different methods of analysing gene expression were evaluated and it was concluded that commercial microarrays were the method of choice. Where appropriate microarrays are not commercially available then SAGE (Serial Analysis of Gene Expression) is the preferred alternative.

For proteomic analysis it was concluded that there is no viable alternative to 2D-gel electrophoresis. Although 2D-gel analysis has a well-deserved reputation for lack of robustness, one contractor showed that it could be made very reproducible by paying exquisite attention to experimental detail. Interpretation of the results of 2D-gel analysis requires advanced software and two suitable programmes were identified.

Both NMR (Nuclear Magnetic Resonance) spectroscopy and mass spectrometry (MS) were found to be suitable for metabolomic analysis. NMR is relatively simple and quick and long-term instrument stability is not a problem. However, it is much less sensitive than mass spectrometry. Many different kinds of mass spectrometer are available and usually they are coupled to other separation systems such as GC (Gas Chromatography) or HPLC (High Performance Liquid Chromatography). The stability of some of these hyphenated systems was found to be problematic and rigorous quality assurance systems had to be applied to generate meaningful data. Direct

injection of unfractionated extracts into an electrospray mass spectrometer to generate metabolic fingerprints proved to be a very useful high throughput method. It was able to provide sufficient data for many applications and could be supplemented with other forms of mass spectrometry as appropriate. Key variables in the design of metabolomics experiments were identified (e.g. sample extraction procedure, sample randomisation, etc.).

A generic method of data analysis was developed but use of the method requires a detailed understanding of multivariate statistics. Where differences could be shown between GM and non-GM samples it was considered essential to determine the identity of the factors causing these differences. In evaluating differences, a distinction needs to be made between separations caused by a large shift in the concentration of one factor versus those caused by a large number of small changes in many factors.

Key results from contractors

All the contractors successfully detected intended and unintended effects in transgenic plants. However, in most cases the magnitude of the changes was small, even in a plant line with a defect in a key biosynthetic step, and seldom exceeded twofold. By contrast, the range of natural variation in metabolite levels seen in landraces and common cultivars was much greater and frequently exceeded fivefold. Similarly, when the same test plants were grown in geographically separate regions or in different years (different climatic conditions), the differences caused by environmental factors far exceeded the differences between GM and non-GM lines.

A key observation made by a number of contractors was that somaclonal variation induced by passage through tissue culture rather than transgenesis *per se* was responsible for some of the cluster separations observed between GM and non-GM lines.

None of the contractors observed new metabolites in any of the transgenic plants examined. One contractor identified a number of metabolites in non-GM potatoes that had not previously been described in crop plants and this indicates the power of untargeted metabolomic analysis.

Implications for the Food Standards Agency

The genomics methods evaluated in the G02 programme could be applied to the safety assessment of novel, non-GM foods.

The lessons learned in the G02 programme; especially the value of different experimental approaches and the utility of the quality approach that was used could be applied very usefully in other Agency-funded projects involving metabolomics and proteomics.

2 BACKGROUND

2.1 Introduction

The G02 programme 'Safety Assessment of Novel Foods' was a three-year research programme that was launched in September 2001. The aim of the programme was to:

- Refine the then current safety assessment procedures for GM foods to cover the next generation of GM plants;
- Explore the feasibility and practicability of using emerging techniques in genomics, proteomics and metabolomics in the safety assessment process.

The programme was set up as a result of a number of reviews on the safety assessment of GM foods including that of the Food Standards Agency Board (June 2000) and an FAO/WHO expert consultation (May 2000). This consultation recognised:
'That there were presently no alternative strategies that would provide a better assurance of safety of genetically modified foods than the appropriate use of the concept of substantial equivalence. Nevertheless, it was agreed that some aspects of the steps in the safety assessment process could be refined to keep abreast of developments in genetic modification technology. New methodologies, such as profiling techniques, offer the means of providing a more detailed analytical comparison. However, it was recognised that much more developmental work was necessary before such methods could be validated'.

2.2 Potential risks to consumers of GM foods

In GM crops, new genes (transgenes) that confer some desirable agronomic or nutritional property are inserted into the plant genome. Simplistically, these transgenes are of two types: neutral transgenes and metabolically active transgenes. Neutral transgenes encode proteins that have no enzymatic activity, e.g. cereal storage proteins, the *Bacillus thuringiensis* insecticidal protein, etc. In theory, their presence in host cells should not alter the metabolism of the cell. However, the site of insertion may interrupt the coding sequence of a host gene with two potential outcomes. First, a novel hybrid protein could be created leading to a change in protein profile that could have unexpected toxicity, e.g. allergenicity. Second, if the gene that is disrupted has enzymatic activity, its inactivation could lead to significant metabolic changes.

By definition, metabolically active transgenes will alter the metabolism of the host cell. The extent to which cellular metabolism is altered will depend on the enzymatic properties of the transgene product and the level of expression. However, in considering the potential risks associated with metabolic changes in GM foods it is important to note that at the outset of the G02 programme there was very little information on:

- The differences in metabolite content between different cultivars of the same plant species.

- The changes in metabolite content that occur within a single cultivar when it is grown in different soil types.
- The effect of seasonal variation (early versus late planting) and weather on the metabolite content of a particular plant cultivar.
- The effect of pathogen/pest attack and any control measures used.

2.3 Project selection

A call for proposals was made in late 2000 and 24 proposals were received. These proposals were reviewed by a panel comprising:
Dr Jon Bell (then Deputy Chief Executive, Food Standards Agency)
Dr Geraldine Schofield (Unilever Research, Colworth House)
Mr Nick Tomlinson (Food Standards Agency)
Dr Clair Baynton (Food Standards Agency)
Dr Sandy Primrose (Independent Programme Advisor designate)
Of the 24 proposals submitted, a total of 6 were selected for funding and the details of these are summarised in Table 1 below.

TABLE 1: Details of projects supported by the G02 programme

PROJECT TITLE	CONTRACTOR	VALUE
G02001 Transcriptome, proteome and metabolome analysis to detect unintended effects in genetically modified potato.	Prof. Howard Davies Scottish Crop Research Institute Invergowrie, Dundee	£988,319
G02002 Methods for the analysis of GM wheat and barley seed for unexpected consequences of the transgene insertion.	Prof. John Snape John Innes Centre Norwich	£386,862
G02003 Comparison of the metabolome and proteome of GM and non-GM wheat: defining substantial equivalence.	Prof. Mike Beale Long Ashton Research Station Bristol (later transferred to Rothamsted following closure of Long Ashton)	£556,419
G02004 Development and comparison of molecular profiling methods for improved safety evaluation using GM brassicas.	Prof. Mike Gasson Institute of Food Research Norwich	£911,123
G02005 The application of metabolic profiling to the safety assessment of GM foods.	Prof. Peter Bramley Royal Holloway College University of London Egham	£338,116
G02006 Metabolome technology for the profiling of GM and conventionally bred materials.	Prof. John Draper University of Wales Aberystwyth	£933,362

The plant species and methodologies selected by the successful applicants are shown in Table 2 below. Although *Arabidopsis thaliana* is not a crop species it was selected for study by two of the groups because it is widely used as a model dicotyledenous plant and at the time the G02 programme was initiated it was the only plant whose genome had been completely sequenced.

TABLE 2: Plant species analysed and methodologies used in the various G02 projects

PROJECT	PLANT SPECIES	TISSUE	METHODOLOGIES
G02001	Potato	Tuber	Transcriptomics, proteomics, metabolomics
G02002	Wheat, barley	Grain	Transcriptomics, proteomics, metabolomics, DNA analysis
G02003	Wheat	Grain	Proteomics, metabolomics (transcriptomics funded by BBSRC)
G02004	Broccoli, *Arabidopsis*	Flower, Whole plant	Transcriptomics, proteomics, metabolomics
G02005	Tomato	Fruit	Metabolomics
G02006	Potato, *Arabidopsis*	Tuber, Whole plant	Metabolomics

A number of specific methodologies selected by the successful applicants were of particular interest to the review panel in addition to the use of protein and metabolite profiling techniques. In particular:

- The use of the SAGE (Serial Analysis of Gene Expression) technique instead of the more usual microarrays for analysis of transcription patterns (G02002, JIC);
- The analysis of junction sequences to determine if transgenes have inserted into other genes (G02002, JIC);
- The use of differential labelling of proteins with stable isotopes for the qualitative and quantitative comparison of protein species in two different samples (G02001, SCRI);
- The use of sophisticated statistical and mathematical techniques for the analysis of very large data sets (G02001, SCRI and G02006, Aberyswyth).

2.5 Key points to note about the G02 programme

In reviewing the achievements of the G02 programme, it is important to bear in mind the state of the art at the time the project was initiated (April 2001). In particular:

- There was virtually no information on the junction sequences for virtually any transgenic plant lines because of the difficulty of getting this information.

- Only a very limited number of Affymetrix microarrays were available and there were none for any of the major crop species. The quality of some of the arrays was also a key issue.
- SAGE was a relatively untested procedure, particularly for the use to which it would be put in the G02 programme.
- 2D-gel electrophoresis was the standard method for proteomics but was not a robust technique (and to some extent still is not robust).
- Metabolomics was in its infancy, the initial papers on plant metabolomics being published in 1999 and 2000.
- There was no clear idea how the vast amounts of 'omics data, particularly that generated by metabolomics studies, might be analysed sensibly.

3 QUALITY ISSUES

3.1 Introduction to key issues

During the initial review of the proposals, the panel was cognisant of a number of factors that could have a negative impact on the success of the programme. These included:

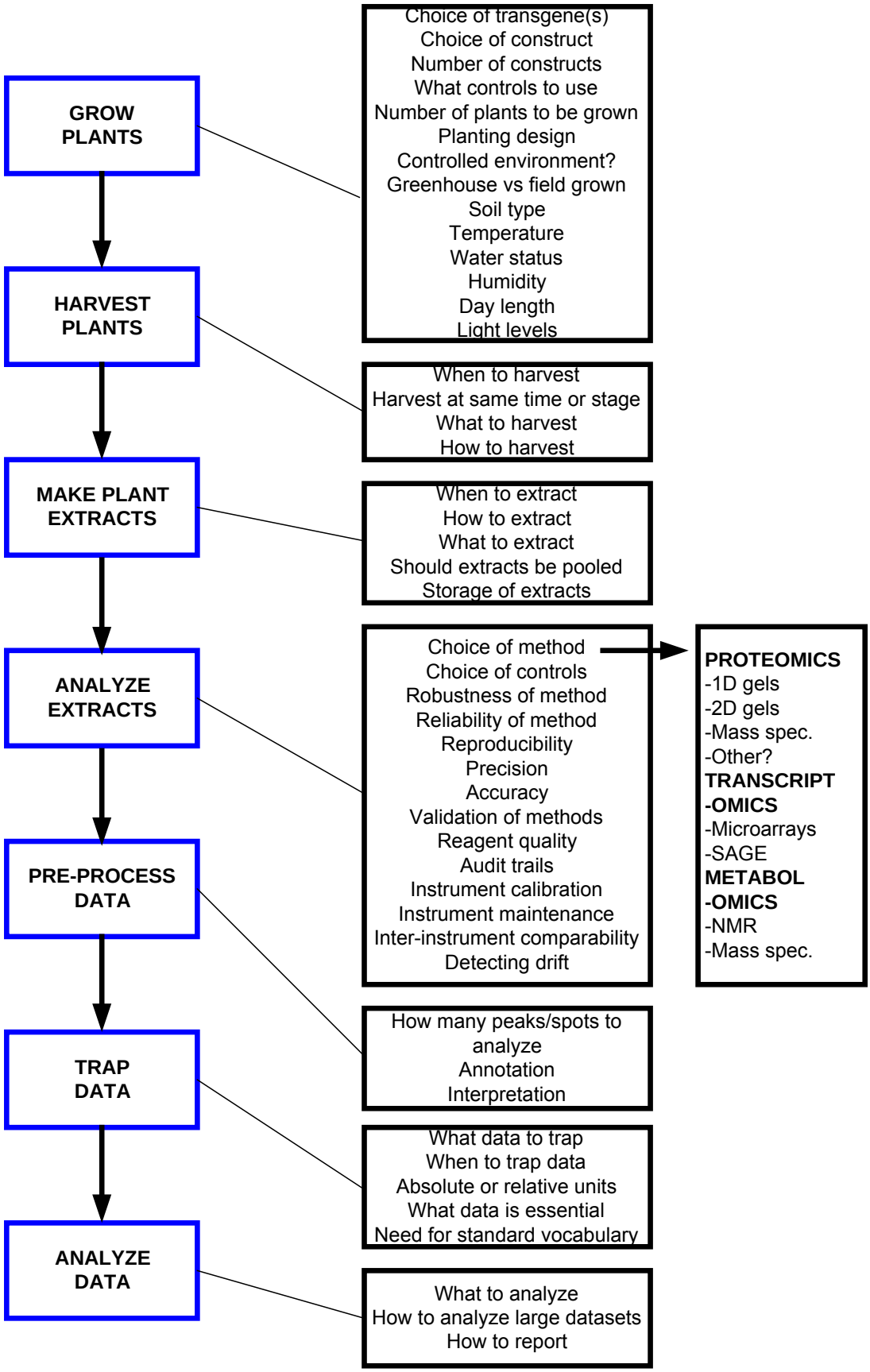
- The amount of data that would be generated (hence the support for the data analysis modules of G02001 SCRI and G02006 Aberystwyth);
- The development of *robust analytical* methodologies which could be utilised widely throughout the scientific community.

As the programme progressed it became apparent that the scope of the projects were very ambitious, not just for the FSA but in terms of global plant research. Furthermore, the complexity of the projects was far greater than first imagined. There were many issues that, if not actively managed, could have resulted in failure to deliver some or the entire project and/or programme objectives. These issues are discussed in more detail below. It could be asked why the initial Review Panel did not foresee these issues. However, it is the view of the Programme Advisor that, in hindsight, the panel could not have foreseen most of the issues that arose.

3.2 Complexity

To understand the complexity of each project, it is necessary to consider the potential variables that have to be born in mind in order to obtain meaningful results in any comparison of GM versus non-GM plants. These variables are shown in the figure below and some are discussed in more detail later (section 3.3). Field grown crops take one year before material is available. Therefore decisions regarding the experimental design often are taken without the benefit of hindsight. Compounding, this was the requirement to develop and assess the utility of different analytical techniques. For some techniques it is possible to minimise experimental variability by developing and validating standard operating procedures (SOPs). For others, SOPs are inadequate to handle variance and the issue can only be managed through the use of statistically sound experimental design. For many of the techniques, the appropriate balance between the use of SOPs and statistical experimental

design could not be made for 18-20 months when analysis of the first year crops had been completed.
Consequently, the G02 programme was extremely ambitious in developing and assessing analytical techniques, trapping data, developing data handling and analysis methodologies and undertaking full quality assurance within a 3 year programme.


GROW PLANTS
HARVEST PLANTS
MAKE PLANT EXTRACTS
ANALYZE EXTRACTS
PRE-PROCESS DATA
TRAP DATA
ANALYZE DATA
Choice of transgene(s)
Choice of construct
Number of constructs
What controls to use
Number of plants to be grown
Planting design
Controlled environment?
Greenhouse vs field grown
Soil type
Temperature
Water status
Humidity
Day length
Light levels
When to harvest
Harvest at same time or stage
What to harvest
How to harvest
When to extract
How to extract
What to extract
Should extracts be pooled
Storage of extracts
Choice of method
Choice of controls
Robustness of method
Reliability of method
Reproducibility
Precision
Accuracy
Validation of methods
Reagent quality
Audit trails
Instrument calibration
Instrument maintenance
Inter-instrument comparability
Detecting drift
PROTEOMICS
-1D gels
-2D gels
-Mass spec.
-Other?
TRANSCRIPT -OMICS
-Microarrays
-SAGE
METABOL -OMICS
-NMR
-Mass spec.
How many peaks/spots to analyze
Annotation
Interpretation
What data to trap
When to trap data
Absolute or relative units
What data is essential
Need for standard vocabulary
What to analyze
How to analyze large datasets
How to report

3.3 Some examples of key variables

As an illustration of the complexity of the experimental design of each project, some of the choices accompanying *each* variable are described below. Note that this list is not exhaustive.

Choice of transgene and/or construct. Any transgene encoding a plant growth regulator is likely to have pleiotropic effects following its insertion into the plant genome. Similarly, insertion of a 'neutral' transgene will have pleiotropic effects if it interrupts the expression of a plant growth regulator. In both cases, the transgenic progeny are likely to exhibit significant differences in their content of proteins and small molecules when compared with their parents. Clearly, the choice of transgene and its site of insertion are very important.

Time of harvest. The key issue here is that transgenic lines and their non-GM parents may not reach the same developmental stage at the same time. The problem is confounded by the fact that the growth habit of the transgenic lines may be different from that of the parent. Tomatoes provide a good example of this problem. At what stage should the fruit be harvested? This simple question provoked a long debate and no clear consensus when it was raised at a G02 Programme Workshop. For example, a comparison of tomato colour is highly subjective and leads to complications in comparing fruit, but simply taking fruit after a certain number of days is also problematic. Furthermore, if the fruit from transgenic plants is harvested at a different time from that of the parents, should the earlier harvest be stored until the later harvest is available and, if so, how can we minimise artefacts created by storage? However, if the fruit is to be analysed immediately upon harvesting, how can we be sure that the analytical instruments are performing identically (see Section 7.3)?

Extraction. Should plant material for analysis be extracted immediately or should it be stored under 'normal conditions' before analysis? Potatoes provide a good example of this problem. Except when home grown, potatoes usually are stored in cool conditions for several days to several months before being cooked and eaten. Therefore, at what stage should potatoes be analysed: immediately or after several weeks of storage? Cereals represent a different aspect of the storage problem. As is well known, grain can be stored for very long periods and still be suitable for either sowing or for processing but this does not mean that it does not undergo metabolic changes. So, for the purposes of the G02 programme, should grain be generated anew or can 'old' material be used?

Another aspect of extraction is what tissue or tissues should be extracted and/or analysed. For example, with cereals, should the flour be separated from the husk and, if so, should both be analysed or only the flour?

Yet another aspect of extraction is whether separate extractions should be done for analyses of RNA, protein and metabolites, using optimal procedures for each, or should only a single, 'universal' extraction method be used. In this context, it should be noted that there have been no comparisons of extraction techniques that show high reproducibility, robustness and recovery for all

classes of compounds from plant tissues of varying complexity and degrees of hardness.

In order to minimise the effects of natural variability it is necessary to analyse material from a large number of plants of each type. However, if the sheer number of analyses is not to become unmanageable it may be necessary to pool materials. If pooling is to be done, should it be done before extraction and what materials should be pooled? On the other hand, if large numbers of different samples are not analysed separately then no information will be obtained about the extent of natural variability (range, mean and median). In this context, it should be noted that contractors frequently observed that within-plant and between-plant variability was 10 times greater than instrument variability!

Finally, if large numbers of extracts are made it is unlikely that all of them will be able to be analysed immediately. This certainly is true if, for example, 2D-gel electrophoresis is to be undertaken. So, how should the extracts be stored to ensure that no significant changes occur on storage? For example, work undertaken in project G02003 (Rothamsted) showed that the proteins in grain extracts degrade unless the extract is heat-shocked prior to storage.

Growth conditions. If experimental plants are field grown, they are subject to the vagaries of the British weather. Thus plants can be exposed to one or more of the following during a single growing season: drought stress, waterlogging, heat stress, cold stress, low light levels. Depending on the nature of the modification, certain GM plants may be better able to cope with such stresses. The extent to which plants grown in a greenhouse are free of environmental stresses will depend on how well the greenhouse can be environmentally controlled and the care with which it is managed. A number of the contractors (e.g. G02003, Rothamsted) noted that there was a strong effect of environment (year of growth, location) that generally was much greater than that seen between GM and non-GM.

3.4 Analytical methodology

With any analytical method, it is expected that appropriate standards will be used on a regular basis for calibration purposes. Thus, if the fructose content of a large number of samples were being determined by HPLC, say, then fructose standards would be included at the beginning and end of the analyses and at various other points depending on the number of samples. The use of such standards permits the determination of the precision and accuracy of the measurements and facilitates the detection of instrument drift. The use of standards also eliminates between-operator and between-instrument variability. However, when trying to compare plant extracts using 2D-gel electrophoresis, NMR or mass spectrometry, it is necessary to detect differences in the amount of material in large numbers (50-1000) of spots or peaks. In this situation there is no obvious standard. How then can the experimenter detect instrument drift, differences in instrument sensitivity

before and after maintenance or column changes, or differences between two supposedly identical instruments?

A different problem is the selection of the most appropriate methodology. In the case of 2D-gel electrophoresis it is well known that membrane proteins and very basic or acidic proteins are poorly represented. Should the analysis of such proteins be ignored and, if not, how should they be quantified? In the case of metabolomics, many different analytical methodologies could be used (NMR, HPLC, LC-MS, MS-MS, etc) but which are appropriate? One determining feature is the availability of appropriate spectral libraries for each type of instrument. Without such libraries, the identification of peak components is not possible and the results of analyses are less interpretable. Another determining feature is an understanding of the likely metabolic consequences of transgene insertion. Since no one analytical methodology will detect all metabolites, it is necessary to make a reasoned judgement about the metabolites whose quantities might change. This in turn demands an understanding of both the transgene *and* the location of its insertion into the genome. To put it another way, if you do not know what you are looking for, you might never find it.

Finally, when large numbers of samples have to be analysed using instruments it is common practice to use autosamplers. However, this means that there could be long time periods between the analysis of the first and last samples. In these circumstances, it is possible that sample degradation occurs and/or the instrument drifts during the waiting time. As a minimum precaution, samples for analysis should be statistically randomised. Indeed, in the G02001 project (SCRI), it was shown that different results are obtained with the same samples that have been randomised and not randomised on the autosampler. If large numbers of samples are being loaded manually then operator fatigue can introduce systematic errors unless randomisation is used. *This illustrates the point that good experimental design goes far beyond the way in which control and test plants are arranged during field or greenhouse growth.*

3.5 Sources of experimental variability

Many of the analytical methods described in the previous section are very sophisticated and very sensitive. Consequently, it is possible for the experimenter to introduce variability that was not present in the original material. As shown in Table 3, there are 8 sources of experimental variability.

TABLE 3: The principal sources of variability in laboratory experiments

SOURCE OF VARIABILITY	*EXAMPLES*
Pipettes	Are pipettes regularly calibrated and only used if within recommended tolerance? Cheap pipette tips do not always fit properly leading to pipetting errors. Releasing agents from the manufacturing process may contaminate pipette tips and lead to errors, etc.
Chemicals/ reagents	Are they of a suitable quality and what proof does the experimenter have? Is there batch to batch variability and is it important?
Water	Is it fit for purpose? Is the source properly maintained? How is water stored after preparation?
Equipment	Do instruments used have the analytical resolution expected of them? Does a qualified person regularly maintain the equipment? How and how often are instruments calibrated? How are instruments affected by changes in temperature, humidity, etc?
Procedures	Are procedures written in an unambiguous way? Are procedures free of subjective statements such as ‘heat’, ‘boil’, ‘stir’, ‘mix’, etc? When large numbers of samples are to be analysed on the same day, are the samples randomised to eliminate bias from operator fatigue, instrument drift or sample degradation?
People	Have laboratory personnel been properly trained in the procedures that they are expected to do?
Strains	Are the plant varieties being used really what the experimenter thinks that they are?
Records	Have the appropriate data been recorded correctly and stored safely?

All of the above examples are perfectly obvious when someone draws your attention to them. However, if you do not realise that certain factors can adversely affect your results, you do not account for them in your experimental design. To ensure that contractors were aware of these issues, and others like them, a number of workshops were held that were devoted to

best practice. The first of these was held in November 2002 and following this particular workshop it was clear that a number of the contractors had given much thought to the potential sources of operator-induced variability and how to eliminate/minimise them.

3.6 Data trapping and data management

At the outset of the G02 programme it was not clear what data needed to be trapped. For example, it is obvious that the genotypes of all the plants used needs to be recorded as well as the growing conditions. However, if the plants were field grown, should the daily rainfall, hours of sunshine and temperature fluctuations also be recorded? Similarly, if the plants were grown in a greenhouse, what environmental parameters need to be recorded? At the opposite end of the scale, when making analyses, how important is it to record the serial number of the instrument, the elapsed time since last serviced, the identity of the analyst, the serial number of the column, the batch number of the standard(s), the room temperature and humidity, etc? The short answer is that it is necessary to trap every piece of data until such time as it is known what is and is not important. A key part of the G02006 (Aberystwyth) project was the development of a pro-forma template for trapping all metadata.

A key issue in all programmes was sample size. This has two dimensions: number of components (e.g. tomatoes) sampled per plant and the number of plants of each type (GM and non-GM) that are sampled. Clearly, the more of each that are sampled; the better will be our knowledge of within-plant and between-plant variation. However, the number of analyses that need to be made on each sample, and the resource requirements (time, labour and consumables) limits the number of samples that can be analysed. One solution is to pool samples but what method of pooling should be used and will this result in loss of essential data or compromise the statistical analysis? Several contractors addressed this issue, e.g. G02003 (Rothamsted).

Each of the analytical methods used generates a large amount of data. For example, 2D-gel electrophoresis can resolve 1-2000 individual protein ‘spots’ and a similar number of peaks might be resolved by NMR analysis of low molecular weight metabolites. How should these data be analysed and interpreted and is it necessary to annotate all the spots/peaks before analysis? In reality, many of the spots/peaks that were resolved were not fully annotated and this added a degree of complexity to the data analysis step. Although it is possible to analyse complex data in an abstract way, i.e. without any knowledge of the identities of the individual components, it does mean that there is a tendency to give every component equal weighting. However, if one knows that certain NMR peaks are derived from alkaloids in potatoes, for example, and others from sugars then additional analyses should be done in which more weighting is assigned to the alkaloids as these are known to be toxic.

3.7 Workshops

Once the true complexity of the G02 programme became apparent it was decided that the best way of dealing with many of the technical challenges was to hold a series of highly focussed workshops (Table 4) in addition to regular visits to the contractors by FSA staff and the programme advisor.

TABLE 4: Details of G02 workshops

DATE	TOPIC	COMMENTS
April 2002	Proteomics	Review of available methods
June 2002	Analytical methods for metabolomics and data trapping	Review of pros and cons of different analytical methods (mostly mass spectrometry) including choice of instrument, software, detectors, etc.
November 2002	Best practice	Annual meeting
April 2003	Experimental design and data handling	Small workshop dedicated to statistical methods for designing experimental methods (best practice) plus methods for analysing very large data sets.
July 2003	Proteomics	Review of what methods are proving most suitable in terms of resolution, reliability, etc (i.e. best practice).
September 2003	Progress and problems	Critical review of results to date
October 2004	Methods review	Critical review of the advantages and disadvantages of the different methods for transcriptomic, proteomic and metabolomic analysis.

SECTION B: EXPERIMENTAL METHODOLOGIES

4 ANALYSIS OF JUNCTION SEQUENCES

4.1 Introduction

There are three general methods for introducing transgenes into plants. The first method exploits the natural ability of certain bacteria of the genus *Agrobacterium* to naturally transfer DNA to the genomes of infected plant cells. This usually results in the stable transformation of the infected cell and the transferred DNA behaves as a new genetic locus. Initial limitations with respect to the host range of *Agrobacterium* (only selected dicotyledenous plants) prompted research into alternative methods based on direct DNA transfer. The most widely used alternative technique has been biolistics where plant cells are bombarded with microprojectiles coated with DNA. This can result in both transient and stable transformation. A major disadvantage of biolistics is that it frequently results in multiple transgene insertions. As methods have been developed for extending the host range of *Agrobacterium* to cereals (monocots) the use of biolistics has declined. Finally, plant viruses can be used as vectors for transgene delivery. However, the viruses of plants never integrate into the genome so stable transformants cannot be obtained. In all six G02 projects the transgenic material used was generated using *Agrobacterium* or biolistics.

Junction sequences are the DNA sequences that delimit the insertion of a transgene into the host genome. The ability to analyse junction sequences is crucial to the molecular characterisation of transgenic crops as the information can be used to establish:

- The position of the transgene in the genome,
- Whether any functional genes are disrupted and
- Whether any new open reading frames (ORFs) are created resulting in the synthesis of novel proteins.

At the outset of the G02 programme there was virtually no information available for the junction sequences in any transgenic plant and it was generally recognised that obtaining this information was very difficult. Of the six G02 projects, only one (G02002 JIC) investigated methods for determining junction sequences.

4.2 Evaluation of methods for analysing junction sequences

Three different methods for determining junction sequences were investigated in the G02002 (John Innes) project. These were:

- Inverse PCR,
- Thermal Asymmetric Interlaced PCR (TAIL PCR) and
- Adapter Ligation PCR (also called Adapter Mediated PCR).

Inverse PCR involves digestion of the genomic DNA with a restriction endonuclease and then ligating the resultant fragments to circularise them.

The circularised DNA is then used as a template for PCR. The unknown sequence is amplified using two primers, specific to the known transgene sequence but running in opposite directions, and the resulting amplicon sequenced. Experimental workers elsewhere have used inverse PCR to determine junction sequences but the method does not work routinely. The biggest problem with this technique is the initial cleavage of the genomic DNA. The choice of restriction enzyme depends on the position of cleavage sites relative to the junction sequences but in the absence of sequence information it is difficult to select an enzyme!

TAIL PCR utilises three long T-DNA specific nested primers together with a smaller arbitrary degenerate primer. The relative differences in primer length, and hence annealing temperatures, permit the amplification efficiencies between specific and non-specific products to be controlled thermally. An advantage of the TAIL PCR method over inverse PCR is that it does not involve an initial digestion of genomic DNA. However, the TAIL PCR method involves three different cycles of PCR plus three sets of primers and three sets of annealing conditions. This makes the method very complex and means that it will not be robust. It has been used for high throughput analysis following a single insertion event (e.g. in plant breeding trials) but is not particularly suited to the analysis of many different transgenic plants.

Adapter ligation PCR uses the ligation of adaptors to digested transgenic DNA so that PCR amplification can take place using primers complementary to the adaptors and primers complementary to known sequences. Two rounds of PCR are carried out; the second round using nested primers to ensure that a specific sequence is generated.

Adapter ligation PCR has proved to be a relatively simple and robust technique for the characterisation of junction sequences in barley and wheat. It worked best with *Agrobacterium*-transformed lines where only a single copy of the transgene is present. However, some success has been obtained with lines (mostly barley) produced using biolistics and which tend to have multiple transgene inserts. Since the G02 programme was initiated there have been a number of papers published describing the use of adapter ligation PCR for identifying junction sequences in *Arabidopsis* and rice.

4.3 Summary of results

Three methods for analysing junction sequences were evaluated and Adaptor Ligation found to be the most suitable. Adaptor ligation has been shown to be a relatively simple method for the analysis of junction sequences in transgenic barley and wheat lines, particularly if only a single transgene is present. Work done outside of the G02 programme has shown that the method is suitable for use with other plant species (*Arabidopsis* and rice).

5 TRANSCRIPTOMICS

5.1 Introduction to transcriptomics

The full complement of RNA molecules produced by the genome has been dubbed the transcriptome. However, it is important to realise that the transcriptome is potentially more complex than the transcribed portion of the genome. This is because one gene can produce different mature transcripts by alternative splicing and polyadenylation or RNA editing. Different transcripts can produce different proteins. Nevertheless, all the different transcripts will be represented when the total RNA content of a sample is analysed. Complex as the transcriptome is, it is never seen as a complete system *in vivo* because there is no situation in which all the genes in the genome are simultaneously transcribed. Cells transcribe a basic set of *housekeeping* genes, the resultant proteins being required at all times for essential cellular functions, and other *non-housekeeping* genes that are expressed in a regulated manner. Three of the six projects within the G02 programme had a transcriptome component. It should be recognised, however, that there are two fundamental weaknesses in transcriptomic analysis. First, the quantity of a particular mRNA may not correlate with the quantity of the corresponding protein for no cognisance is given to either the rate of RNA turnover or the rate of protein turnover. Second, nucleic acid hybridisation techniques do not always detect low abundance oligonucleotides and hence low-level expression may not be detected. On the other hand, transcriptomics has an inherent advantage over proteomics and metabolomics in that the potential coverage is much greater.

There are currently two basic methodologies used for global RNA expression analysis:

- Hybridisation of extracted mRNA to comprehensive DNA sequences immobilised on microarrays;
- Direct sampling of sequences from source RNA populations.

There is a significant difference between these two methods. Microarrays are closed systems. That is, you can only detect those transcripts for which there is a complementary oligonucleotide or cDNA sequence on the microarray. By contrast, sequence sampling is an open system in that no assumptions are made at the outset about which transcripts will be present.

5.2 Transcriptome analysis using microarrays

For a DNA microarray to be of value, it needs to be prepared from cDNA sequences derived from the organism of interest or, failing that, a closely related species. For example, a barley microarray can be used for analysis of gene expression in wheat. However, in such circumstances sensitivity is reduced due to sequence heterogeneity between species. In addition, tissue-specific and developmental-stage specific genes are required for such microarrays depending on the tissues under study. A further complication is that many arrays only carry sequences derived from a proportion of the transcribed genes and important genes may be missing. The leading provider of DNA sequence microarrays is Affymetrix and at the start of the G02

programme only a very limited range of plant genome microarrays was available. The G02002 (JIC) and G02004 (IFR) projects benefited from the availability of barley and *Arabidopsis* microarrays respectively. However, for the G02001 (SCRI project a potato microarray had to be generated *de novo*.

The *de novo* generation of microarrays is not without its problems. First, large numbers of cDNAs have to be generated and this is very time consuming especially if a subsequent redundancy check is performed. Second, microarrays developed in research laboratories are never of the same quality as commercial arrays because of inherent problems in arraying equipment. Third, the cDNAs vary in length from ~500-1500 nucleotides and this can lead to differential hybridisation efficiency of mRNA. By contrast, commercial microarrays consist of large numbers of short oligonucleotides (~20-25 nucleotides). These factors make the use of commercial microarrays, where these are available, preferable to laboratory-generated cDNA arrays.

Despite the problems associated with cDNA arrays, differences were seen between GM and non-GM lines of potato. However, these differences were limited and there are three *possible* reasons for this:

- Only large differences could be observed because the arrays were sub-optimal,
- Changes induced by a genetic modification are not represented to their full extent in the transcriptome of a dormant tissue type such as potato tubers,
- Not many changes were induced by the genetic modifications under study.

Quality checks showed that the arrays used were of an acceptable quality. By contrast, metabolomic analyses strongly suggested that not many changes had been induced and this underscores the need for complementary analyses to transcriptomics.

Regardless of whether commercial oligonucleotide microarrays or laboratory-made cDNA arrays are used for the primary transcriptomic analysis it is advisable to confirm the differences detected using another technique. In the G02001 (SCRI) project, the differences in transcript levels seen with microarrays were checked using quantitative PCR and the Luminex system. Both these systems confirmed the *relative* changes in transcript levels but the *absolute* changes differed by a factor of 5-10.

5.3 Transcriptome analysis by direct sequence sampling

Expression profiling by direct sequence sampling involves the large-scale random sampling of sequences representing a given mRNA source. For example, a cDNA library that has not been normalised is representative of the mRNAs in the source population used to prepare it. Some mRNAs are likely to be highly abundant and some extremely rare. If 5000 sequences were picked at random from a library, abundant transcripts would be more frequently represented among the sequences than rare transcripts. Statistical analysis of these results would allow relative expression levels to be determined. Comparison of libraries from different sources (GM versus non-

GM) should facilitate the identification of differentially expressed genes. A number of methods are available for sequence sampling and the most widely used is SAGE (serial analysis of gene expression). This method was evaluated in the G02002 (JIC) project.

SAGE is a digital method for large-scale gene expression analysis and is based on two principles. Firstly, a short (14bp) oligonucleotide fragment, or SAGE tag, produced by restriction enzyme digest is sufficiently unique to identify a specific transcript. Secondly, concatamers of SAGE tags subcloned into a suitable cloning vector, serve as templates for automated sequencing and significantly increase the efficiency of sequence-based gene expression analysis. Consequently, a single sequencing reaction can provide data for as many as 25-30 transcripts. SAGE results are interpreted using software which creates a digital profile of gene expression, based on the frequency of occurrence of each tag species, which is a direct representation of the abundance of the respective mRNAs in the tissue or cell type under study.

A major objective of the G02002 (JIC) project was to examine the ability of the SAGE technology to detect significant changes in the transcriptome of transgenic barley plants when compared to non-transformed controls and null segregants. In this regard, SAGE unambiguously detected transgene expression and provided a quantitative measure for the level of this expression. In addition, the method permitted the identification of those genes differentially expressed between transgenic and non-transgenic plants. For example, in one transgenic barley line, 58 transcripts had a greater than 5-fold change in abundance and in most cases the corresponding gene could be identified.

5.4 Microarrays versus direct sequence sampling

In the G02002 (JIC) project a direct comparison was made of the ability of SAGE and commercial barley microarrays to quantitatively measure gene expression and identify differentially expressed genes in barley. Good overall comparability was observed and both methods could be used for detecting changes in the transcriptome. As an open system, SAGE allows the analysis of any transcriptome without the need for prior knowledge of the genome sequence but extensive EST databases are necessary for data interpretation. Microarrays can only detect the genes that are represented on the chip used but have a much higher throughput than SAGE. Results can be obtained from microarrays within days of having an RNA sample. By contrast, the construction of SAGE libraries is very time consuming and technically very challenging due to the multiple enzymatic reactions and the intricate manipulations involved in the process. Moreover, considerable amounts of sequencing are required to obtain adequate information from SAGE libraries. Overall, SAGE is highly quantitative and very sensitive, especially for detecting rare transcripts, while microarray data suffer from a lack of sensitivity at very low expression levels due to background noise.

5.5 Quality issues

The construction of cDNA arrays technically is very challenging – from the isolation of mRNA for conversion to cDNA all the way to reproducible array spotting. Therefore, from a quality point of view, cDNA arrays should be used only when absolutely necessary. Commercially available oligonucleotide microarrays are much preferable since they are subject to extensive quality control during manufacture although the end user does need to do their own quality checks. SAGE is very laborious and technically demanding and so by definition is error prone. If SAGE is to be used then detailed SOPs coupled with adequate training are absolutely essential.

5.6 Summary of utility of transcriptomic methods

All of the methods for transcriptome analysis evaluated during the G02 programme proved to be capable of detecting changes in transcript levels as well as detecting new transcripts. Both SAGE and commercial microarrays proved to be robust and reliable, at least for analysis of transgenic barley cultivars. As more microarrays for crop species become commercially available, transcript analysis should become easier. However, regardless of which method is used, transcript analysis is very expensive. However, once whole transcriptome microarrays are available for major crop species they could be used to determine how many transcripts are changed in a transgenic crop with selection being made for those lines with the fewest changes. A determination of the identity of the changed transcripts taken together with the nature of the crop species could provide useful information for safety assessment. For example, potatoes are a higher risk than barley because of the presence of natural toxins.

6 PROTEOMICS

6.1 Introduction to proteomics

There are 4 *theoretical* risks associated with the consumption of genetically modified food. As a result of transgene insertion into a crop species one or more of the following could have occurred:

- If a foodstuff contains a sub-critical level of a toxic or allergenic protein then the level of this protein could be increased above the no-effect threshold.
- A new protein could be created that is either toxic or allergenic.
- The level of a toxic metabolite could be increased significantly such that it no longer is destroyed by cooking or rises above the no-effect threshold.
- Novel, toxic metabolites are synthesised in the transgenic plants.

There is a clear link between the first two risks and proteomic analysis and the requirement is the detection of any new proteins made in transgenic plants or the identification of any proteins whose levels have increased significantly.

The link between the second two risks and proteomics is less obvious. To appreciate the link it is important to realise that in cells many metabolites

undergo multiple transformations. If the level of one of the enzymes participating in a metabolite transformation increases then the flux of that metabolite through other pathways may decrease. The net effect is that a change in the level of one enzyme might change the small molecule composition of the cell. This effect alone could change the levels of toxic molecules already present in the cell (e.g. alkaloids in potato tubers). Similarly, an increase in the level of a particular metabolite may result in it being metabolised in a novel manner by pre-existing enzymes. Although the role of metabolomics is to detect changes in metabolite composition for example as a consequence of genetic modifications, analysis of protein changes can provide very useful supplementary information. A good example of this was provided by the G02004 project at IFR.

Of the 6 projects that formed the basis of the G02 programme, 4 had a proteomics component. The methodologies that they opted to evaluate are listed in Table 5.

TABLE 5: Proteomics methodologies evaluated in the G02 programme

	METHODOLOGY	**COMMENT**
G02002 (John Innes)	1D gels	Very simple to run but of low resolution
G02003 (Rothamsted)	2D-gels	Exceptional progress made in developing highly reproducible technique
G02004 (IFR)	2D-gels + protein identification by mass spectrometry	Preliminary assessment also made of MudPIT
G02001 (SCRI)	I-CAT and similar tagging techniques	Includes comparison with 2D-gel data

6.2 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) is a simple, cheap and potentially high-throughput technique for analysing protein mixtures but it has a low resolution when compared with two-dimensional analysis (see below). Thus the utility of SDS-PAGE is restricted to the analysis of protein mixtures of low complexity. Such low complexity is seldom found in foodstuffs but one major example is mature cereal grains where storage proteins account for ~50% of total protein. The John Innes group (G02002) found that SDS-PAGE was very useful for detecting changes in the relative proportions of the different protein subunits. No new proteins were detected in any of the transgenic cereal plants that were analysed but it is difficult to ascertain whether a low level of expression of a novel protein would have been detected even if its molecular weight were significantly different from the major storage protein subunits. However, use of SDS-PAGE would be appropriate if analysis of junction sequences (section 5) indicated that no novel proteins should be synthesised.

6.3 Two dimensional gel electrophoresis (2D-gels)

The standard method for comparing the protein content of different samples is to use 2D-gel electrophoresis. The method involves isoelectric focussing in the first dimension and separation on the basis of size in the second dimension. Size fractionation is achieved by equilibrating the isoelectric focussing gel in a 2% solution of the detergent sodium dodecyl sulphate (SDS). The SDS binds non-specifically to all proteins and confers a uniform negative charge. After fractionation, the protein gel is stained. The commonly used stains are Coomassie Blue or silver salts but a number of improved stains are becoming available. The outcome is a unique pattern of dots, each dot representing a protein. Individual spots can be picked from the gel and the proteins in them identified by mass spectrometry.

Problems with 2D-gels

Although 2D-gels are the most widely used method for studies on comparative proteomics, there are a number of technical disadvantages. These include:

- The technique is very time consuming and labour intensive and this reduces throughput.
- The maximum number of spots that can be resolved is typically in the order of 1-2,000. If each spot represents a different protein then only about 10% of the proteome is represented. However, up to 25% of the spots on a typical gel may be post-translational variants of proteins represented elsewhere on the gel.
- Many proteins cannot be resolved by 2D-gel electrophoresis, e.g. membrane proteins and proteins that are very acidic or very basic.
- Very abundant proteins (e.g. ribulose bisphosphate carboxylase, mitochondrial proteins) generally are not of interest but can obscure many proteins that are of interest. A partial solution to this problem is to fractionate the total protein sample prior to electrophoresis and to use a narrower pH gradient in the isoelectric focussing step.
- It is very difficult to get good gel to gel and day to day reproducibility with this technique. One way of minimising variability is very strict attention to detail (see sections 3.3 and 6.6) but this greatly reduces throughput.
- A common requirement is to directly compare the 2D-gel electropherograms produced by two different samples. In practice, this involves looking at up to 1000 spots on each of two gels and trying to identify those spots whose intensity is significantly different. This is very difficult, very tiring and very subjective. Software programmes are available that can do comparisons but have many limitations. It is possible to directly compare two samples by first labelling all the proteins in each sample with different fluorescent dyes and then running the two samples on the same gel. However, the very high cost of these fluorescent dyes meant that they could not be used in the G02 programme.

Because of the differences associated with 2D-gel analysis, a variety of other approaches were evaluated (see sections 6.4 and 6.5). However, none of these alternatives proved to be suitable and hence 2D-gels currently remains the only technique with the resolving power that is required for safety assessment. In considering the use of 2D-gels for proteomic analysis it is extremely important to realise that the quality of the data obtained is dependent on five factors:

- The method used to extract proteins from the test samples,
- The choice of gel running conditions,
- The reproducibility of the gels,
- The choice of protein stain,
- The gel analysis software that is used.

One contractor (G02003, Rothamsted) devoted a lot of effort to optimising proteomic analysis using 2D-gels, understanding the sources of variability, and eliminating them. Their success is quite remarkable and should be easily transferable to proteomic analysis of other crop species. (See section 13.2)

Extraction of proteins

The ideal method for extracting proteins from samples will depend on the nature of the test material and hence no generalisations can be made. In the G02003 project (Rothamsted) an evaluation was made of seven different methods for extracting proteins from cereal grains. The criteria used to select the method of choice were:

- Reproducibility
- Compatibility with 2D–PAGE
- Practicality (ease of use)
- Number of protein spots resolved on 2D-PAGE

By contrast, the IFR group (project G02004) has a standard protocol, originally developed for analysis of tomatoes, that they always use. However, this method, which worked well with tomatoes and *Arabidopsis*, gave poor results when applied to cereal grains at Rothamsted. Clearly, a method that has been optimised for a particular plant tissue will give better results but there is a need to balance the development effort required against the particular questions being asked.

Gel running conditions

There are many options when selecting the gel running conditions, particularly in the choice of pH gradient. However, in the end, a judgement has to be made between resolving a large number of proteins over a broad pH range and obtaining more detail over a narrow pH range. Given that there is no single set of conditions that will resolve all the proteins present in a sample, the choice of running conditions will depend on the questions being asked. For example, in the G02004 (IFR) project the decision was made to use 15cm gels and a pH 4-7 gradient. However, proteins encoded by the transgene could not be detected in green tissue extracts without removal of the most abundant proteins. In the G02003 project, they used 24 cm pH3-10 gradients which are much more informative but much more difficult to run reproducibly.

In this case, it was possible to detect transgenic proteins without prior fractionation.

Gel staining

In the G02003 project at Rothamsted, a detailed comparison was made of the performance of the different protein stains that are available. Although the methods were assessed using a limited range of samples the results that were obtained (see Table 6) are likely to be of much broader relevance. Based on these results the Rothamsted group selected Sypro Ruby as the stain of choice for proteomics analysis. Independently, the IFR group (project G02004) selected Sypro Ruby because it was simple to use, gave reproducible results and had the best dynamic range.

TABLE 6: Results of evaluation of different 2D-gel staining method

PROTEIN STAINING METHOD	ADVANTAGES	DISADVANTAGES
Colloidal Coomassie Brilliant Blue-G	Quick and easy to use Relatively inexpensive Compatible with mass spectrometry	Insensitive (limit of detection ~1 microgram)
Silver staining	Extremely sensitive (can detect picograms of protein)	Multi-step procedure. Development time very subjective. Staining to detect minor proteins results in over-staining of abundant proteins. Reproducibility difficult to achieve. Background staining makes image analysis difficult. Incompatibility with mass spectrometry (without method variation).
Colloidal Coomassie Blue plus silver staining	Can detect low abundance and high abundance proteins simultaneously	Very poor reproducibility
Blakesley stain	Similar to colloidal Coomassie Brilliant Blue stain	Similar to Coomassie Brilliant Blue but time consuming to prepare stock solution.
Fluorescent Sypro Ruby	Proteins cannot be overstained. Can detect low and high abundance proteins simultaneously. Background staining minimal. Staining artefacts easily detectable. Highly reproducible.	Gels need to be handled in the dark High cost (~£67/gel) Reduced sensitivity compared to silver staining (but much better than Coomassie Brilliant Blue)

Gel analysis software

The analysis of the protein spots on an individual gel and the comparison of two or more samples run on different gels require the use of image analysis software. A number of proprietary software programmes are available but most do not live up to the claims of their vendors. Two software packages were assessed in the G02003 (IACR/ Rothamsted) project: PD Quest (from Bio-Rad) and Phoretix 2D Expression (from Non-Linear Dynamics). PD Quest was found to have a number of serious defects that were non-resolvable by the supplier including:

- Different results obtained when the same gel was scanned multiple times
- Generation of artefacts
- Some highly desirable functions did not work
- Software is very complex making operator training lengthy
- Master images had no statistical validity
- Images were of poor quality when compared with the actual gel that was imaged.

By comparison with PD Quest, the Phoretix 2D Expression software had many advantages including:

- Ease of use
- No false positive spots
- Consistent spot counting
- Potential to 'warp' gels to enable direct comparisons between gels that had run slightly differently (which is inevitable)
- Minimal manual intervention required
- Data easily exportable for statistical analysis

In the G02004 (IFR) project the software used was ProteomWeaver. This package was selected because it permits the comparison of one gel with another without the need for a reference gel. It also is particularly good for matching protein spots on different gels.

Identification of protein spots

In both the G02003 (Rothamsted) and GO02004 (IFR) projects, 2D-gel analysis revealed changes in the levels of proteins in some transgenic plants when compared with null segregants. For risk assessment purposes, it is essential to know the identity of the proteins whose expression level has changed. At IFR (project G02004), proteins were successfully identified by mass spectrometry after trypsin digestion. The only limitation of this technique is that a protein excised from a gel may be matched with an unnamed protein in the various databases. In such cases, the function of the protein may be putatively assigned or might be totally unknown. That is, the problem is with the database content and not the technique itself. However, the information in the databases is doubling annually and so the problem should be much less of an issue in the future.

6.4 Liquid chromatography coupled with mass spectrometry

An alternative methodology to 2D-gels is the use of mass spectrometry coupled with sample specific labels. This involves labelling proteins with a mass label, digesting them with trypsin, separating the resultant peptides by liquid chromatography and then subjecting them to electrospray MS. The sequences of the different peptides are determined from their molecular masses in conjunction with a user-generated peptide mass library and these permit identification of the proteins from which they were derived. To compare two different samples, say a GM line and its non-GM equivalent; the proteins are labelled with isotope-coded affinity tags (I-CAT). One of these tags has 8 hydrogen residues and is used to label the proteins in the control sample. The other tag has 8 deuterium residues and is used to label the proteins in the test sample. The two protein samples are mixed, digested and subjected to LC-MS thereby permitting simultaneous analysis and quantitative comparison. At the outset of the G02 programme the ICAT technique had not been critically assessed for its utility in large-scale proteomics studies and such an assessment formed part of the G02001 project at SCRI. The key findings of this assessment were:

- The technique works very well when tested with model proteins;
- The technique does not work as well when used with complex protein mixtures such as those extracted from potatoes. In the case of potato extracts, patatin swamps the chromatogram that is obtained because patatin is a very abundant protein;
- Good resolution of peptides probably requires multiple chromatographic dimensions (see MudPIT, described below);
- The technique has a low throughput;
- The technique is very expensive with the reagents costing in excess of £1,000 per experiment.

Because of the high cost of the ICAT reagents, alternative protein-tagging techniques for quantitative profiling were evaluated as part of the G02001 (SCRI) project. One alternative to ICAT is mass-coded abundance tagging (MCAT). The MCAT technique is similar to ICAT except that proteins are treated with O-methylisourea to convert lysine residues to homoarginine thereby generating a 42 atomic mass unit difference. In practice, reciprocal labelling is undertaken. That is, the test sample is treated and the control sample is untreated (test labelled) and then the experiment is repeated where the test sample now is untreated and the control sample is treated (control labelled). In the G02001 (SCRI) project the following advantages and disadvantages of the MCAT technique were noted:

- The technique is very economical (£1-5 per sample) compared with ICAT.
- Peptides from treated and untreated samples often do not co-elute.
- Overlapping of unrelated peaks was observed frequently.

Another variation of the ICAT technique is to differentially label the test and control samples with SATA (N-succinimidyl-S-acetylthioacetate) and SATP

(N-succinimidyl-S-acetylthiopropionate). In the G02001 (SCRI) project it was noted that:

- The SATA/SATP method worked well with model proteins.
- The effectiveness of the method declined rapidly as the number of proteins and/or peptides to be analysed increased. The problems that were encountered were largely due to inconsistent reproducibility in labelling with the light (SATA) tag.

The final variation on the ICAT technique that was explored was differential labelling with iodoacetamide (IA) and acetamide (AA). This method is extremely cheap to use (<£1 per sample). However, peptides were not labelled with the same efficiency in reciprocal labelling experiments thereby rendering the method unusable.

The key conclusions from the analysis of the different dual labelling approaches are:

- Labelling must not compromise chromatography. Basically, this restricts the method to tags that substitute deuterium for hydrogen (ICAT) or ones with an additional $–CH_2-$ group in the label (SATA/SATP).
- The label must have a unique purification advantage but not to the degree that removal from the purification medium compromises the chromatography (as occurred with SATA/SATP).
- Currently, plant protein databases are still in their infancy and any search for peptide hits following labelling still yields many unknown peptides and/or proteins.

Multidimensional protein identification technology (MudPIT) which was used in the G02004 (IFR) project is another approach that may alleviate some of the disadvantages of 2D-gels. MudPIT uses two chromatography steps interfaced back to back in a fused silica capillary. The advantage of this is that the band broadening (loss of resolution) associated with many chromatographic steps is avoided. Also, the capillary can be placed directly into the ion source of a mass spectrometer thereby maximising sensitivity. Chromatography proceeds in steps with increases in salt concentration used to free peptides from a cation exchange resin after which they bind to a reversed phase resin. A reversed phase gradient of increasing hydrophobicity then is applied to progressively elute peptides from the reversed phase packing into the mass spectrometer.

6.5 Other methods of proteomic analysis

Two alternatives to 2D-gels were assessed in the G02003 project (Rothamsted): the Agilent capillary electrophoresis system and the DeltaDot protein analysis technology. The Agilent system was easy to use but suffered from a number of serious defects:

- The sensitivity of the system was low and was unable to detect changes in minor proteins. The number of proteins detected was less than with single dimension SDS-PAGE gels;

- Misidentification of components of the molecular weight ladder was a common problem and made quantitation and molecular weight measurements impossible;
- There was drift in resolution across a single chip such that the same sample in different positions on the chip gave different results.

The DeltaDot system is still under development and is not yet commercially available. An early prototype was evaluated and was able to distinguish non-transgenic samples from transgenic samples with good reproducibility. However, for substantial equivalence work, the sensitivity would need to be increased.

6.6 Quality issues

The three contractors who used gel electrophoresis for proteomic analysis developed standard operating procedures (SOPs) and found the use of these SOPs beneficial. However, the Rothamsted group (project G02003) made an extraordinary effort to understand and eliminate the sources of variability in 2D-gel analysis. The outcome of their efforts was an intensive staff-training programme and detailed and carefully written SOPs and the benefits were:

- Greater than 95% of the 2D-gels that were run generated useful data even under the most demanding circumstances (24 cm gels, pH3-10 gradient).
- Identical results were obtained when different individuals ran the same sample on different days.
- It became feasible to undertake an experiment requiring 288 separate gels to be run!

The achievements at Rothamsted have set a new, and very high, standard for 2D-gel analysis that should be adopted by anyone undertaking proteomic analysis and not just in the area of safety assessment.

6.7 Summary

Currently, 2D-gels are still the best method for proteomic analyses even though under the best conditions they permit only 10% of the proteome to be analysed on a single gel. Even so, they have been used to detect changes in the levels of proteins in transgenic plants, including low abundance proteins. As will be seen later, the analytical power of the technique probably is sufficient when knowledge of junction sequences and levels of expression are taken into account.

2D-gels have a reputation for not being reproducible but the Rothamsted group has shown this problem can be overcome by paying strict attention to detail and by optimising protein extraction for the plant tissue being analysed.

To detect changes in protein expression between transgenic and non-transgenic plants, it is necessary to directly compare the levels of all the proteins in the two samples. One way of facilitating this comparison is to use liquid chromatography coupled with mass spectrometry and isotope coded affinity tags. This technique is very expensive. Now that it is possible to run

2D-gels reproducibly an easier and cheaper method is to compare the two samples run on different gels using one of the better proprietary software packages.

The identity of proteins, whose levels have changed between transgenic and non-transgenic plants, can be ascertained by mass spectrometry following tryptic digestion. The major limitation of this method is the amount of information on known proteins in the appropriate databases.

7 METABOLOMICS

7.1 Introduction to metabolomics

The metabolome comprises all the low molecular weight (i.e. non-polymeric) compounds found in a cell or tissue. The various components of the metabolome can be viewed as the end products of gene expression and define the biochemical phenotype of the source material. The major limitation of metabolomics is its current inability to comprehensively profile all of the metabolome in a single analysis due to:

- The chemical complexity of the metabolome;
- The biological variance inherent in most living organisms (discussed earlier in the context of extraction);
- Dynamic range limitations of most instrumental approaches.

The complexity of the metabolome can best be appreciated by comparison with the genome, the transcriptome and the proteome:

- The genome and transcriptome are linear polymers of four nucleotides.
- The proteome is a mixture of linear polymers of 22 amino acids, many of which can be separated by a single technique (electrophoresis).
- The metabolome comprises thousands of different compounds of many types: ionic inorganic species, hydrophilic carbohydrates, hydrophobic lipids, complex natural products, etc. that cannot be separated by a single method.

Dynamic range defines the concentration boundaries of an analytical determination over which the instrumental response as a function of analyte concentration is linear. However, the dynamic range is severely limited by the sample matrix and/or the presence of interfering and competing compounds. Of particular relevance to plants, high concentrations of primary metabolites such as sugars can interfere with the ability to analyse secondary metabolites such as flavonoids. For this reason, effort was expended in the G02005 project (Royal Holloway) in developing a method for reducing the interference caused by sugars.

Given the chemical diversity of the metabolome, there is no single analytical methodology that will suffice and even the use of multiple methodologies represents a compromise. The three most important techniques to have emerged for metabolomics studies are gas chromatography (GC), high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR). Normally GC and HPLC are combined with mass spectrometry (MS).

These methods are capable of resolving and quantifying a wide range of compounds in a single sample. In addition, high sample throughput is possible with all of them although with NMR this can be achieved only with very powerful instrumentation.

All six projects within the G02 programme had a metabolomics component and all 6 utilised mass spectrometry and/or NMR spectroscopy, albeit using different types of equipment. In some cases, additional techniques were used, e.g. HILLIC-MS that is particularly good for the analysis of secondary metabolites. *In reviewing the metabolomics component of the G02 programme it is essential to keep in mind that at the outset of the programme the methodology was in its infancy and there were only a very limited number of published papers describing experimental plant metabolomics.*

7.2 Nuclear magnetic resonance spectroscopy

NMR is quick and easy to perform because it requires no sample clean-up, no fractionation, no chromatography and no derivatisation; i.e. all metabolites potentially can be detected. Although it is a quantitative method, the analysis of complex extracts results in overlapping peaks thereby reducing it to a semi-quantitative method. This, coupled with its relative insensitivity, means that NMR usually is used to generate metabolomic fingerprints.

The strengths of NMR for metabolomics are:

- You can detect a wide range of compounds;
- The method is very reproducible and robust;
- The data is easily transferable for statistical analysis;
- You get good compound identification. If the compound of interest is not in the database you can apply 2D – NMR;
- Separation of metabolites is not related to size or charge;
- It is good for rapid screening;
- It is non-destructive;
- It is good for structural characterisation;
- NMR is complementary to LC-MS (but not particularly complementary to GC-MS);
- NMR instruments are very stable (unlike MS instruments).

The weaknesses of NMR are:

- The method is of lower sensitivity than mass spectrometry;
- You get multiple peaks per compound. If there are a lot of compounds present the peaks can overlap;
- The method cannot be used for absolute quantification but can be used for relative quantification;
- In order to get high resolution it may be necessary to throw away data;
- To obtain optimal results the method needs to be tailored to each crop species;
- High throughput requires instruments with powerful magnets.

All the project groups that used NMR used a similar methodology but at the detailed level there was no standard methodology. There were two reasons for this. The first reason was the need to tailor the exact methodology to each crop species. The second reason relates to the choice of instrument available, i.e. probe type, field strength and sample temperature. Essentially, the choice of instrument is a trade off between resolution (high field strength and high cost) and sensitivity (use of cryoprobe).
The newer NMR instruments that are coming on the market, but which were not available at the start of the programme, will increase the utility of NMR. For example, LC-NMR will allow small amounts of standards to be prepared.

Despite the simplicity of NMR as a means of metabolic profiling, sample preparation is a key issue. For example, in the G02002 (JIC) project, over 6 months was spent on optimising the extraction procedure for barley and wheat grains and key issues were:

- The use of sonication versus agitation;
- The need to ensure sample homogeneity;
- Controlling the mass of material for analysis;
- The presence of interfering high molecular weight materials.

The uniformity of the NMR response from all proton-containing metabolites permits a direct comparison between the metabolite profiles obtained from transgenic test samples and null segregants or wild type controls. This uniformity of the NMR response also is the key to studying the relationship between the concentrations of metabolites from a single data set. There is a direct correlation between the peak intensity in the NMR spectrum and the concentration of the compounds from which individual resonances arise. Unlike the mass spectrometer, the NMR spectrometer gives a uniform response to concentration for all metabolites.

During the course of the G02002 (JIC) project, a software package (CSL-Metabolab) was developed that enables clear visualisation of NMR data alongside the results of statistical tests. These statistical tests were performed to highlight areas of the spectral data that are able to discriminate between the test sets. For example, using the software it was possible to identify a peak associated with a component of all plants carrying the *bar* gene.

In the G02002 (JIC) project a brief study was made of the transferability of NMR measurements between different instruments. The same extracts were analysed on two instruments operating at the same frequency but with different probes. Although one instrument had far greater sensitivity the overall profiles were similar and statistical analysis indicated no significant difference between them.

In the course of the G02001 (SCRI) project an assessment was made of extraction and instrumental repeatability between years. Principal components analysis showed that repeatability was satisfactory and the minor differences observed could be ascribed to retuning of the instrument in the intervening period. The fact that subtle differences could be identified gives confidence in

the power of the technique to detect similarities and differences between GM and non-GM materials.

7.3 Mass spectrometry

Introduction to mass spectrometry

A mass spectrometer is an instrument that measures the mass to charge ratio (m/z) of individual molecules that have been converted into ions, i.e. electrically charged. The sample to be analysed enters the vacuum chamber of the mass spectrometer through an inlet and is usually a connection from another separation system such as a gas chromatograph (GC/MS) or liquid chromatograph (LC/MS). The sample is ionised and volatilised in the ion source and the gas phase ions are sorted in the mass analyser according to their m/z ratios. A detector collects the sorted ions and the ion flux is converted to a proportional electrical current. Finally, the data system records the magnitude of these electrical signals as a function of m/z and converts the information into a mass spectrum. Many different types of mass spectrometer are available with the variation being in the means of ionisation and the analyser (see Table 7).

TABLE 7: Summary of the mass spectrometer variants used in metabolomics research

MEANS OF IONISATION	*TYPE OF ANALYSER*
Electrospray Matrix assisted laser desorption ionisation (MALDI) Electron impact Chemical ionisation	Quadropole mass filters Quadropole ion traps Time of flight (TOF) Fourier transform ion cyclotron resonance (FT-ICR)

The various types of analysers differ in their resolving power, the accuracy with which m/z ratios can be determined and their throughput. For example, time of flight (TOF) analysers for GC-MS instruments permit rapid scanning (500 spectra per second) as opposed to the one spectrum per second achievable with quadropole mass spectrometers. Four out of the six contractors used mass spectrometry in their metabolomics studies and each used one or more different instruments with no two contractors using an identical instrument. As part of the G02006 project (Aberystwyth), the same GM and non-GM potato samples were analysed on different instruments in two research centres (Golm, Germany and Aberystwyth) and similar results were obtained. A similar observation was made in the G02001 project when the same potato extracts were analysed by LC/MS at the Scottish Crops Research Institute and the Institute for Food Research. Furthermore, the same variety of potato (Desiree) was used in the G02001 (SCRI) and G02006 (Aberystwyth) projects and similar metabolites at similar levels were found despite the different growing environments and the use of different instruments. Similarly, in the G02005 (Royal Holloway) project on tomatoes,

similar results were obtained when the same material was analysed on a quadropole instrument and a TOF instrument. Thus, data is transferable between instruments.

Instrument operating issues

A key issue with MS instruments is instrument tuning/calibration and cleaning. Different instruments have different requirements. For example, in the G02005 (Royal Holloway) project, a mass spectrometer operating in the electron spray ionisation mode with a quadropole ion trap was tuned prior to use on a daily basis and this was a relatively simple task. However, in the same project, a skilled operator was required to tune another instrument with the same ionisation mode but a time of flight analyser. Also, tuning had to be done several times per day. In terms of inlet cleaning, the first sample in every batch of runs was a hydrocarbon standard and inspection of the profile would enable the operator to establish if the inlet system required attention. Typically, a hydrocarbon profile would drop off in intensity towards the higher mass components when the inlet and front of the GC column became dirty.

In the G0O2006 (Aberystwyth) project, even greater attention was paid to optimising the sensitivity and selectivity of time of flight (TOF) instruments. As well as using retention time standards, quality control charts based on statistical process control were used on a daily basis. The objective of these charts was to ensure that chromatograms were not accepted which had lost low abundance peaks. This allowed chromatograms to be compared reproducibly and consistently. As part of the standard operating procedure for TOF instruments, up to 25% of the samples run on a daily basis were quality control standards of one sort or another. A key finding from this work on optimisation was that it is not possible to compare directly results obtained before and after column cleaning or instrument servicing without a further level of data pre-processing. A similar conclusion was reached in the G02001 (SCRI) project.

Variations in retention times in gas chromatography can result in misidentification of components even with mass spectrometry providing identification of components, as compounds with similar chemical structures will have similar mass spectra. One approach to this problem is to use retention time locking. This technique works through control of the column head pressure and calibrates the retention time relative to the head pressure. Retention time locking is particularly useful once a method is developed but is not convenient during method development. An alternative method is to use retention time calibration and the G02 contractors found this to be essential for quantitative studies. For this, a calibration mix is run with each batch of samples and is used to generate a relative retention time for analytes of interest.

Choice of system

Although most of the contractors undertook some LC-MS work, the general conclusion was that it was suitable for the analysis of particular plant

components, e.g. potato glycoalkaloids, but not suitable for global metabolite profiling. The reasons for this are:

- There are no established, validated and generally accepted retention index markers;
- LC-MS peaks are usually wider than GC-MS peaks thereby hampering chromatographic resolution;
- Peak finding and mass spectral deconvolution are inherently noisier in LC-MS;
- Ionisation is not carried out under standard conditions (unlike GC-MS) and, when coupled with matrix effects, means that the same molecule gives different spectra on different instruments;
- To date, all software solutions are incapable of fully extracting the information from MS/MS runs under positive/negative switching.

In undertaking metabolomic analysis, the investigator has two options:

- Molecules in a sample can be resolved from each other by chromatographic techniques (LC, GC) before they are measured in the mass spectrometer in order to develop a *metabolite (peak) profile*.
- The crude extract can be injected directly into the mass spectrometer without prior separation of metabolites to generate a *metabolite (ion) fingerprint*.

The advantages of fingerprinting techniques is that they are much more rapid and are not limited by problems associated with trying to separate individual molecules prior to detection. They thus produce a more comprehensive picture of total metabolite content. Consequently, in the G02006 (Aberystwyth) project, an assessment was made of the reproducibility and sensitivity of fingerprinting as a first pass screen for discriminating between GM and non-GM crops. This study showed that as far as potatoes are concerned, fingerprinting is indeed a very useful screening tool especially when GC-MS is used subsequently to identify the metabolites responsible for compositional differences. Work done outside the G02006 project has shown that the same protocol works in other species (*Arabidopsis*, tobacco and grasses).

Extraction protocols

As with NMR studies, the protocol for preparing extracts for mass spectrometry need to be tailored to the plant species being studied. All the contractors were aware of this and the preliminary studies undertaken by each covered such issues as choice of solvent, effect of repeated extraction of the same sample and the reproducibility of the extraction process when tested on aliquots of the same material. Generally the extraction methods were very reproducible but in the G02005 (Royal Holloway) project, it was noted that the efficiency of extraction of certain metabolites in tomatoes was much more variable than observed for the metabolite pool as a whole.

Derivatisation of metabolites

Many of the metabolites found in plant cells are not volatile and so cannot be analysed without first being derivatised. The key variables in derivatisation are:

- The efficiency of derivatisation
- The reproducibility of derivatisation
- The stability of derivatised metabolites.

In project G02005 (Royal Holloway) it was noted with tomato extracts that the variation in derivatisation for most compounds was less than 10% but one metabolite was found to have a variance of 23%. The stability of derivatised metabolites is particularly important since samples may be stored for up to 24 hours in autosamplers prior to analysis. Again, in the G02005 (Royal Holloway) project it was satisfying to note that all the derivatives appeared to be stable.

Interfering substances

With some plant tissues, certain metabolites are so prevalent that they mask or interfere with the detection of less abundant metabolites. For example, hexoses are very common in fruits and frequently cause problems. In the G02005 (Royal Holloway) project, a generic method was developed for selectively removing these sugars.

7.4 Reference materials

A major problem in metabolomics is the identification of the metabolites that are detected. The standard way of identifying unknown compounds is to compare their spectra with those of pure compounds stored in mass spectral libraries. The quality and breadth of mass spectral libraries are essential parameters if identification of metabolites is to be successful. If good libraries are not available then most of the compounds analysed will remain unidentified and the significance of their presence in plant extracts will remain unknown. Unfortunately, the classes of compounds for which spectra are lacking are the very compounds of greatest interest, e.g. secondary metabolites. For this reason, one of the projects (G02005 Royal Holloway) also involved the generation of improved mass spectral libraries. In total, spectra for 235 compounds derivatised for GC/MS and 149 compounds derivatised for LC-MS were determined.

Because most metabolomic approaches use different technology platforms (i.e. different models of mass spectrometer or NMR instrument), and these vary in their range of measured metabolites, accuracy, resolution, dynamic range and sensitivity, spectral libraries on their own are not enough. Reference materials also are required. The generation of reference materials was not part of any of the G02 projects but one of the G02 contractors (Rothamsted) now is generating a set of reference materials for *Arabidopsis* using funding from another source (BBSRC).

When the analysis of large numbers of experimental samples results in the detection of large numbers of unidentified metabolites there is a need for a system of nomenclature so that the presence of the same compound in different samples can be recognised. This issue was noted on many occasions during the G02 programme and three of the contractors contributed to a draft nomenclature system proposed by an international consortium of 14 laboratories (Bino et al 2004, *Trends in Plant Sciences* **9**, 418-425).

7.5 Quality issues

Metabolomics was in its infancy when the G02 programme was conceived. By focussing the G02 contractors on quality issues at an early stage in the programme, they collectively were able to identify the key sources of variability and to manage them. The principal sources of variability are:

- Efficient extraction of metabolites. The method used needs to be tailored to the plant material, the intended derivatisation step and the analytical instrument.
- The derivatisation step (GC-MS only) needs to be optimised and the stability of derivatised metabolites determined.
- The appropriate analytical instrument needs to be selected with careful thought being given to sensitivity and selectivity.
- The performance of the instrument needs to be assessed constantly, particularly where mass spectrometry is used, and appropriate internal and external standards used in a judicious manner. Two of the contractors (G02001, SCRI and G02003, Rothamsted)) also used reference extracts (from potato and flour respectively).
- Where large numbers of samples are being run it is important to ensure that ‘new’ peaks are shown to be genuine and are not the result of instrument drift. That is, data pre-processing is essential before data analysis is undertaken.

All these sources of variability have been discussed in more detail in earlier sections. In addition, Bino et al (*Trends in Plant Sciences* **9**, 418-425, 2004) have suggested a checklist of the information necessary to provide context for metabolomics data that is to be published. This is known as MIAMET (minimum information on a metabolomics experiment) and three of the G02 contractors participated in its preparation. MIAMET has been developed into a database (called ArMet) and has received wide endorsement from the plant metabolomics community.

7.6 Summary

Metabolomic profiles can be generated for plant extracts using NMR and mass spectrometry and both methods have been shown to be capable of detecting unintended effects in transgenic crops. Nevertheless, neither method is capable of separating/identifying more than a subset of the metabolites present in any cell or tissue.

NMR is much simpler to use than mass spectrometry but is less sensitive. Although NMR data collection is straightforward to perform once instrument set-up has been optimised, developing an optimised extraction procedure is

very time consuming and is an essential pre-requisite. A software package has been developed that permits multiple statistical analyses to be undertaken on data sets to highlight unintended effects.

Mass spectrometry is much more sensitive than NMR but places much greater demands on the analyst in terms of instrument calibration and maintenance, the need for an optimised derivatisation process (GC-MS) and the need for spectral libraries of a wide range of compounds. As with NMR, metabolite extraction is a key step and needs to be optimised. A mass spectrometry fingerprinting method has been developed that permits rapid screening of samples to identify those that deserve a more detailed examination.

The G02 contractors identified most, if not all, of the sources of variability associated with metabolomics and managed these variables in their experimental programme. Thus, the results generated by them are beyond question and thus only the interpretation of these results can be questioned.

8 DATA ANALYSIS

8.1 Overview of data analysis

With all three of the methodologies described above, each experiment produces large amounts of data. For example, an NMR spectrum of a single extract contains hundreds of peaks representing signals derived from bonds in common to many different metabolites. Similarly, a 2D electropherogram of a single sample can exhibit over 1000 protein spots and a microarray simultaneously examines thousands of different RNA species. Given that each experiment generates multiple samples, how does one analyse the data? Bearing in mind that the data for a single experiment can be generated over a period of months, the need for consistency in procedures and correction for machine drift become crucial quality issues.

Another important issue with the use of the ‘omics’ approach is that data comparisons cannot be made in the absence of a complete set of metadata (i.e. data about data). With this in mind, a key part of the G02006 (Aberyswyth) project was the development of a data model for plant metabolomics known as ArMet (architecture for metabolomics). This encompasses the entire experimental time line from experiment definition and description of biological source material, through sample growth and preparation to the results of chemical analyses. ArMet recognises that ‘omics data in general and metabolomics data in particular are expensive to collect and may be analysed in ways that were not originally imagined. The resulting data descriptions specify the full experimental context and enable:

- Comparison of data sets
- Proper interpretation of experimental results
- Permit exact repetition of experiments
- Provide a basis for the design of systems for data storage and transmission.

The use of ArMet has been supported by scientists from 20 institutes around the world (Jenkins et al 2004, *Nature Biotechnology* **22**, 1601-1606) and has been made an essential requirement by BBSRC for their new initiative on plant and microbial metabolomics.

The classical method of data analysis is a univariate one. It seeks to establish significant differences between positive and control group mean values, or to quantify the levels of random variation from these sources, on a compound by compound or peak by peak basis. In the G02 programme univariate statistics (ANOVA and t-tests) were used in only one project (G02002) and this required the development of a suitable data representation package (Metabolab). Other groups opted to use multivariate analysis ('chemometric methods') to test for 'substantial equivalence' of metabolic profiles. Simplistically, this involves:

- Combining in one set the metabolic profiles from GM and non-GM cultivars;
- Clustering this set on the basis of the similarity of the metabolic profiles;
- Determining if the GM cultivars consistently cluster with the non-GM cultivars (substantially equivalent) or cluster separately (non-equivalent).

The GM and non-GM samples should cluster only when the *expected* metabolic changes resulting from the transgene are removed from the data. Note that 'clustering' is also known as 'unsupervised learning' in machine learning literature and as 'classification' in statistical literature.

Many different statistical clustering methods exist. They differ in their assumptions about the form of the data and the type of similarity expected, and there is no best clustering algorithm. The clustering method that is best known, and used by all the contractors, is principal component analysis (PCA). This reduces the original data (intensities for hundreds of data points or metabolites for each sample) to a set of 'scores' on a much smaller number of principal component axes for each sample. The scores are first used in an exploratory way to look for clusters of similar samples (e.g. via scatter plots) and then in a more formal fashion to construct classification models. No attempt is made to pre-assign clusters: rather, they are generated directly from the data. One practical consequence is that a sample might be classified as 'very different' from the norm on the basis of its overall composition even though no single metabolite exceeds a prescribed safety limit.

Another issue with PCA is that it can give undue weighting to variables whose absolute values may be small but whose variance may be large. Such variables contribute only noise to the analysis and the effect of their removal needs to be examined. Suitable statistical methodology is available but its use requires the input of a statistician with a good understanding of the experimental objectives and methodologies of the G02 programme. This statistical input was available to four of the six contractors.

Although PCA is the most widely used method of clustering it does have a number of disadvantages including those noted above. Many other

multivariate data analysis methods based on discrimination of sample classes or sample class prediction are available. These include the use of machine learning and neural nets. Such supervised learning methods involve the use of training sets before being applied to test data. Another approach is Inductive Logic Programming (ILP) in which the examples, background knowledge and models are all described as computer (or logic) programmes. The advantage of ILP for clustering metabolomic data is that both chemical structure and toxicity data can be specifically included if desired. This is not possible with other clustering programmes. One contractor (Aberystwyth, G02006) had as a major part of their programme the objective of evaluating the utility of these alternative clustering methodologies and the delivery of a set of metrics that properly define substantial equivalence. They found that supervised learning methods such as Decision Trees and Discriminant Function Analysis were the methods of choice for comparison of metabolome data. These gave excellent classification/ discrimination accuracy and generated easily interpretable models of compositional similarity with clear and simple metrics. Many other data mining methods give good sample classification but fail to identify the metabolites responsible for the discrimination.

8.2 Generic data analysis

The statistician associated with the G02001 (SCRI) project was given the challenge of developing a generic method that could be used to analyse all the data emanating from 'omics' experiments. This he did but there are two caveats associated with his method. First, the use of standard operating procedures is essential. Second, experimental design is crucial. In particular, the aim of the experiment needs to be defined clearly with attention being given to defining each treatment and the number of levels, determining if interactions are important, sample sizes and randomisation.

The proposed generic method has three levels of analysis:

- Plotting of data;
- Analysis of individual features;
- Simultaneous analysis of all features.

It is recommended that the data be plotted using scatter matrix plots since these give an exceptional amount of information for little effort.

The second level of analysis involves an analysis of variance of individual features. These features include microarray probes, protein spots on a gel or particular metabolites. This analysis of variance is done by deriving the probability (p) values and the false discovery rate (expected percentage of false predictions). The cut-off value of p then is selected based on the false discovery rate.

PCA is recommended as the method for analysing all of the features simultaneously. However, it is essential to plot all scores against all scores and not just the first few principal components. Then the component loading and scores are plotted against the original features. The objective of this is to detect which combinations of features summarise the variation in the

samples. Once this is complete, cluster analysis can be performed to see if there are clear groupings of samples with similar features.

Similar conclusions about a generic approach to data analysis were made in the G02006 project at Aberystwyth and in the analysis of metabolomics data undertaken at the Central Science Laboratory as part of the G02002 (JIC) project. Standard operating procedures for data analysis have been produced at Aberystwyth and now are being evaluated with additional datasets as part of the BBSRC Plant and Microbial Metabolomics initiative.

8.3 Summary

A range of statistical methods and data analysis tools has been evaluated for their suitability for analysing the very large and complex datasets produced when 'omics techniques are used for the safety assessment of novel foods. A suitable generic method of data analysis has been developed which can be supplemented by additional procedures. A format for the trapping of essential data also was developed and now has been accepted by a broad range of scientists involved in plant metabolomics.

9 CONCLUSIONS FROM METHOD DEVELOPMENT PORTION OF THE G02 PROGRAMME

A number of methods for determining junction sequences have been assessed and a suitable generic method has been identified.

A number of methods are available for analysing gene expression. Where suitable commercial microarrays are available (e.g. Affymetrix chips) these are the method of choice. Although they are expensive, they are convenient and relatively easy to use and are manufactured to known quality standards. By comparison, the generation of specific cDNA arrays is extremely labour intensive and time consuming and quality assurance is a major issue. SAGE also is time consuming and labour intensive but probably requires less effort and has fewer quality issues associated with it than cDNA arrays. Consequently, SAGE is the preferred alternative when commercial microarrays are not available.

For proteomic analysis there currently is no alternative to 2D-gel electrophoresis. It is labour intensive and has a well-deserved reputation for lack of robustness. However, it can be made very reproducible but this demands very careful attention to detail. Two key variables are the choice of extraction method and the choice of stain. Interpretation of the results of 2D-gel analysis requires advanced software and two suitable versions are Phoretix 2D-Expression and ProteomWeaver.

Both NMR and mass spectrometry are suitable for metabolomic analysis. NMR is relatively simple and quick and long-term instrument stability is not a problem. However, it is much less sensitive than mass spectrometry. Many different kinds of mass spectrometer are available and usually they are coupled to other separation systems such as GC or HPLC. The stability of

these hyphenated systems can be problematical. Shifts in sensitivity after column cleaning or instrument servicing and instrument drift are particularly common and rigorous quality assurance systems need to be applied if meaningful data is to be generated. Direct injection of unfractionated extracts into an electronspray mass spectrometer can yield very useful metabolic fingerprints that may provide sufficient data for many applications. This high throughput method can be supplemented with other forms of mass spectrometry as appropriate. Whatever method is used (NMR or MS), great care needs to be exercised in selecting a sample extraction procedure and randomisation of samples prior to analysis is essential.

A generic method of data analysis has been developed which should be suitable for most applications. However, the use of this method does require a detailed understanding of multivariate statistics and in most cases will demand the involvement of a professional statistician. Where samples can be shown to be different, it is essential to determine the identity of the factors causing the difference. This is possible with only a limited number of data analysis procedures. Also, a distinction needs to be made between separations caused by a large shift in concentration of one factor versus those caused by a large number of small changes in many factors.

SECTION C: ANALYSIS OF CONTRACTORS' RESULTS

10 CHOICE OF TEST COMPARATORS

All the contractors made comparisons between their specific transgenic crops and relevant controls. These were either null-segregant controls that were identical to the genetically modified line but had segregated without the transgene or empty vector controls. The inclusion of such controls is essential in order to rule out effects induced by tissue culture and the transformation process. In many cases the contractors also tested the parental lines from which the transgenic and null-segregant lines were derived. Some contractors also tested the extent of natural variation by including a variety of commercial cultivars in the test samples.

If the methods developed during the G02 programme are to be used in the future for the safety assessment of GM foods then it will be essential that the proper comparators be used. With crops that reproduce vegetatively, comparative analyses would include the parental variety used to produce the transgenic line. With crops that reproduce sexually then the comparators should be the near-isogenic lines. Where F1 hybrids are used, analyses will need to be made on backcrossed breeding material.

11 ANALYSIS OF TRANSGENIC POTATOES AT THE SCOTTISH CROPS RESEARCH INSTITUTE (PROJECT G02001)

11.1 Transgenic material

The GM potato lines used in the G02001 (SCRI) project are shown in Table 8.

TABLE 8: Potato lines derived from the cultivar Desiree

LINE	DESCRIPTION
W2	Contain a gene for a fungal glucan-branching but this is not expressed in the potato lines used because of co-suppression.
MAL 1 sense	Potato MAL 1 gene (encoding α–glucosidase) in sense orientation under the control of a CaMV promoter. Plants have no obvious phenotype.
MAL 1 antisense	Potato MAL 1 gene in antisense orientation under the control of a CaMV promoter. Plants have the following phenotype: Extremely stunted in the field but less so in containment; reduced activity of glycoprotein type II processing enzyme in tubers; distinctive changes in leaf morphology caused by changes in cell wall structure.
SAMDC	Potato S-adenosylmethionine decarboxylase gene in sense (patatin promoter) or antisense (patatin or CaMV promoter) orientation. Antisense constructs under the control of the CaMV promoter had a stunted phenotype with reduced tuber numbers and dry matter content due to modified ethylene/ polyamine metabolism following down regulation of SAMDC activity. Sense constructs showed increased tuber numbers.
SAMTET	Potato S-adenosylmethionine gene under the control of the tetracycline-resistance promoter. These lines were not exposed to tetracycline at any time and can be considered as controls.

Some of these lines contain genes whose expression would be expected to modify key metabolite biosynthetic pathways in potato tubers (c.f. the potato constructs used in the G02006 (Aberystwyth) project).

The control lines used included wild-type tubers, tubers generated from non-GM plants produced via tissue culture and transgenic tubers transformed with empty vectors.

A number of non-GM varieties of potato also were studied in the G02001 project in order to get an understanding of the natural variation in metabolites that can be expected. These varieties included a number of landraces originating from Chile that pre-date any alien gene introgression and 14 cultivars of maincrop potatoes of known pedigree.

11.2 Transcriptomic analysis

The approach used in the G02001 (SCRI) project was to detect changes in gene expression using microarrays developed in-house and then to confirm any significant changes in expression using quantitative RT-PCR and the proprietary Luminex system. Both intended and unintended effects were detected using the microarrays but the magnitude of the changes was relatively small. The GM line that showed the greatest transcriptomic changes was line 47, a SAMDC antisense line, and this line also was picked out as different in metabolomic studies (see next section).

11.3 Metabolomic analysis

In the G02001 (SCRI) project, metabolomic analysis was undertaken using NMR, HPLC/MS and GC/MS. Both intended and unintended metabolomic effects were detected. The utility of the methodology was shown by one unintended effect involving field plot 4 in year 2001. All lines, GM and non-GM, grown in this plot in 2001 differed from those in neighbouring plots but this effect was not noted in other years. This effect presumably arose because of inadvertent differences in fertiliser application.

NMR analysis

Analysis of the metabolomics data generated by NMR showed that the MAL1 antisense lines and the SAMDC antisense line 47 consistently were distinguished from the rest of the samples analysed. The changes in these lines were distributed across a wide range of compounds and in each case the magnitude of the change was small (twofold or less). In general, the changes seen were smaller in effect for plants grown under controlled conditions compared with those grown in the field. Also, for field grown potatoes the magnitude of the effects varied by year of growth. Both these observations confirm that, as expected, weather affects the metabolomic profile.

Principal components analysis of the NMR data from the different cultivars and landraces showed that the Desiree lines (transgenics and controls) separated from the other varieties which in turn could be sub-divided into breeding varieties and modern varieties. Contrary to expectation, no new metabolites were detected by NMR but some commercial cultivars contained up to seven times more glucose than Desiree.

HPLC/MS analysis

HPLC/MS analysis was used to generate metabolomic profiles for all the non-GM potato varieties grown in the field in 2002. This revealed extensive variations in metabolite concentrations. All the compounds measured showed at least a 5-fold range in mean values across the 26 varieties studied. If individual measurements are taken into account, as opposed to mean values, the differences were even greater. Since these results pertain to a single region of Scotland in just one year, the true extent of natural variation probably is much greater.

Detailed analysis of control samples of the Desiree cultivar showed that the within-cultivar variation is much less than the between-cultivar variation. This was expected. From the 150 control samples analysed, the standard deviation of values for individual metabolites was in the order of 20-30% of the mean for major peaks although this could rise to 60% for minor peaks.

Relatively little extra variation was observed when transgenic lines were compared with controls. In most cases the differences were less than twofold. Only SAMDC antisense line 47 showed any changes of substance and most of these were two- to fivefold higher levels of certain metabolites such as caffeoyl and feruloyl putrescine, dihydrocaffeoyl polyamines and certain flavonols and glycoalkaloids. Since SAMDC is involved in polyamine biosynthesis, some of these changes were expected but others can be considered as unintended. Although the glycoalkaloid content of line 47 was enhanced this would not be of concern since the parent strain naturally has a lower glycoalkaloid content than other varieties.

Four new potato metabolites were detected by HPLC/MS analysis in all the GM and non-GM lines tested. These were identified as bis- and tris-dihydrocaffeoyl derivatives of the polyamines spermidine and spermine. The bis-derivatives, also known as kukoamines, have been reported from only one other plant (*Lycium chinense*) while the other two have never been reported. Compounds of this type are known to have pharmacological effects.

GC/MS analysis

As with NMR and HPLC/MS analysis, GC/MS analysis of polar compounds highlighted line 47 as being significantly different from the other lines. The levels of several amino acids were elevated and some of these changes could be explained by the ‘defect’ in SAMDC synthesis. As such, these changes can be considered as intended. However, there was no obvious explanation for some of the other changes and these must be considered as unintended.

Although the levels of some amino acids were elevated in line 47 when compared with controls, most were within the range of natural variation seen in the various cultivars and landraces examined. However, the levels of some (asparagines, phenylalanine, and tyrosine) were either at the extreme high end or even beyond the range of natural variation.

11.4 Summary of G02001 results

Transcriptomic and metabolomic analyses successfully detected intended and unintended effects in transgenic potatoes. However, in most cases the magnitude of the changes was small, even in a line with a defect in a key biosynthetic step (polyamine biosynthesis), and seldom exceeded twofold. By contrast, the range of natural variation in metabolite levels seen in landraces and common cultivars was much greater and frequently exceeded fivefold. This natural variation was measured in plants grown in one location in one season and when environmental variation is taken into account is likely to be even greater.

Analysis with NMR and GC/MS detected no new compounds but four novel polyamines were detected in the various cultivars and landraces of potato by HPLC/MS.

Data suggested that somaclonal variation induced by passage through tissue culture rather than transgenesis per se was responsible for some of the cluster separations observed between the wild type Desiree, GM and control lines (vector only and tissue culture derived lines). Glycoalkaloid levels were generally lower in these lines compared with the wild types. The latter observation also was made in the EU GMOCARE project using targeted analysis and using plants grown under containment in different years.

12 ANALYSIS OF TRANSGENIC WHEAT AND BARLEY AT THE JOHN INNES CENTRE (PROJECT G02002)

12.1 Transgenic material

The plants used in the G02002 project consisted of barley and wheat lines that had been transformed with one or more of the following marker genes: Luc (luciferase), Gus (glucuronidase), Hyg (hygromycin resistance), Bar (herbicide PPT resistance) plus one of the following ‘test’ genes: glucoamylase, phytase, low molecular weight gluteninin, green fluorescent protein or the DapA gene (involved in leucine biosynthesis).

12.2 Analysis of junction sequences

Analysis of the junction sequences in 13 barley lines and 10 wheat lines showed that in most of them the transgene was inserted into gene-rich regions or retrotransposons. This result does not necessarily mean that transgenes are more likely to insert into gene-rich regions. Any transgenes inserted into inactive regions of the genome would be silenced and therefore the lines containing them never isolated.

A range of different genes was found to have been disrupted and a number of these genes were identified. For example, transgene insertions into disease resistance loci were seen and these could have implications for plant breeders considering use of the material. Another transgenic line had a disruption in a gene involved in iron metabolism and could have implications for the nutritional quality of any food derived from it. Similarly, disruption to seed storage protein loci could have implications for nutritional quality and the functional properties of grain.

In addition to providing information about the genomic regions flanking transgene insertions, the analytical method utilised permits the determination of the precise insertion of the *Agrobacterium* T-DNA at the right border. This is of importance in safety assessments because of concerns over the presence of additional unwanted sequences within transgenic lines. None of the *Agrobacterium*-mediated barley lines contained any sequence from outside of the T-DNA at the right border. By contrast, five out of eight wheat lines had up to 150bp.

12.3 Transcriptome analysis

Transgenic barley line HC12 carries two reporter genes *(luc* and *bar*) and a fungal glucoamylase gene under the control of a wheat α–amylase promoter. SAGE was used to compare the transcripts found in line HC12, its non-GM parent and a null segregant. Transgene expression was detected unambiguously in line HC12 and could be quantified. More importantly, the gene expression profiles in the transgenic plants differed from the two controls with 58 genes showing a >5-fold difference at a significance level of $P<0.001$. The function of most of the genes whose expression has changed is known and at this stage there is no obvious reason for the changes. However, in line HC12, the transgene has inserted into the chalcone synthase gene and the presumption must be that the changes are due to the resultant changes in metabolite fluxes.

Some of the changes in gene expression in line HC12 deserve further comment:

- The magnitude of the changes observed can be very large. For example, the expression of one protein (putative phospholipid transfer protein) was increased 30 fold and that of two proteins (globulin-2 precursor and oleosin 2) was reduced by 100 fold.
- The expression of a protein belonging to the BetvI allergen family was increased 15-fold.
- The expression of two storage protein precursors was significantly down regulated and this was mirrored by decreased amounts of the storage proteins in the cereal grain.
- The expression of the gene for 2-oxoglutarate 3-deoxygenase was increased 18-fold. This enzyme is part of the anthocyanin biosynthetic pathway, as is chalcone synthase whose gene is the site of transgene insertion.

The Affymetrix barley gene chip was used to analyse two additional transgenic lines, HC1 and HQ2A. The analysis showed that expression levels of the probe sets corresponding to the transgenes used in both lines were highly significant. This highlights the ability of the microarray approach to detect the expression of transgenes even when they are expressed at low levels. This is especially important given that the *luc* gene product could not be detected in extracts of line HC1. The other transcripts in lines HC1 and HQ2A did not appear to differ more than twofold from the controls and hence were not considered of biological significance. However, the Affymetrix arrays carry probes for only about 45% of barley genes and changes may have occurred in transcripts whose sequences are not represented.

12.4 Protein analysis

SDS-PAGE analysis of seed storage proteins did not reveal any novel proteins formed as a result of transgene insertion in any of the lines but this is not the most sensitive analytical procedure. Changes in intensity of many protein bands were seen between most of the transgenic lines and their corresponding controls. In some cases there were changes in all of the storage proteins whereas in others there were changes in individual bands. These changes are of no significance from a food safety point of view but would be of concern to plant breeders.

12.5 Metabolomic analysis

Analysis of both barley and wheat lines clearly showed that there was far greater variation between the metabolite profiles of the transgenic and null segregant plants when they are compared to the wild type control than when compared to each other. Nevertheless, line-specific changes could be seen in metabolites between transgenic plants and their corresponding null segregant controls, particularly in barley, but almost all of them were in levels of common metabolites, e.g. alanine.

A metabolic marker was found in barley that appears to be related to the presence and expression of the *bar* gene as it was found only in transgenic plants. The *bar* gene encodes the PAT protein that confers resistance to the herbicide PPT. It was not possible to establish the identity of this metabolic marker. Nor was it found in transgenic wheat lines expressing the *bar* gene.

Overall, there were few differences in the metabolome that could be ascribed to the presence of the transgene. This was particularly the case for wheat and it could be that the redundancy afforded by the hexaploid wheat genome might buffer the effect of transgenesis when compared with the diploid barley genome.

12.6 Summary of G02002 results

Analysis of junction sequences showed that in most of the transgenic lines that were selected that insertion had occurred in gene rich regions and had led to gene disruption. None of the barley lines had any DNA from outside of

the T-DNA at the right border whereas five out of the eight wheat lines did have extraneous DNA. These findings are important for safety assessment of transgenic crops.

Transcript analysis was undertaken on only a limited sub-set of the transgenic lines. SAGE showed that in one line there were 58 genes whose expression was up- or down-regulated more than 5-fold with some genes showing changes as great as 100-fold. As yet, no explanation can be provided for these changes. Use of Affymetrix arrays with two other lines did not identify any significant changes in gene expression except for the transgenes themselves.

Analysis of seed storage proteins showed changes in the intensity of all protein bands in some lines and changes in individual protein bands in other lines. These changes are not significant in the context of food safety.

Metabolomic analysis using NMR showed that:

- There are far greater differences between transgenic lines and their corresponding null segregants when compared to wild type controls than when compared with each other. Thus somaclonal variation accounts for most of the metabolomic changes rather than the transgenes themselves.
- No noteworthy changes in metabolites were seen.
- The presence of the *bar* gene can be detected in barley but not wheat by analysis of the metabolome but the identity of the metabolite detected could not be determined.
- The presence of the *bar* gene can be detected in barley but not wheat by analysis of the metabolome but the identity of the metabolite detected is not known.
- It is possible that the ploidy of the host genome could influence the extent of the changes seen in transgenic plants since the changes observed in hexaploid wheat were far less than those seen in diploid barley.

13 ANALYSIS OF TRANSGENIC WHEAT AT ROTHAMSTED RESEARCH (Project G02003)

13.1 Transgenic material

The wheat lines tested had been genetically manipulated to alter their glutenin composition and hence bread-making properties. The lines tested were:

- 4 GM lines (B73-6-1, B102-1-2, B102-1-1, B72-8-11b)
- 1 null segregant (B72-11a)
- 2 non-GM parents (L88-6, L88-31)

13.2 Proteomic analysis

One-dimensional electrophoresis techniques including capillary electrophoresis were tested but found to be unsuitable for the kinds of analysis that were deemed necessary for wheat flour. Successful comparative

imaging of replicate 2D - protein gels from GM and non-GM lines was seen as more important for substantial equivalence work than building a catalogue of protein identifications by mass spectrometry. This led to the development of a standard operating procedure (SOP) that gave highly reproducible gels as described in section 6.3.

The 2D-gel SOP has been applied to the comparison of lines B73-6-1 (GM) and L88-6 (non-GM) grown in two locations (Long Ashton, Bristol and Rothamsted, Hertfordshire). At the time of writing this report the analyses were still on going but sufficient data was available to make a preliminary analysis. Initial attempts to investigate the data using Principal Components Analysis (PCA) suggested that no obvious patterns existed. Since PCA is an unsupervised method, an attempt was made to model the data using the supervised method PLS-DA (Projection to Latent Structures – Discriminant Analysis). This supervised method was able to separate the transgenic line from the non-GM control and the protein spots responsible for the differences have been identified. The identity of these protein spots will be determined by mass spectroscopy of tryptic digests.

13.3 Metabolomic analysis

NMR analysis showed that polar extracts of white flour were much less complex than green tissue extracts. The major components of white flour were carbohydrates and, at a much lower level, amino acids. For any one line the metabolic profile of flour varied by site and by year. These environmental effects generally were greater than that seen between GM and non-GM lines for a particular year and site.

Based on NMR analysis of flour, three of the GM lines and the null segregant showed the same metabolite levels as the non-GM parents whereas the fourth GM line (B73-6-1) consistently was different. Analysis of the data indicated that increased levels of maltose were responsible for line B73-6-1 separating from the other lines on PCA. Based on NMR analysis of green extracts, all the GM lines except B73-6-1 presented the same metabolic profile as the parent However, the two parent lines differed from each other in their sucrose levels and line B73-6-1 exhibited decreased levels of glucose, glutamine and γ–butyric acid.

NMR fingerprinting is an attractive technique because it takes contributions from all of the extractable hydrogen-containing compounds. The disadvantage is that fingerprints are dominated by major metabolites and this is an issue with wheat flour. Consequently, flour samples from one harvest (year 2000) were examined by direct infusion of extracts into an electronspray mass spectrometer. In contrast to the results obtained by NMR spectroscopy, PCA analysis did not show any differences between material derived from the two sites. However, differences were seen when PLS-DA was used and these differences were due to changes in the levels of various sugars (glucose, maltose/sucrose, raffinose, gluconic acid and galacticol/sorbitol). As with the NMR analysis, transgenic line B73-6-1 was not equivalent to its parent line

and sugars plus some unidentified ions were responsible for these differences.

Some targeted analysis (amino acids) was undertaken by GC/MS. This did not reveal any differences between GM and non-GM lines.

13.4 Summary of G02003 results

Proteomic and metabolomic analysis has revealed unintended effects in white flour in one out of four transgenic wheat lines genetically engineered to have a different glutenin content.

Metabolomic analysis showed that the GM line that was found to be different from its parent had higher sugar levels. However, the differences between this GM line and its parental control generally were smaller than that seen between sites or between years for any particular line whether it was GM or non-GM.

14 ANALYSIS OF TRANSGENIC BRASSICAS AT THE INSTITUTE OF FOOD RESEARCH (Project G02004)

14.1 Introduction

Project G02004 differed from the other projects in that a principle objective was to use the 'omics techniques to analyse a number of independent lines carrying the same transgenes but with vastly differing levels of expression. This work was undertaken in the model plant *Arabidopsis thaliana*. This plant, which is a Brassica, was selected because its genome had been sequenced thereby making a lot of molecular data available. A second objective was to determine the transferability of the methods to another Brassica species (broccoli) that is grown as a crop. However, as only two generations of the transgenic broccoli could be produced, little progress was made with this second objective.

14.2 Transgenic material

The model transgenes used in *Arabidopsis* were as follows:

- The *E. coli* GUS (β-glucuronidase) gene. Synthesis of GUS was expected to have no effect that was predictable, as there is no known substrate for GUS in plant cells. That is, it should be a neutral transgene.
- The *Pseudomonas fluorescens* HCHL (hydroxycinnamoyl-CoA hydratase/lyase) gene. Expression of this gene was expected to generate a strong pleiotropic response because of its effect on key intermediates in the phenylpropanoid pathway. Changes to lignin and cell wall biosynthesis and flavonoid and anthocyanin synthesis were expected.
- The *Datura stramonium* ODC (ornithine decarboxylase) gene. The sole function of ODC is to decarboxylate ornithine to putrescine. Putrescine

is the precursor for polyamine biosynthesis, a biosynthetic pathway with an extremely robust homeostatic control system.

- The mammalian SSAT (spermine/spermidine acetyltransferase) gene. SSAT does not exist in plants and this construct was included as a test of the 'omics techniques.

All work was undertaken with homozygous T3 generation transgenic plants and their corresponding null segregants. The genetic transformation method used was the floral dip method since this avoids the need for tissue culture and the associated problem of somaclonal variation.

14.3 Effect of light on gene expression

Analysis of microarray data for HCHL, ODC and SSAT control plants suggests that light has a major effect on gene expression. There were relatively few changes in transcript levels when the ODC and SSAT control plants were grown in temperature controlled greenhouses with similar light levels. By contrast, when the HCHL control plants were grown in the same greenhouse when the light levels were much higher there was a 5- to 10-fold increase in the number of genes whose transcript levels were significantly changed. Many of these transcripts are derived from genes involved in stress responses.

14.4 Analysis of transgenic plants expressing GUS

A total of 68 lines expressing GUS were isolated and a line with an intermediate level of expression was selected for proteomic analysis. No new proteins other than GUS were detected but different isoforms of GUS were observed.

14.5 Analysis of transgenic plants expressing HCHL

Eleven different transgenic lines of *Arabidopsis* carrying the HCHL gene were isolated. Northern blotting was used to determine the level of HCHL expression and 6 lines were selected for further study. One of these lines had no detectable expression of HCHL and the others varied in their expression level by a factor of ~50.

Transcriptomic analysis showed that in the line that did not express HCHL the levels of two transcripts were altered, one up regulated and one down regulated. The two lines showing the highest level of expression of HCHL had changes to 636 and 318 transcripts. The other three lines with intermediate levels of HCHL expression had changes in the levels of 45-65 transcripts.

Proteomic analysis was undertaken on two of the HCHL lines, one expressing intermediate levels of HCHL and one expressing high levels. Some proteins showed a clear correlation between increases in steady state mRNA abundance and increases in protein abundance. Others showed no changes at the transcript level but clear changes at the protein level. This could be due

to differences in the rates of RNA and protein turnover. As with the GUS constructs, multiple isoforms of HCHL were observed.

The presence of HCHL was expected to result in the formation of hydroxybenzoic acids and four such compounds were detected, the levels being proportional to the level of expression of the HCHL gene. However, one of these hydroxybenzoic acids was a novel glycoform and is an example of an unintended effect. Metabolomic analysis also showed:

- There was an inverse correlation between levels of the hydroxybenzoic acids and the levels of flavonoids and sinapoyl malate. The latter compound is the major 'sunscreen' for the chloroplast and reductions in its concentration would be expected to trigger stress responses:
- There was an increase in indole glucosinolates in the transgenic plants;
- The amino acid profiles of the more strongly expressed HCHL lines were markedly perturbed

14.6 Summary of G02004 results

No unintended proteomic effects were seen in transgenic *Arabidopsis* plants expressing the 'neutral' gene GUS but this needs confirmation with transcriptomic analysis.

In plants expressing a gene for a novel enzyme (HCHL) that is metabolically active, the extent of the changes in RNA and protein species and metabolites is directly proportional to the level of expression of the transgene. The activity of the HCHL led to the synthesis of new metabolites in *Arabidopsis*.

Changing light levels can lead to very significant differences in the numbers of genes expressed.

15 ANALYSIS OF TRANSGENIC TOMATOES, POTATO AND SOYA AT ROYAL HOLLOWAY COLLEGE (Project G02005)

15.1 Introduction

The G02005 study was the smallest of the six projects that were funded. Most of the effort in this project was expended on method development rather than analysis of transgenic crops *per se*.

The analysis of tomato lines was to provide answers to the following questions:

- Does the transformation process alone affect metabolic profile of tomato fruit?
- Does the production of a latent (non-functional) enzyme affect the metabolic profile of tomato fruit?
- Does manipulation of a biochemical pathway affect unrelated pathways?
- Does the level of transgene expression have an effect on unrelated metabolites?

- Does the number of transgene inserts affect the metabolite profile of tomato fruits?
- What is the extent of biological variation in tomatoes?

The analysis of potato and soybean lines was to assess the transferability of the analytical methods that had been developed for use with tomatoes.

15.2 Transgenic material

For studies on tomatoes a variety of well-characterised lines with enhanced nutritional properties were used. These lines had altered levels of carotenoids, tocopherol, phytosterols and flavonoids, compounds that are derived from the common precursor isopentenyl diphosphate.

The potato lines used carried carotenoid biosynthetic genes as the effector genes designed to convey phenotype.

Soybean analysis was done on herbicide-resistant (Roundup Ready) and herbicide-sensitive varieties.

15.3 Metabolomic analysis of tomatoes

A number of different lines and their corresponding null segregants were analysed using mass spectrometry. Principal components analysis showed that the null segregants and the transgenic lines cluster as two groups close together under many different plots within the PC matrix. These results suggest that the differences are small and that no one compound is responsible for the separation of the groups. Unfortunately, in this study no comparison was made with the corresponding parental (wild type) line(s).

Analysis of two lines that differ only in the level of expression of the transgene (2-fold difference) did not reveal any significant differences in metabolic profile. Similarly, the presence of one or two copies of a transgene did not make any significant difference to the metabolomic profiles obtained.

When the metabolomic profiles for any one line were examined the spread of results was large. This could be due to natural variation or analytical variation. Further analysis showed that there was considerable variation in the profiles of tomato fruits from different plants but that the within-plant variation was low.

Two different statistical analyses indicated that the compounds responsible for most of the differences observed were seven amino acids (alanine, valine, leucine, glycine, serine, beta-aminobutyric acid and aspartic acid).

15.4 Metabolomic analysis of potatoes and soybeans

The objective of the potato and soybean analysis was to determine how easy it would be to transfer the GC/MS profiling method developed for tomatoes. The outcome was that transference was straightforward but two issues are worth noting:

- With potatoes, the differences between individual tubers are as big as the differences between lines.
- With soybeans, the method of sample preparation greatly affected the outcome. When samples were prepared by grinding with a pestle and mortar, analysis of GC/MS data indicated that samples did not cluster relative to type. When a different method of preparation (freeze milling) was used the samples now clustered as expected.

15.5 Summary of G02005 results

The GC/MS methods developed for profiling transgenic tomatoes were capable of detecting unintended effects but the changes observed were small and due principally to alterations in the levels of amino acids.

The profiling methods used for tomato analysis were easily transferable to soybeans and potatoes but care had to be exercised in the extraction protocol to avoid meaningless results.

Between-plant variation was much greater than within plant variation.

16 ANALYSIS OF TRANSGENIC POTATOES AT THE UNIVERSITY OF WALES, ABERYSWYTH (Project G02006)

16.1 Project objectives

The aim of this project was to determine which combination of metabolomic analytical routines, coupled with bespoke data storage and advanced data analysis was the most appropriate for routine use to demonstrate 'substantial equivalence' for food raw materials. Initially it was planned to use coulometry and mass spectrometry but the former technique was dropped at an early stage because of the lack of an adequate database of standard spectra and general problems with peak resolution and reproducibility of HPLC when using a complex matrix.

16.2 Transgenic material

The lines analysed were derivatives of the potato cultivar Desiree that had been engineered to produce fructans and inulins because of the potential beneficial effects on gut microflora. The transgenic lines fell into two classes:

- Single transgenic lines carrying a gene for the enzyme sucrose:sucrose transferase (SST) that results in the production of a trisaccharide (1-kestose) and oligo-fructans up to 5 degrees of polymerisation.
- Double transgenic lines carrying the gene for SST and fructosylfructose transferase (FFT), the latter encoding an enzyme that converts kestose and fructans to inulins.

In addition to the transgenic lines, analyses also were done on a range of conventional cultivars (Agria, Granola, Linda and Solara) and two Desiree lines (one propagated via tubers and one via tissue culture).

16.3 Metabolomic analysis of potatoes using electrospray mass spectrometry

Principle components analysis (PCA) of electrospray mass spectrometry (EMS) data showed that three major genotype classes (meta-classes) could be identified: cultivars, single transgenics (SST) and double transgenics (SST + FFT). These results suggest that the transgenic lines have distinct differences, both from each other and from the cultivars at the level of the metabolome. It was not unexpected since the transgenic lines had been engineered to produce fructan molecules that are not known to occur naturally in potatoes.

Discriminant function analysis (DFA) of the same data as used for PCA revealed a better separation of the individual classes. Specifically, double transgenics were well separated from single transgenic, which clustered close to the progenitor Desiree genotype. Furthermore, all the cultivars, including Desiree, were well separated from each other indicating that each has a distinct metabolome. Of particular importance is the fact that these separations were seen when data from two different harvests (years 2001 and 2003) were analysed independently. Similarly, identical patterns of discrimination were seen when the same material was analysed on two different instruments operated by different individuals in different countries. These results are the best proof of the transferability of mass spectrometry methodology but it is essential to note that transferability is dependent on the use of appropriate quality assurance and quality control procedures.

When the EMS data was analysed using confusion matrices the classifications obtained were similar to those seen using DFA. Different single transgenic lines were confused with each other and with their progenitor genotype Desiree, indicating their similarity in metabolomic content. Within the group containing the conventional cultivars, significant confusion occurred only between the two Desiree genotypes. This again suggests that each cultivar has a distinct metabolome.

The transgenic potato lines were expected to contain new metabolites such as fructans and inulins that would have a strong influence on the classifications obtained using PCA and DFA. This raised the question as to the separation that would be achieved when these new metabolites were removed from the dataset. Analysis of all the metabolites whose concentrations had changed significantly in different samples showed that only a very small number made a major contribution to the differences in the metabolomes of GM and non-GM plants. Those that did make a difference were found to be fructans. When these metabolites were removed from the dataset the separation of the GM and non-GM genotypes was greatly reduced but there still was a general grouping into cultivars, single transgenics and double transgenics. This suggested that further metabolic differences could

exist that might be revealed only by a more comprehensive profiling method such as GC/MS.

16.4 Comparison of GM and non-GM potatoes using GC/MS

Extensive analysis of data sets revealed that, as with EMS fingerprinting, profiles obtained on material from different harvests and on different instruments (GC-TOF and GC-quad) were comparable. GC/MS also confirmed that the transgenic lines have distinct differences, both from each other and from the cultivars at the level of the metabolome.

The highest-ranking metabolites that represent differences between traditional cultivars were found to be principally specific amino acids that have been linked to flavour development during cooking as well as the major sugars glucose, fructose and sucrose. When comparisons were made between GM and non-GM lines the major discriminants were fructan-containing disaccharides and trisaccharides. The advantage of GC/MS is that it was able to differentiate between disaccharides such as levanbiose, inulobiose and sucrose that EMS cannot. Only the first two of these compounds was found in GM lines. Similarly, GC/MS was able to distinguish between the trisaccharide fructans and raffinose, the latter being found in all potatoes.

When the all the fructans were removed from the GC/MS dataset, analysis showed that it was very difficult to discriminate the GM lines from the progenitor Desiree cultivar whereas all the different cultivars separated clearly. This result confirmed that the side effects of the genetic transformations could be localised to the low molecular weight fructans that arise from the enzymic activities resulting from transgene expression. That is, the transgenic potatoes are substantially equivalent to the non-transgenic parent.

16.5 Glycoalkaloid levels in transgenic potatoes

The metabolomics analyses described above strongly suggested that fructans were the only novel metabolites in the GM lines examined. However, the inability of current analytical methodology to identify all the metabolites in a sample meant that changes in the levels of known toxins could not be ruled out. Since glycoalkaloids are the major toxins present in uncooked potatoes, an analysis was made of the glycoalkaloid levels in the various lines using appropriate methodology. The levels of glycoalkaloids found in the GM crops were within the ranges seen in non-GM cultivars.

16.6 Summary of G02006 results

A hierarchical metabolomics approach was used successfully to separate GM potatoes (carrying genes for fructan and inulin synthesis) from non-GM potatoes although single transgenic lines (fructan synthesis only) were very similar to the parental Desiree cultivar.

The transgenic potato lines were no more different from the parental Desiree cultivar than the different cultivars were from each other.

The metabolites responsible for the separation of transgenic lines from the parental cultivar were shown to be a range of fructans. When these fructans were removed from the datasets the transgenic lines clustered with the parental cultivar.

Different methods of generating potato metabolomics data (electrospray MS fingerprinting, GC/MS profiling) and different methods of data analysis (Principal Components Analysis, Discriminant Function Analysis, etc) gave essentially the same results.

No unintended changes in alkaloids were detected.

SECTION D: IMPLICATIONS OF G02 PROGRAMME FOR THE FOOD STANDARDS AGENCY

17 SAFETY ASSESSMENT OF GM FOODS

17.1 Introduction

An evaluation of a GM crop involves comparison with the parental line as a starting point for assessment of safety. This not only provides a comparator with history of safe food use but also gives a basis for assessing the intended modification. Once differences are identified and quantified, a further safety assessment would be carried out. This would include studies of allergenic potential, toxicology and the nutritional assessment of the overall composition of the plant. As an example, consider one of the GM potato lines used in the G02006 project (but note that they were not intended for commercial production). One of these lines was modified to synthesise relatively high levels of inulin. In addition to any safety studies, an assessment of this line would have to take into account any potential effects related to increased dietary intake of inulin. Given the perceived prebiotic effect of inulin, such an assessment of the effect of increased inulin intake could include an investigation of the effect on the bacterial flora of the gastrointestinal tract.

The presence of unintended alterations in classically bred cultivars of GM lines is not in itself potentially unsafe. Nor is it a new phenomenon. The emerging analytical techniques that have been developed in this programme could be used to identify unintended effects resulting from the genetic modification. They could also serve to place these in the correct context as in many cases classical breeding practices alter, directly or indirectly, the compositional profile of plants to improve flavour or pigmentation.

The approach used to date in the safety assessment of foods derived from genetically modified organisms was developed in collaborative work with international agencies such as OECD and FAO/WHO. It involves the concept of substantial equivalence whereby the characteristics of the modified food/crop are compared to an existing food/crop with a history of safe use. This usually is the parent crop from which the modifications were made. The process involves a targeted compositional analysis (profile of major nutrients and toxicants) and the expected intake and role in the diet. This comparison provides the basis on which to focus further toxicological requirements for safety assessment.

Concerns have been raised that the current approach of using targeted analyses to compare the composition of GM crops to their traditional counterparts is biased and does not take into account the possibility of unintended effects. Such unintended effects could arise in the following ways:

- If the transgene is inserted into a functional gene then a novel, chimeric protein could be produced and this new protein could be allergenic.

- If the transgene is inserted into a gene whose product is involved directly or indirectly in a metabolic pathway then a metabolic shift could occur leading to the formation or increased synthesis of a toxic metabolite.
- Expression of the transgene could cause a major metabolic shift leading to the formation or increased synthesis of a toxic metabolite.

The possibility of such effects needs to be taken into account when assessing risk but at the outset of the G02 programme suitable methods did not exist for analysis at the transcriptomic, proteomic or metabolomic level. Thus there was no guidance for crop producers on which parameters should be measured for comparative purposes, what analytical methodology should be used and which sampling procedures should be followed to provide statistically sound analyses. The principal output from the G02 programme is a set of analytical and quality assurance procedures whose suitability for detecting unintended effects has been rigorously assessed.

17.2 Detecting unintended effects

All of the contractors were able to detect genuine, unintended effects using a variety of methods applied to a range of plant species (tomato, potato, wheat, barley, *Arabidopsis*) carrying a variety of transgenes. This finding shows the power of the technology but it does have a major shortcoming. That is, the methods developed for proteomics and metabolomics can detect only 10-20% of the proteome and metabolome respectively. By contrast, transcriptomic methods have a much higher coverage (>80%). However, there are a number of ways that this shortcoming can be overcome.

1. If transcriptomic analysis reveals that only a limited number of genes show a significant change (+/- 5-fold or more) in expression level then the unintended effects are likely to be minimal. This conclusion could be reinforced if the protein products of the relevant transcripts can be identified and their cellular function shown to be inconsequential for safety assessment.
2. If proteomic and metabolomic analysis show relatively few changes versus the relevant controls then it is highly likely that the total proteome and metabolome have not changed significantly. The more information that there is about the molecules that have changed the more definitive that one can be.
3. Where data analysis shows that a transgenic crop is different from its parent then the molecules responsible for the differences can be identified. If these are mostly common metabolites (e.g. amino acids as seen in transgenic tomatoes) then the risks are minimal.
4. If expected new metabolites are removed from datasets and subsequent analysis does not separate the transgenic plant from the parent (as in the transgenic potatoes used in the G02006 project) then the unintended effects are likely to be minimal.
5. If the extent of the differences between the transgenic plant and its parent are less than that seen between different cultivars or less than known environmental effects then the risks are likely to be minimal. However, where a plant is known to contain toxins (e.g. potatoes) then

the analytical methodology used must be able to separate molecules related to the toxin.

The G02 programme has yielded methods for *detecting* unintended effects. However, it also has provided additional tools for *assessing* the likelihood of unintended effects. The nature of the transgene always has been a consideration and the significance of transgene function is well illustrated by the results obtained in the G02004 and G02006 projects. The identification of a generic method for determining transgene junction sequences has two implications. First, it now is possible to assess if a novel, chimeric protein is likely to be synthesised as a consequence of transgene insertion. The synthesis of a chimeric protein will only be of concern if it is allergenic but the standard proteomic methods give no indication of allergenicity. However, other work funded by the FSA has shown that certain protein motifs are associated with allergenicity and a search for such motifs could form part of the safety assessment.
Second, junction sequence analysis will indicate if a host gene function is likely to be inactivated and, if so, whether this might have metabolic consequences. The significance of gene inactivation is likely to be tempered by the ploidy of the host. Thus, in the G02002 project, metabolic effects were easier to discern in diploid barley compared with hexaploid wheat. Similarly, the unintended effects seen in potatoes in the G02001 and G02006 projects were minimal and could be because potatoes also are hexaploid.

17.3 Natural variation

In making assessments of unintended effects in transgenic crops due consideration needs to be given to natural variation. Prior to the G02 programme little information was available. Work undertaken as part of the G02 programme has shown that:

- Greater than fivefold variations in amounts of common metabolites are seen within a range of potato cultivars grown in one location in one season (G02001).
- The nutritional and/or irrigation status of the soil can significantly affect the metabolome of potatoes (plot 4 effect in G02001).
- The metabolic profile of potato cultivar Desiree is quite different from that of other potato cultivars (G02001 and 2006).
- Greater variation in common metabolites was seen for a single cultivar grown in two locations or in different years than was seen between a GM and a non-GM line of wheat (G02003).
- Varying light levels can significantly affect the transcriptional activity of plants (G02004).
- Inter-plant variation in tomatoes is far greater than intra-plant variation (G02005).
- The concentrations of different metabolites can vary markedly within a single wheat grain (G02003) or, especially, a single potato tuber (G02001). Pooling strategies can mask such variation.

17.4 Outstanding issues

The major outstanding issue is the lack of comprehensive compound libraries and spectral databases, especially for the secondary metabolites that normally are found in plants. However, this is a recognised problem and the plant metabolomics community already has taken the necessary action.

17.5 Value for money

Overall, the G02 programme has met the objectives that were set for it. In addition, much of the work undertaken in the programme has been presented to the international plant molecular biology community and the UK now is seen as having a leading position, particularly in the field of plant metabolomics.

18 APPLICABILITY TO OTHER FOOD STANDARDS AGENCY PROGRAMMES

18.1 General applicability

Transcriptomic, proteomic and metabolomic approaches are being used in other projects funded by the Food Standards Agency, e.g. food authenticity, nutrition, toxicology, food microbiology and TSE diagnostics. In the G02 programme, all of the current analytical methods were assessed with particular emphasis being placed on quality assurance, especially understanding and controlling sources of variability. Thus, the analytical methods that were deemed most suitable represent best practice and should be considered for new research projects and programmes within the Agency. In most cases, best practice has been documented as Standard Operating Procedures and these should be transferable to other laboratories/ contractors.

18.2 Food authenticity

In an ideal world the adulteration of a premium foodstuff, or its substitution with an inferior product, would be detected by the presence of one or more specific marker substances. In reality, this ideal seldom is achieved. The Agency, and MAFF before it, has funded many projects to identify markers of fraudulent practices and most of these have been unsuccessful. The basic problem is that finding such markers, assuming that they exist in the first place, is rather like looking for a needle in the proverbial haystack.

A different way of approaching the issue of food authenticity is to look for ‘substantial equivalence’. That is, is the sample in question substantially equivalent to authentic material? Thus the proteomics and metabolomics methodologies developed in the G02 programme should be used to address long-standing authenticity issues such as:

- Adulteration of olive oil with hazelnut oil;
- Confirming the PDO status of selected foods;
- Distinguishing organic food from non-organic food;

- Distinguishing free-range eggs from battery eggs.

An added advantage of this approach is that at the data analysis stage it may be possible to identify those compounds that are important in sample classification and this in turn could lead to the development of specific tests.

It is worth noting that chemometric methods have been investigated in the past. However, in these projects the experimental methods and the data analysis procedures were very crude by comparison with those used in the G02 programme. In addition, suitable quality procedures are an essential component of the analytical instrumentation if meaningful data is to be generated and the importance of these only now is being recognised.

18.3 Nutrition

For many people an inappropriate choice of diet leads to metabolic imbalances and an enhanced risk of diseases such as type 2 diabetes, food allergies and intolerances, gastrointestinal disorders, etc. The solution is to choose a better diet but what is a better diet? The genetic and phenotypic variation among humans is so wide that a diet that might be optimal for one individual could predispose another to disease. A prerequisite for empowering individuals to make better food choices is proof that particular diets are beneficial.

For a long time, nutritionists have attempted to show the relationship between diet and health by understanding the interaction of nutrients with metabolic pathways. To date, the tools for doing this have been relatively crude but the 'omics technologies should permit more incisive experiments. The American Society of Nutritional Sciences (ASNS) has recognised this and has identified the framework of basic knowledge that is required. For them, the essential 4 steps are:

- Technologies will need to be developed and implemented to measure metabolites in humans and experimental animals quantitatively and comprehensively.
- Databases will have to be assembled that contain data from a representative subset of the population in which individual metabolic data are arrayed against a wide range of phenotypic endpoints.
- Causal relationships will have to be drawn between metabolism and phenotype.
- The biochemical relations between diet and metabolism will have to be established with sufficient mechanistic understanding to make quantitative predictions of how to guide metabolism.

As a result of the G02 programme the Agency now has personnel in house and access to contractors with expertise in each of the 4 steps recognised by ASNS. Nobody has a technique that can measure metabolites comprehensively but the issues associated with quantitative measurements are well understood and G02 contractors would have the relevant analytical skills. A more important issue would be the selection of the appropriate tissues to be sampled, e.g. epithelial cells from the colon, bladder, bucchal mucosa or mammary duct. As far as databases are concerned, the metadata

will be even more important than in the G02 programme as it will need to be able to accept additional metadata (e.g. SNP profiles) as these become available. The ArMet database developed in project G02006 could well fit this need. The ability to establish causal relationships between metabolism and phenotype is at an early stage and some G02 contractors achieved only limited success. Nevertheless, some of the contractors have the expertise to make the necessary advances.

Two key issues were not raised by the ASNS. The first of these is experimental design. What the G02 programme demonstrated at an early stage is that failure to consider every source of variability from choice of target to data analysis *before* the first experiment is initiated will result in meaningless output. In essence, one can replace experimental crops with laboratory animals since both are inbred. Once work is initiated on humans, an outbreeding population, experimental design and trapping of metadata are even more important. The second key issue is quality assurance. In the G02 programme, the quality standards demanded of contractors were far higher than those enshrined in the Code of Practice to which the Agency is a signatory. A similar standard to that adopted in the G02 programme should be mandatory for nutrition studies.

The US LIPID MAPS project

The sequencing of the human genome has provided the impetus for building a comprehensive picture of a mammalian cell. The goal is a complete map of the genes, gene products and their interacting networks in a functioning cell. Thereafter, the task will be to move from the individual cell to a tissue and then an organ and eventually the whole animal. This is generally referred to as ‘systems biology’. Given the current state of knowledge it could be argued that it is too early to consider applying the ‘omics technologies to research in nutrition. An alternative approach that has been taken by a US consortium (Lipid Metabolites and Pathways Strategy, LIPID MAPS) is to focus on the lipid section of the metabolome. Their argument is that lipids play such an important role in many diseases that understanding what lipids are present and, more importantly, how they shuttle between different pathways is essential before engaging in nutrigenomics. The techniques that will be used by the LIPID MAPS consortium are no different from those developed in the G02 programme and so the Food Standards Agency easily could adopt a similar approach.

19 GLOSSARY

Biolistics A method for introducing foreign DNA into plant cells. The technique involves coating metal microspheres with the DNA of interest and firing them at the plant cells with a miniature shotgun.

Metadata This is the term used to describe data about data. For example, a library catalogue contains information (metadata) about the publications (data) that are stored. In the context of the G02 programme it describes who

collected the data, how they collected it, when they collected it, what the data contains and how it is stored.

Pleiotropic An adjective used to describe mutations, which result in multiple phenotypic effects.