VALUE ADDED WHEAT CRC PROJECT REPORT

Review of

Program 1: Diagnostics

and

Program 3: Genomics and Proteomics

Convened by:
Drs Neil Howes and Peter Sharp

Compiled by: Clare Johnson

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7 November 2002

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Contents

Summary *Clare Johnson*

In his introduction, Neil Howes described input and output links between the Diagnostics Program and other VAWCRC Programs.

Project 1.1.1 - Protein-composition analysis - suffered a delayed start because of the delay in GRDC funding the postdoctoral positions. Aims were revised and the CRC funded one postdoctoral position. The capillary electrophoresis instrument has been commissioned. Dr Dilek Sivri's work on protein damage, conferred early in-crop by a "bug" prevalent in Turkey, much of Europe and Central Russia, was of interest to Arnotts (see slides). Dr Batey is consulting the entomologists at CSIRO's Stored Grain Research Laboratory on this.

In Project 1.1.2 - Antibody-based diagnostics - the group has divided its function between core technology development and target identification. Their use of MAP peptides (multiply antigenic peptides, i.e. multiple copies of a peptide on a lysine core, with or without a lipid attachment) overcomes cross-reactive background issues. They have found peptides 20 amino acids in length confer more specificity than 14-mers.

A set of waxy mutants, assembled by Xiaochun Zhao during his PhD was made available by PBI Cobbitty, and has enabled screening of the antibodies. Where polyclonal antibody results were promising, monoclonal antibodies (MAbs) were developed. Consequently, VAWCRC now has MAbs which detect Wx-4A and 7A, respectively, and the team is in the process of developing an antibody for Wx-7D.

In collaboration with the proteomics project (3.1.2) the group has also developed a MAb for the hardness marker, puroindoline A. This is present in Rosella (cracker and noodle quality) but absent in Bowie (cookie quality), and would be useful to screen WA varieties, Lorikeet etc. A 50KDa protein is another target for varietal identification, on the criterion of occurrence in 50% of the population. It was absent in 5/5 soft varieties tested, and present in 6/13 hard varieties. Its peptide sequence defines it as a starch granule-associated LMW glutenin.

The group has thus already developed several antibodies diagnostic for soft wheats and waxy wheats.

ACTION NH, PS: resolve requirement for more resources on protein sequencing.

Advances in Project 1.2.3 – Diagnostics delivery – include redesign of the WheatRite card and monitor and redefinition of the look-up table, and there is

also a new hand-held detector with a PC interface for in-field use. Kits for 1RS/1BS translocation and GBSS Null 4A are now available at $1/test, with plans for an LMA test in the next 6 months, potential pesticide residue and mycotoxin tests, and tests for varietal and quality type identification using the antibodies described in project 1.1.2.

In project 3.1.1 - Markers and Mapping - SSR marker development to enable the cost-efficient production of informative markers, which are amenable to high throughput assays, is progressing strongly. Sequence tag profiling (STP) technology is being used to develop a large number of mapped simple sequence repeat (SSR) markers for high throughput analysis, and to enable the discovery and high-throughput analysis of single nucleotide polymorphisms (SNPs). In a joint project with the John Innes Center (JIC), and the Australian Winter Cereal Molecular Marker Program, each party is to generate ~1000 mapped sequence-tagged microsatellites, resulting in a consensus SSR map of bread wheat, relevant to current Australian and European germplasm, and fully integrated with published genetic maps.

In the Diversity Array Technology (DArT) work, CAMBIA is addressing both the need to discriminate presence/absence of markers, and the need to quantify the DNA. Using a Mathcad file embedded in Excel, they measure the green/red signal ratio and use this to normalise the output. Within 2 weeks, they will have developed and screened another 1500 clones. The current constraint is lack of hardware. Cluster analysis of outputs discriminated well between varieties. Miniature Inverted Repeat Transposable Elements provide a promising source of variation. Database and analysis software systems are currently being planned in detail.

In the proteomics project (3.1.2), soft wheat and Puroindoline A results have provided crucial input to project 1.1.2, reported above. Further, in our comparison of Bowie and Rosella, we found two other +/- polymorphisms for potential cultivar discrimination, an alpha-amylase/subtilisin inhibitor, and a perioredoxin. Serine protease inhibitor (serpin) polymorphisms are also being characterised, with a good +/- discrimination target found by screening 7 cultivars. The proteomics of wheat germ tissue has been well characterised by the PhD student.

In Project 3.1.3, targeted mutagenesis, has commenced. Initial work will be focussed on tissue culture and bioloistic delivery. Proof of concept will involve transient assays, followed by a selectable system (herbicide tolerance) producing whole regenerated plants with the desired mutation. We will then target genes of relevance to quality. We also envisage provision of a service generating mutants, to help researchers identify the function of newly-discovered genes, by comparing the mutants with wild-type.

Program 1 Overview

Neil Howes

Program 1: Diagnostics

Aim to develop diagnostic tools and methods for wheat and wheat products

- To measure new quality traits such as starch properties and flour proteins
- Variety identification, grain uniformity, soundness and purity
- Emphasis on development of on-the-spot tests for immediate decision making

Value Chain (Increasing value)

- Germplasm
  ⇦ GBSS,CE
- Varieties(Breeders)
- Seed increase (Seed growers)
- Production (Farmers)
  ⇦ WheatRite®, Variety ID
- Grain Delivery (Grain handlers)
  ⇦ CE, GBSS
- Processors (Millers, Bakers)
- Delivery to Consumers (Retailers)

Diagnostics Projects

1.1.1 Protein Composition Analysis
(fast protein separation methods)
Project Leader: Ian Batey

1.1.2 Antibody-Based Diagnostics
(production of new antibodies)
Project Leader: James Chin

1.2.3 Diagnostics Delivery
(production of user friendly kits for industry)
Project Leader: Felice Driver

Diagnostics- Inputs to other VAWCRC programs

Program 2	Blending (Gel electrophoresis, CE)
Program 3	Site-directed Mutagenesis (antibody screening tools)
Program 4	Germplasm development (GE, CE for gliadins & glutenins; Antibodies for LMA, GBSS null 4A, 1B/1R,.....)

Diagnostics- Inputs from other VAWCRC programs

Program 3	Proteomics (Identification of variety specific proteins)
Program 4	Novel Germplasm (new targets for variety ID)
Program 5	Education and technology transfer

Project 1.1.1:
Protein-composition analysis

Ian Batey

Project 1.1.1
Protein Composition Analysis

Original Objectives

Provision of diagnostic methods based on aspects of grain-protein composition that would facilitate the identification of wheat **varieties** and of **quality attributes**

Project 1.1.1

Milestones

Report on feasibility study – December 31, 2001

Develop a new project charter and apply for external funding – on GRDC schedule (January 18, 2002)

Milestones have been achieved on schedule

Project 1.1.1

Revised Objectives

Undertake feasibility study for conduct of original aims and strategy considering industry needs within Australia, and current developments within Australia and overseas.

Based on the findings, to develop a new project charter to achieve the directions indicated by the findings, bearing in mind the possibility of external funding.

Project 1.1.1
Protein Composition Analysis

Staff involved

SARDI	**CRC - FSA**
Dr Geoff Cornish	Dr Colin Wrigley
Rebecca Tonkin	Dr Ian Batey
	Dr Siri Nakkote
Sabbatical visitor:	Prof Dilek Sivri
Student from old CRC:	Laila Daqiq

Program Leader: Dr Neil Howes

Project 1.1.1

New Improved Revised Objectives

Develop methods, based on analysis of protein composition by CE, HPLC and PAGE, for more efficient identification of quality type, suited to screening breeders' lines, using known protein markers

Develop methods, based on analysis of protein composition and/or protein markers, for more efficient identification of varietal identity and of quality type, deployable beyond the requirements of a laboratory

Identify specific protein markers which are indicative of genotype (variety) and/or of end-use quality for use in novel diagnostic applications

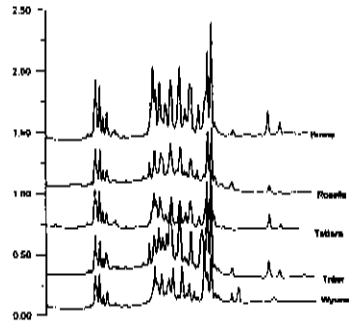
Project 1.1.1
Main Points from Feasibility Study (1)

- There is an industry need for improved methods of identification of variety
- Analysis of grain composition provides valuable opportunities for the identification of variety and/or quality type
- Better methods of protein analysis are potentially available to suit industry needs

Project 1.1.1
Main Points from Feasibility Study (2)

- There is a need to develop simpler methods for the routine analysis of marker proteins
- Better management of the information about varieties and results, taking advantage of advances in IT
- Trialling and implementation of methods developed

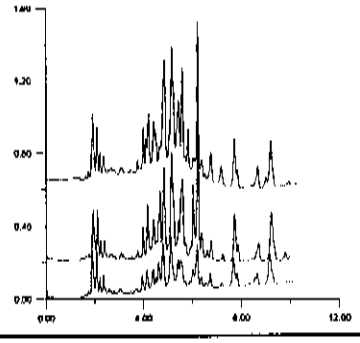
Project 1.1.1
CE Traces of 5 Soft Wheat Varieties



Project 1.1.1
Possible methods (1)

- Capillary electrophoresis with research equipment to screen for quality in a breeding program;
- Capillary electrophoresis with research equipment to screen for variety in a centralized situation, and to provide a basis for simpler CE methodology;

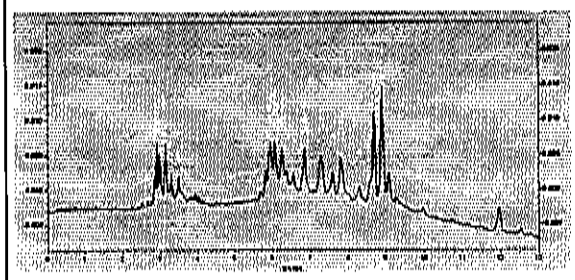
Project 1.1.1
CE Traces of Soft Wheat Variety with different protein content



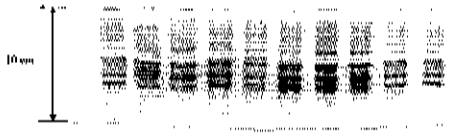
Project 1.1.1
Possible methods (2)

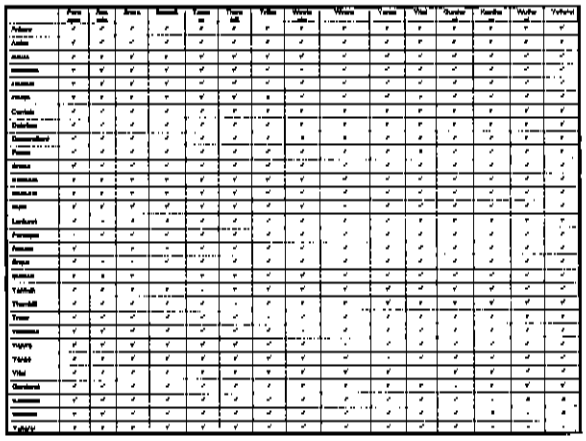
- Capillary electrophoresis with simple equipment for deployment at the regional centers, the labs of grain processors, and possibly in field situations;
- Micro-gel electrophoresis for varietal identification in a regional center;
- Mass spectroscopy for varietal identification in a central laboratory.

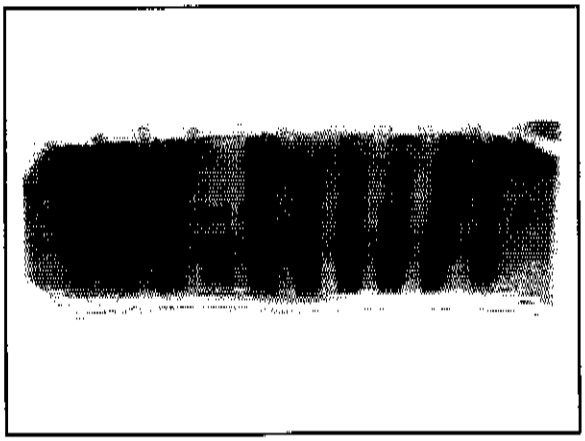
New CE Commissioned!



Distinguishing Australian wheat cultivars using mini gels

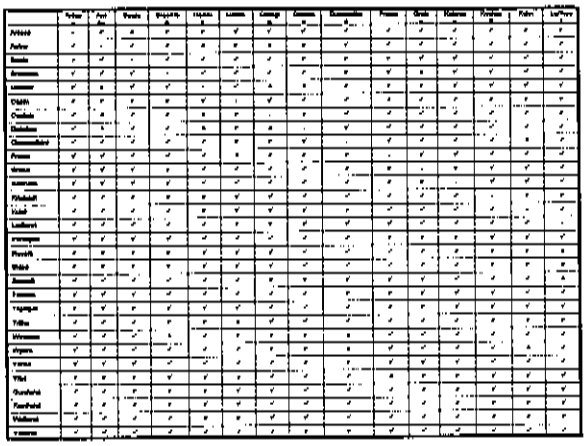






Advantages of mini gels

- Speed
  - Run time 30 min, stain and destain 45 min
- May be used in regional, less well equipped labs
- Equipment relatively cheap



Issues inhibiting progress

- Delayed move into new accommodation (CE commissioned last week)
- No full time staff (post doctoral position just advertised)
- Limited other resources (hoped for grants from GRDC not successful)

Bug protease – visit of Prof Sivri

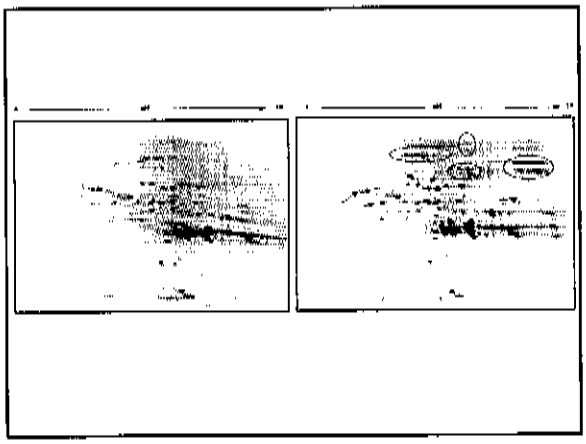
- Sabbatical visitor from Turkey
- Bug damage of grain a serious problem in southern Europe, Russia and NZ
- Insect attacks developing grain, but protease remains in mature grain
- Effect is loss of dough strength
- Need for a diagnostic to avoid admixture

Bug protease

- Proteome approach used successfully to identify degradation products from bug-protease action (large glutenin polymer)
- SE HPLC confirmed reduction in largest glutenin fraction (less UPP)
- Amino acid specificity of bug-protease being determined with synthetic substrates (EMAI collaboration)

Bug protease

- Development of better diagnostics for bug-damaged grain
- Blending studies
  - Suitability of very strong Australian wheats for the Turkey (*et alia*) market. (Relevant to VAW CRC Project 2.1.1)
- Solubilisation of gluten for ingredient use
  - Use of the bug protease to modify gluten properties for food uses. (Relevant to VAW CRC Project 2.1.9)



Bug protease

- Development of better diagnostics for bug-damaged grain
  - Use of photographic film to identify bug-damaged grain
  - Dye-coupled glutenin as a substrate for the protease of bug-damaged grain
  - Proteome analysis to identify marker proteins for bug-damaged grain
  - Development of a test kit for bug damage, using immuno-reaction to the bug protease

Bug protease

- Proteome approach used successfully to identify degradation products from bug-protease action (large glutenin polymer)
- SE HPLC confirmed reduction in largest glutenin fraction (less UPP)
- Amino acid specificity of bug-protease being determined with synthetic substrates (EMAI collaboration)

Future work

- Selection of target proteins for quality
- Development of methods for separation of relevant proteins
- Application of methods to breeders' lines
- Isolation of distinguishing proteins for diagnostics
- PhD project on protein quality determination

Outputs

In past year

- 12 papers/chapters published
- 2 books edited and published
- 13 papers/chapters accepted for publication
- 6 CRC reports published/prepared
- 8 conference/workshop presentations

CRC Reports

Batey, I.L., and Wrigley, C.W. Diagnostics for variety and quality-type identification. Report of Workshops held 18th July and 27th August, 2001.

Batey, I.L., and Wrigley, C.W. Protein-composition analysis to determine variety and quality type: Principles and practice.

Batey, I.L., and Wrigley, C.W. Protein-composition analysis to determine variety and quality type: Principles and practice.

Cornish, G.B., Batey, I.L, and Wrigley, C.W. Australian wheat varieties: grain quality data on recently registered varieties.

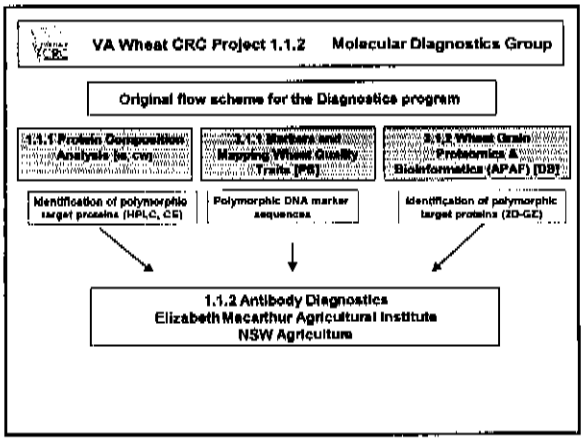
Cooke, R.J., and Wrigley, C.W. Current International Policies on Plant Breeders' Rights

Tonkin, R.E., Cornish, G.B., and C.W.Wrigley, C.W. Rapid electrophoretic verification of varietal identity: application to 30 current Australian wheats.

Wrigley, C.W. Temperature variation during grain growth as a source of quality inconsistency for the Australian wheat industry

Project 1.1.2:
Antibody-based diagnostics

James Chin



ELIZABETH MACARTHUR AGRICULTURAL INSTITUTE
NSW AGRICULTURE

James Chin (Principal Research Scientist)
Thomas Giersch (Research Officer)
Ming Wu (Research Officer)

Araluen Freeman (PhD)
Michelle Powell (MSc)

Louis Duncan (Part-time TO)

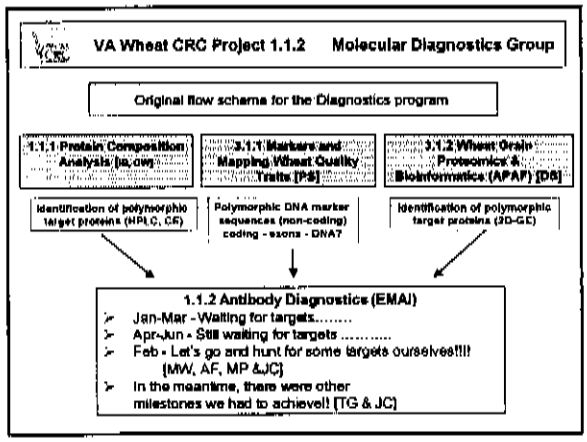
List of Publications – under preparation

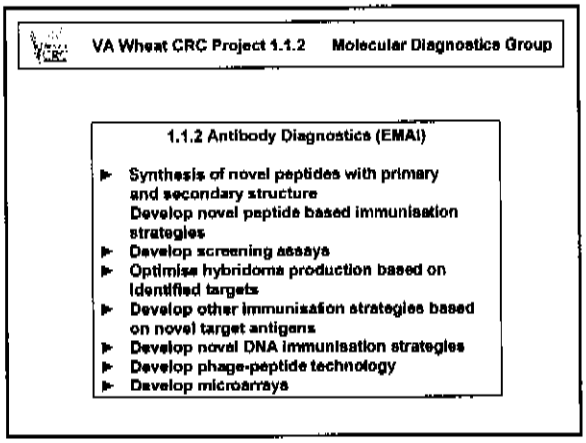
Giersch – Use of novel multiantigenic peptide designs for the production of a Mab specific for GBSS 7A

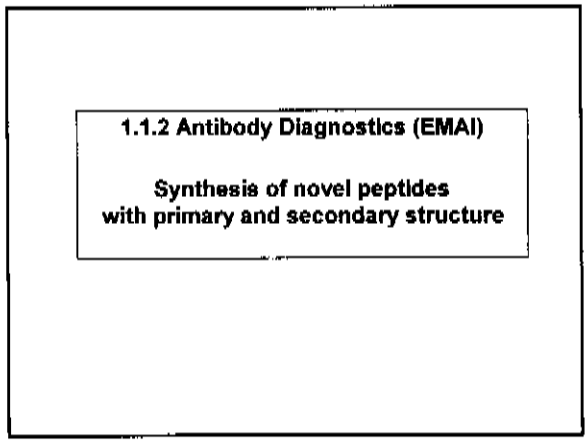
Wu – Members of the Low Molecular Weight Glutenin superfamily present in Starch Granules can be used as markers for some hard wheat varieties

Freeman – New insights into 'surface' and 'internal' proteins of Starch Granules revealed by biotinylation

Skylas – Differentiation between Rosella and Bowie with the Pin A variety marker





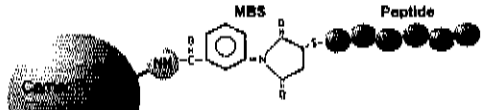


Peptide Based Immunisation Strategies

1. Peptide conjugated to carrier protein (KLH)

Peptide Based Immunisation Strategies

Peptide Conjugation to a Carrier Protein via a bifunctional linker (MBS)



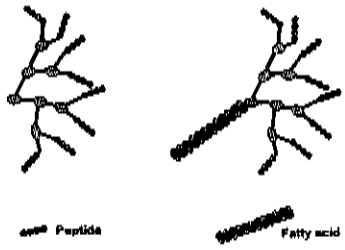
MBS: m-Maleimidobenzoyl-N-hydroxysuccinimide ester

KLH: Keyhole Limpet Hemocyanin

Peptide Based Immunisation Strategies

2. Multiple Antigenic Peptide (MAP)

3. Lipid-MAP

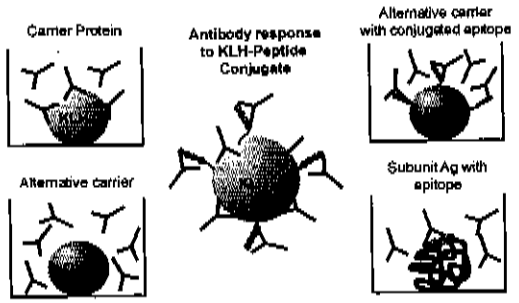


1.1.2 Antibody Diagnostics (EMAI)

Develop screening assays

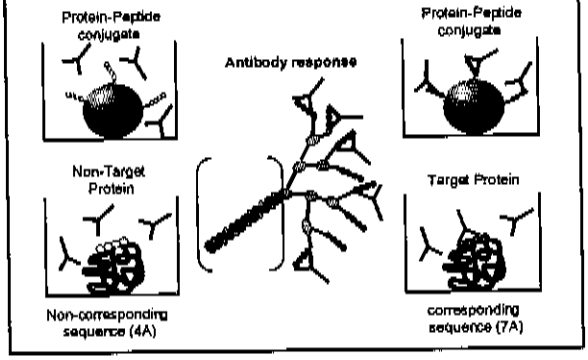
Screening Strategies

For Carrier Protein-Peptide Immunisation



Screening Strategies

For MAP (Lipid-MAP) Immunisation

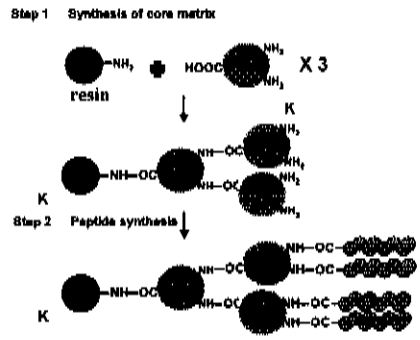


1.1.2 Antibody Diagnostics (EMAI)

Develop novel peptide based immunisation strategies

Multiple Antigenic Peptide (MAP)

Chemistry of MAP Synthesis



Peptide Based Immunisation with GBSS 7A MAP

Why GBSS 7A ?

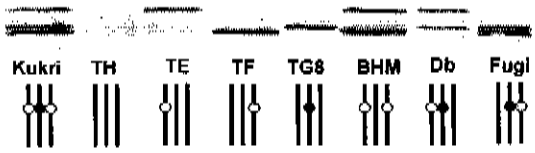
- Sequences of the 3 alleles are known
- Presence/absence in different wheat cultivars
- Demand of breeders for a quick method to identify isoforms (antibody for GBSS 4A available)

```
                                351                 370
      7D 'D' GENOME:    DVSEWDPTKDKFLAVNYDIT
      4A 'B' GENOME:    DVSEWDPAKDKFLAANYDVT
short 7A 'A' GENOME:          PIKDKFLTVNYDVT
long  7A 'A' GENOME:    DVSEWDPIKDKFLTVNYDVT
```

Short 14-aa version of GBSS 7A allele - free MAP and resin-bound
Longer 20-aa peptide of GBSS 7A allele - free MAP

GBSS1-null Wheat Varieties (X. Zhao)

16% SDS-PAGE of Starch Granules (SG)



- ○ *Wx-7A* - Chromosome 7, Genome A (7A)
- ● *Wx-7D* - Chromosome 7, Genome D (7D)
- ◌ *Wx-4A* – Translocated from Chromosome 7, Genome B (7B) to Chromosome 4, Genome A (4A)

Screening Strategies

Antigen extraction from SG

8M Urea

10 mg SG + 400 µL 8M Urea vortex
↓
Incubate 1h at room temperature
↓
Spin 5 min at 13 Krpm
↓
Xt, for ELISA
dilute SN 1:50 - 1:100
in coating buffer (CB)

4% SDS/ME

30 mg SG + 400 µL SDS-sample buffer
4% SDS, 20% glycerol, 5% 2-ME,
120 mM Tris, pH 6.8
↓
Boil 10 min
↓
Spin 10 min at 13 Krpm
↓
Xt, For SDS-PAGE & ELISA

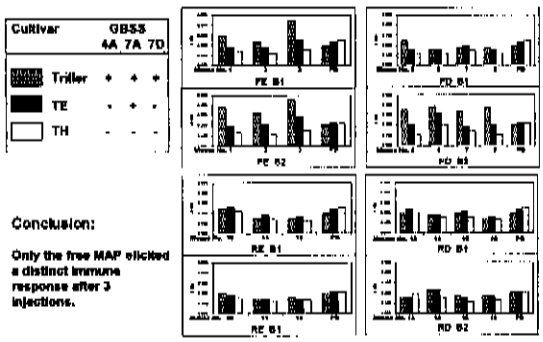
Immunisation Protocol: Short 7A MAP

Route	Subcut - 3 sites, 40 µL/site
Dose	200µg peptide equivalent in 120 µL
Antigen:	Short 7A Free-MAP
	Short 7A Resin-MAP

Time Week	Treatment
0	Pre-Bleed (PB)
1	1st Injection
5	2nd Injection
7	Bleed 1 (B1)
9	3rd Injection
11	Bleed 2 (B2)
17	Booster Injection
18	Euthanise
	Terminal Bleed (TB)
	Fusion

Peptide form \ Adjuvant	Emulsigen	Diluvac
Free MAP	Group FE n=4	Group FD n=4
Resin-MAP	Group RE n=4	Group RD n=4

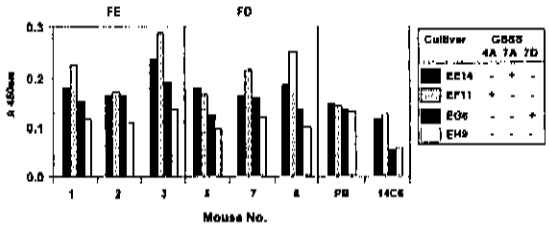
Reactivity of Mouse Sera (B1) x short 7A-MAP against 8M Urea SG extracts



Conclusion:

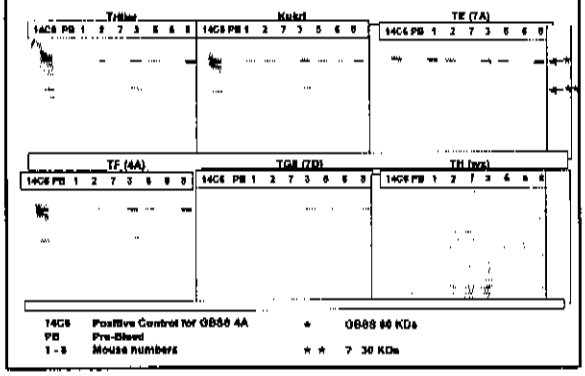
Only the free MAP elicited a distinct immune response after 3 injections.

Reactivity of Mouse Sera (B2) x short 7A-MAP against 8M Urea SG extracts from GBSS null lines



Predominant specificity for the wheat line EF11 with the GBSS 4A allele

Immunoblot Reactivity of Mouse Sera (TB) x short 7A MAP against SDS SG extracts from GBSS null lines



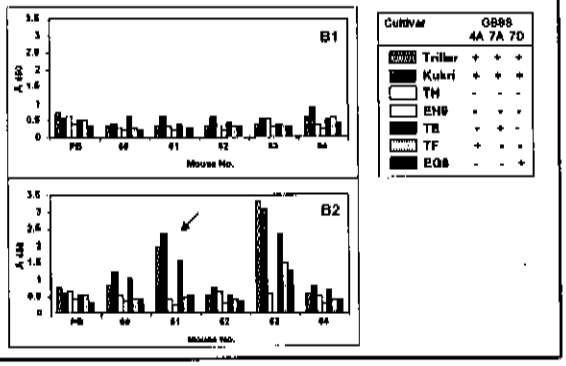
Immunisation Protocol: Long 7A MAP

Route	Subcut - 3 sites, 40 µL/site
Dose	200µg peptide equivalent in 120 µL
Antigen:	Long 7A Free-MAP

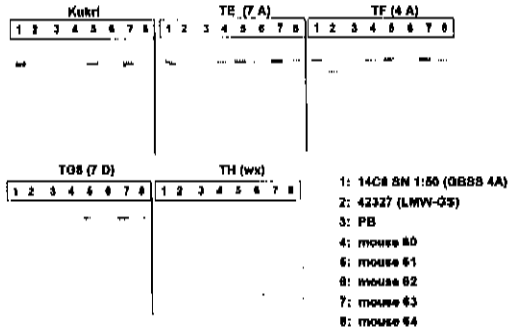
Time Week	Treatment
0	Pre-Bleed (PB)
1	1st Injection
4	2nd Injection
6	Bleed 1 (B1)
7	3rd Injection
9	Bleed 2 (B2)
12	Booster Injection
13	Euthanase
	Terminal Bleed (TB)
	Fusion

Peptide form \ Adjuvant	Emulsigen
Free MAP	Mouse No. 60 - 64

Reactivity of Mouse (B1&B2) x long 7A MAP against 8M Urea SG extracts from GBSS null lines



Immunoblot Reactivity of Mouse Sera (B2) x long 7A MAP against SDS SG extracts from GBSS null lines



1: 14C8 SN 1:50 (GBSS 4A)
2: 42327 (LMW-G3)
3: PB
4: mouse 60
5: mouse 61
6: mouse 62
7: mouse 63
8: mouse 64

Lessons Learnt from the use of MAPs

- Longer (20 aa) is better ? than shorter (14 aa)
- Free MAPs work better than resin-bound MAPs
- Emulsigen adjuvant is better than Diluvac

1.1.2 Antibody Diagnostics (EMAI)

Optimise hybridoma production based on identified targets

Hybridoma Development

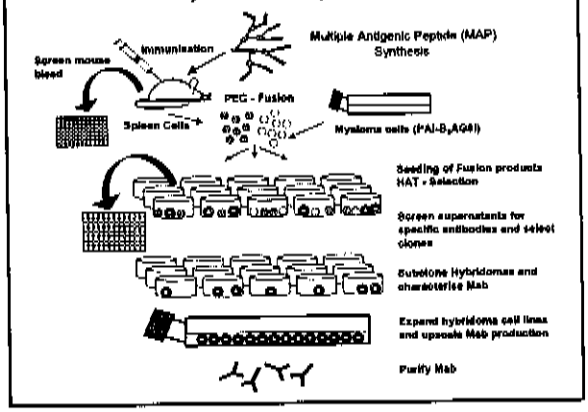
Mice immunised with free GBSS 7A MAP (short + long)

- Selection of mice with highest specific immune response
- Spleen cells fused with the myeloma cell line PAI-B_3AG8I using PEG 1500.
- 2-10% of the fusions plated out for initial screening
- Fusion rates: $1.2x10^{-4}$ to $8x10^{-6}$
- Screening results:
  On average 5% of the tested wells with cell growth reacted strongly with starch granule extracts from Triller and to a different degree as well with the line TH containing no GBSS.

Hybridoma Development

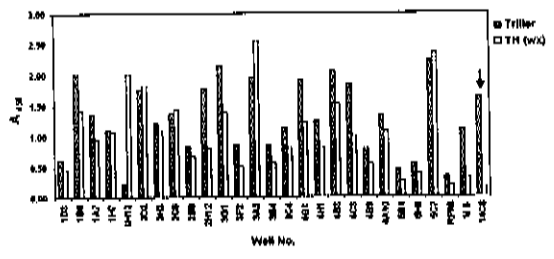
	Stage	GBSS short MAP PIKDKFLTVNYDVT	GBSS long MAP DVSEWDPIKDKFLTVNYDVT
1	Immunisation	2 Adjuvants 2 Forms Free/Resin	1 Adjuvant Free
2	Mouse Screening	ELISA, Immunoblot	ELISA, Immunoblot
3	Cell Fusion with PAI-B_3AG8I	4	2
4	Hybridoma Screening 2x by ELISA	1320 wells (5% of fusions)	900 wells (5% of fusions)

Hybridoma Development



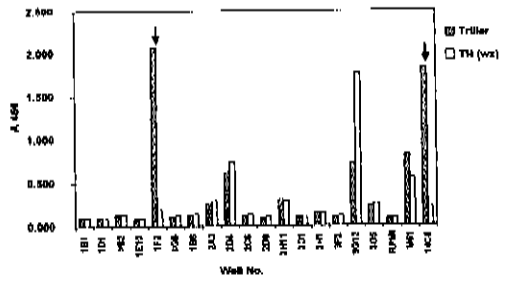
Hybridoma Screening (short 7A pep fusion - M5)

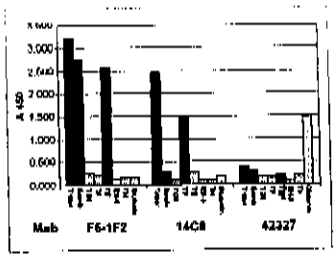
Primary Screen of Supernatants from F1 against 8M-urea SG Xts. from Triller (4A+, 7A+, 7D+) & TH (Triple Null)



Preferred Outcome: see 14C6 = High Triller and low Th reactivity

Primary Screen of Supernatants from F5 against 8M-urea Xts. from SG of Triller (4A+, 7A+, 7D+) & TH (Triple Null)

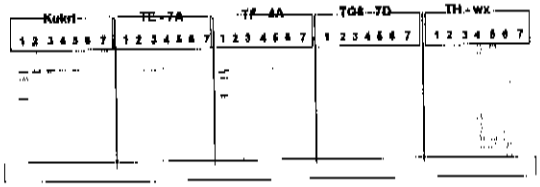




Cultivar	GBSS 4A	7A	7D
Triller	+	+	+
Bowie	-	+	+
TG 8	-	-	+
TF	+	-	-
TE	-	+	+
EH-9	-	-	+
TH	-	-	-
Glutenin	+	-	-

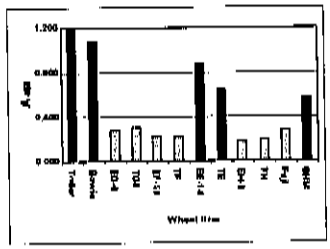
Specificity was assessed against SG extracts from Triller, Bowie and GBSS null lines
Mab 14C6 is 4A specific and reacts x Triller and TF
Mab 42327 is specific x 46 kDa LMWG and does not react against any SG Xts.

Confirmation that F5-1F2 Mab is 7A-specific by immunoblotting against SDS SG Xts. From GBSS null lines



1: 14C6
2: F5-1F2 (original clone)
3: F5-1F2 (expanded clone)
4: M 61 (B2)
5: M61 (TB)
6: Pre-bleed (B0)
7: RPMI

Can the F5-1F2 Mab detect only GBSS 7A in 8M urea Extracts from Whole Grains



Cultivar	GBSS 4A	7A	7D
Triller	+	+	+
Bowie	-	+	+
EG-6	-	-	+
TG 8	-	-	+
EF-11	+	-	-
TF	+	-	-
EE-14	-	+	-
TE	-	+	+
EH-8	-	-	-
TH	-	-	-
Fujimikomugi	+	-	+
Baihuomai (BHM)	-	+	+

Coating: Single wheat grain is crushed & extracted with 400 µl 8M urea for 1 h, diluted 1:100 in carbonate buffer
Antibody: Supernatant from cell line F5-1F2 diluted 1:5

The GBSS 7A Monoclonal Antibody F5-1F2 A VAW-CRC IP !! in 10 months

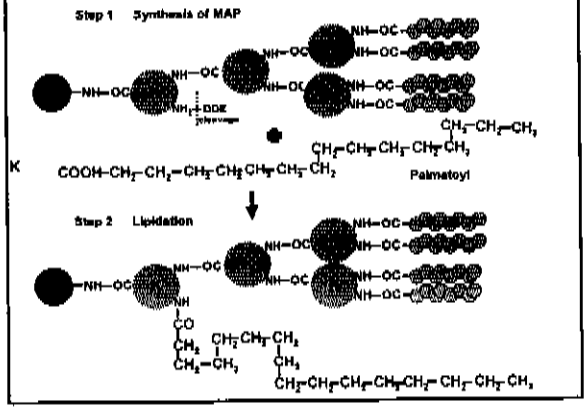
- Mab F5-1F2 can discriminate wheat lines with respect to the presence or absence of GBSS 7A
- Scale up culture supernatant production of Mab F5-1F2
- Validate Mab F5-1F2 against a wider spectrum of wheat cultivars with or without the GBSS 7A (Neil Howes)
- Scale up and purify Mab F5-1F2 for C-Gentec
- Is there a Need for a GBSS Polyclonal Ab or a multi-4A/7A/7D Mab as capture antibody?

1.1.2 Antibody Diagnostics (EMAI)

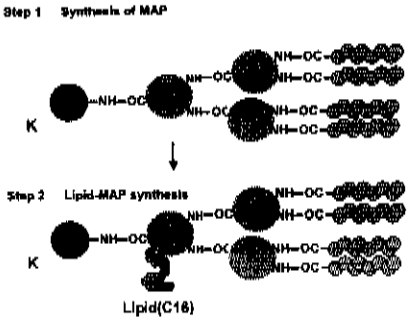
Develop novel peptide based immunisation strategies

Lipid-Multiple Antigenic Peptide (MAP)

Chemistry of Lipid-MAP Synthesis



Chemistry of Lipid-MAP Synthesis



Rationale for using Lipid MAP for Pin A/B

- The Pin A/B peptide is extremely hydrophobic
- It is associated with lipids that are integral or vital to the structure and formation of the starch granules

Immunisation Protocol: R-Pin A-Lipid-MAP

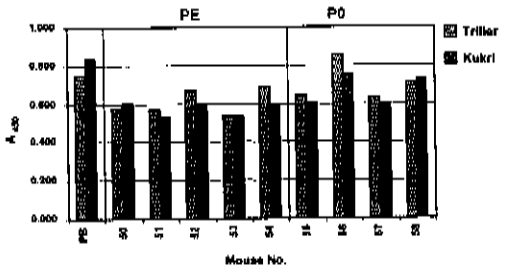
Route: Subcut - 3 sites, 50 µL/site
Dose: 400µg resin equivalent in 55 µg peptide
Antigen: Puroindoline A Lipid-MAP (Resin-bound)

PIN A (14aa) 117 132
DRASKVIQEAKNLPPR
(is absence a specific marker for hard cultivars?)
10% of cultivars worldwide, only a few in Australia

Time Week	Treatment
0	Pre-Bleed (PB)
1	1st Injection
5	2nd Injection
7	Bleed 1 (B1)
9	3rd Injection
11	Bleed 2 (B2)

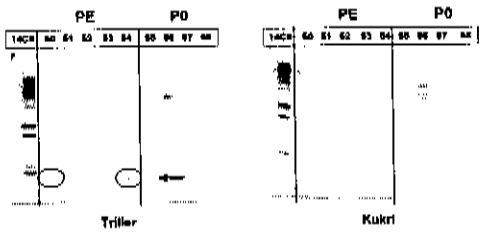
Peptide form \ Adjuvant	Emulsigen	nil
Lipid-MAP Resin-bound	Group PE n=5	Group P0 n=5

Reactivity of mouse x Pin A Lipid MAP (B2) against 8M Urea SG extracts from Triller (Pin A+) and Kukri (Pin A-)



No ELISA specificity - Background antigens in 8M urea extracts
Evident in immunoblots - see next slide

Localisation of mouse x R-Pin A Lipid MAP (B2) against SG extracts from Triller (Pin A+) and Kukri (Pin A-) by Western Blotting



Note blot specificity - Reaction x Triller Pin A at 15 kDa (arrowed)

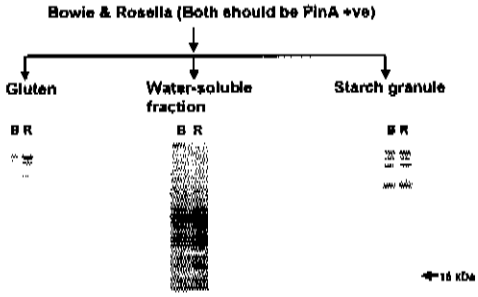
Immunisation: Pin A-Lipid MAP

- Boost mice with Pin A peptide intravenously or Pin A-Lipid MAP
- Confirm reactivity of mouse sera x carrier-Pin A peptide
- Should check for negative reaction x carrier-Pin B peptide
- Fuse mouse spleen cells with PAI-B3 myeloma
- Screen hybridoma supernatants against carrier-Pin A
- Confirm specificity of reactivity of supernatants against 8M urea extracts of hard and soft wheat grains

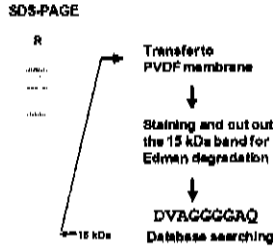
1.1.2 Antibody Diagnostics (EMAI)

Can we differentiate between Rosella and Bowie ?
Both being soft wheat cultivars

Unique Protein Targets Between Bowie and Rosella



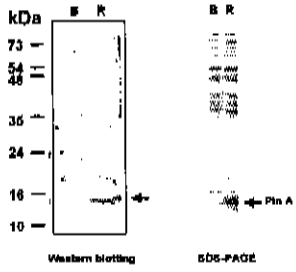
N-terminal AA Sequencing of 15 kDa from Rosella



N-terminal AA Sequence of 15 kDa from Rosella

```
Pin A   MKALFLIGLLALVASTAFAQYSEVV
15 kDa      DVAGGGGAQ
            ||||||||
        GSY DVAGGGGAQQCPVETKLNSCRN
        YLLDRCSTMKDFPVTWRNWKWWKGG
        CQELLGECCSRLGQMPPQCRCNIIQ
        GSIQGDLGGIFGFQR DRASKVIQEA
        KNLPFR CNQGPPCNIPGTIGYYW
```

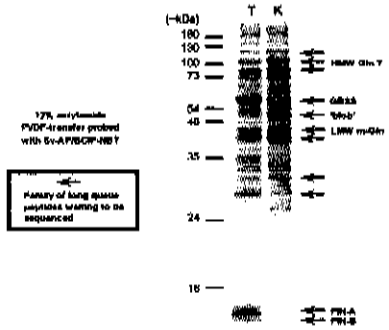
Is the 15 kDa difference between Bowie and Rosella due to a difference in PinA? Probing with M50/54xR-PinA-Lipid MAP



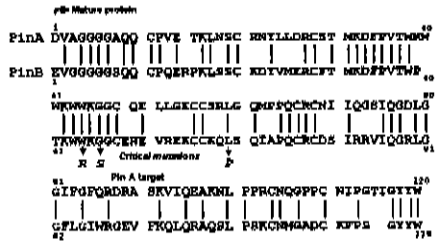
1.1.2 Antibody Diagnostics (EMAI)

Strategy for Generating Antibodies against different epitopes of both Pin A and Pin B for varietal identification of wheat cultivars

Biotinylated SG proteins of Triller & Kukri



Wild-Type AA Sequence of Pin A and Pin B



Softness = coexistence of wild type PinA and PinB
Hardness = critical single mutations of Pin B at 44 (W-R) or at 46 (G-S). The absence of Pin A is also a marker for hardness in 10% of wheat varieties

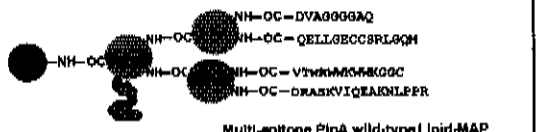
Pin A & B Peptides for Immunisation

PinA
116 131
DRASKVIQEAKNLPPR

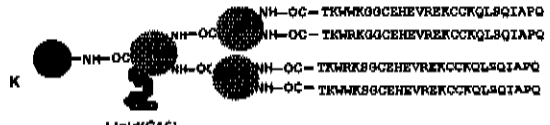
Wild PinB
118 133
GEVFKQLQRAQSLPSK

Mutant PinB
41 61
TKWRKSGCEHEVREKCCKQLS

Lipid MAP - Pin Peptides - The Complete Compendium



Multi-epitope PinA wild-type Lipid-MAP



Multi-epitope PinB Mutant peptide Lipid-MAP

1.1.2 Antibody Diagnostics (EMAI)

Develop other immunisation strategies based on novel target antigens

The search for new targets ……..

Fractionation of Wheat Grain

Soft Varieties	Hard Varieties
Triller Cadoux Datatine Bowie Rosella	H45 Janz Dollarbird Kukri Trident Camm Sunstate Hartog Goldmark Sunvale Frame Diamondbird Kamillaroi Wollaroi

↓

Gluten — Water-soluble fraction Gliadin/Albumin — Starch Granules

Starch Granule Proteins

SG proteins affect end-product characteristics

SG surface protein significance

Influences SG interactions with other substances by virtue of location

↓

Influences characteristics of SG as a whole

↓

Influences characteristics of starch end-products

Why Starch Granules?

- Over 85% of flour by weight is starch
- Presence or absence of Starch granule-associated proteins such as GBSS affects quality traits eg. 4A null - reduced amylose content - udon noodle
- There may be other polymorphisms in starch granule-associated proteins that may have value for quality trait identification and varietal diagnosis

SG extraction

Crush wheat grains

↓

Steep grains in 4% Radiant laundry liquid (1:4 ratio)

↓

Gentle homogenisation, filter (65µm)

↓

Centrifugation to pellet SG

↓

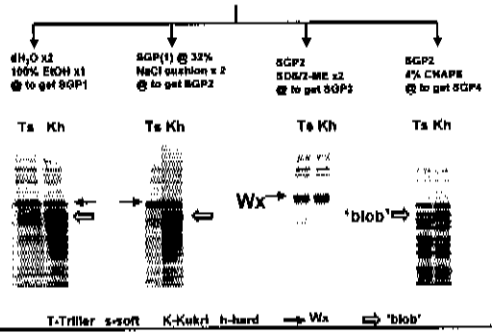
Wash SG 2x in water, 1x in ethanol

↓

Air dry SG

Comparison of Washing Protocols for SG

SG after 65 µm nylon mesh filtration



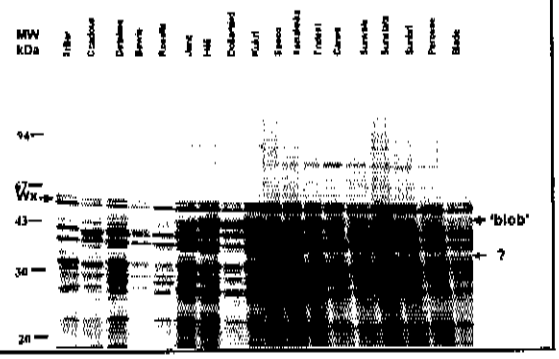
SG Washing Protocols - Results

- Most of the polymorphic SG peptides are not removed by water/EtOH washes
- SDS/ME effectively removes the 'blob' and other low mol.wt. peptides but not GBSS
- The non-ionic detergent CHAPS does not remove either GBSS or the 'blob'
- SDS/ME wash can be easily prepared and used as an assay reagent to screen for 'blob' reactive antibodes
- The requirement for an ionic detergent and a disulphide reducing reagent to remove the 'blob', suggests that this peptide is not a SG surface contaminant

Kukri is a hard wheat variety
50 kDa 'blob' is present in Kukri

How widespread is the 'blob' in other hard and soft wheats?

SDS-PAGE Protein Profile Analysis of Starch Granules from Soft and Hard Wheat Varieties



Distribution of the 'blob' in Hard and Soft wheats

'Blob' is absent in all 5 soft varieties
'Blob' is present in 6 out of 13 hard wheat varieties

'Blob' fulfills Nell's 50:50 split criterion for differentiating between hard wheat varieties

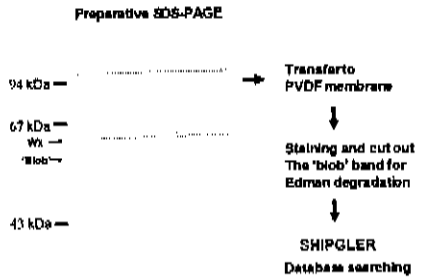
'Blob' will be a target antigen for antibody production of a varietal diagnostic reagent

The Next Step

To characterise the 'blob'

To purify the 'Blob' for immunisation and screening

Characterisation of the 'blob' N terminal AA sequencing



Full Sequence of Low Molecular Glutenin Subunit 's' group (Masci *et al.*, 1998)

'blob' SHIPGLER
GS SHIPGLERPS QQQPLPPQQT LSHHHQQQPI QQQPQQFPQQ
QPCSQQQQQP PLSQQQQPPF SQQQQPPFSQ QQQPVLPQQP
SFSQQQLPPF SQQQQPPFSQ QQQPVLPQQP SFSQQQLPPF
SQQLPPFSQQ QQPVLPQQPP FSQQQLPPFS QQLPPFSQQQ
QPVLPQQPFF SQQQQQPILP QQPPFSQQQQ PVLLQQQIPF
VHPSILQQLN PCKVFLQQQC SPVAMPQSLA RSQMLQQSSC
HVMQQQCCQQ LPQIPQQSRY EAIRAIVVSI ILQEQQQVQG
SIQTQQQQPQ QLGQCVSQPQ QQSQQQLGQQ PQQQQLAQCT
FLQPHQIAQL ELMTSIALRT LPTMCNVNVP LYRTTTRVPF
GVGPGVGGY

Low Molecular Weight Glutenin Subunits
LMW-GS 's' and LMW-GS 'm'

LMW-GS group	N-terminal sequence
's' group	SHIPGLERPS SHIPGLEKPS
'm' group	MET-SHIPGLERPS MET-SCIPGLERPS

Further Confirmation by Q-tof
Internal Peptide AA Sequence of 'Blob'

```
SHIPGLERPS QQQPLPPQQT LSHHQQQPI QQQPQQFPQQ
QPCSQQQQQP PLSQQQQPPF SQQQQPPFSQ QQQFVLPQQP
SFSQQQLPPF SQQQQPPFSQ QQQPVLPQQP SFSQQQLPPF
SQQLPPFSQQ QQPVLPQQPP FSQQQLPPFS QQLPPFSQQQ
QPVLPQQPPF SQQQQQPILP QQPPFSQQQQ PVLLQQQIPF
VHPSILQQLN PCKVFLQQQC SPVAMPQSLA RSQMLQQSSC
HVMQQQCCQQ LPQIPQQSRY EAIRAIVYSI ILQEQQQVQG
SIQTQQQQPQ QLQQCVSQPQ QQSQQQLGQQ PQQQQLAQGT
FLQPHQIAQL ELMTSIALRT LPTMCNVNVP LYR/TTTRVPF
                                   blob    TTTRVPF

GVGPGVGGY
||| ||| |
GVGTGVGAY
```

The Glutenin Enigma

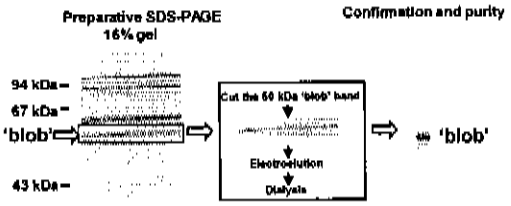
- The requirement for an ionic detergent and a disulphide reducing reagent to remove the 'blob', suggests that this peptide is not a SG surface contaminant
- The 'blob' belongs to LMW-GS 's' group because of Serine as the first aa. However, based on the C-terminal sequence, it is not completely identical to other 's' group glutenins in the database.
- Polymorphism in the 'blob' should be further analysed by full aa sequencing and also by PCR
- Location of 'blob' can not be correlated with any known allele based on available genetic information (CW)

What do we know about the
LMW-GS 's' subunit ?

- It has a theoretical MW of 42 kDa, pI 8.3
- It is one of the most abundant LMW-GS.
- It is the building block of gluten. There are 2 cysteine residues for forming an interchain S-S network, thus extending the glutenin complex.
- Durum wheats also possess a 42 kDa subunit which may play a major role in quality

Masci et al., Plant Physiol. (1998) 116:1147

Purification of 50 kDa 'Blob' Antigen for Immunisation



Purification of 50 kDa 'Blob' Antigen for Immunisation

Starting Material:	2 mg total protein extract from Kukri SG
Yield:	0.1 mg of 'blob'
Purity:	single band by silver staining
Recovery:	5% of total protein

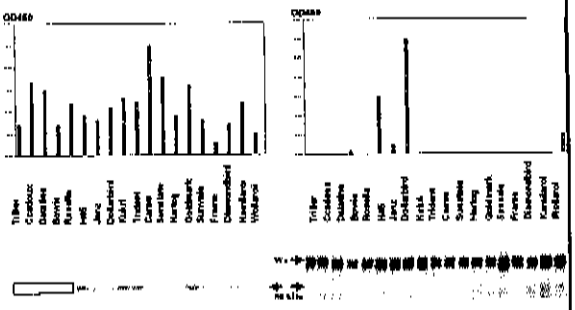
Immunisation Protocol: 50 kDa peptide (blob)

Route: Subcut - 4 sites, 50 µL/site
Dose: 10µg peptide equivalent 200 µL, 50% Emulsigen
Antigen: 50 kDa peptide isolated from SG extract of Kukri

Time Week	Treatment
0	Pre-Bleed
1	1st Injection
3	2nd Injection
6	Bleed 1 (B1)
11	3rd Injection
13	Bleed 2 (B2)

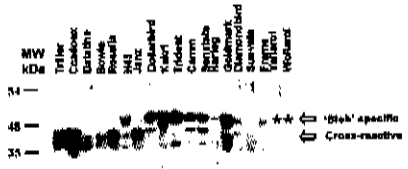
Antigen \ Adjuvant	Emulsigen
50 kDa 'blob'	Mouse No. 65, 66

ELISA Screening of 19 Soft and Hard Wheat Varieties with Mouse Antiserum against 50 kDa 'Blob' protein



4M urea extracts 8M urea extracts

Immunoreactivity of M66 x Kukri blob (B1)



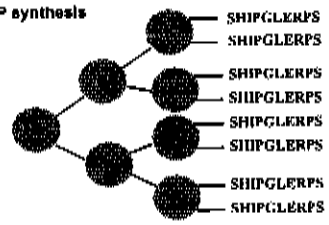
* Note that the blob is weak in SG from Durum wheats

Results of 'blob' Immunisation

Both 'blob' ELISA and Western blotting data show that 'blob' mice are now ready for fusion

Producing a Mab x SHIPGLERPS by MAP Immunisation Our 'blob' Insurance Policy

MAP synthesis



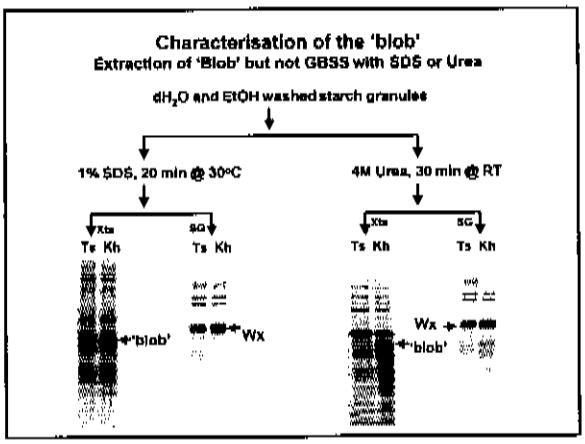
$(SHIPGLERPS)_8K_4K_2K$

Further Investigations to Resolve the blob/Glutenin Enigma

Jekyl Hyde

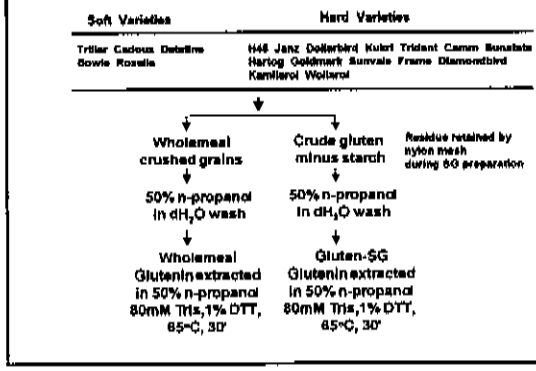
Characterisation of the 'blob'

Extraction of 'Blob' but not GBSS with SDS or Urea



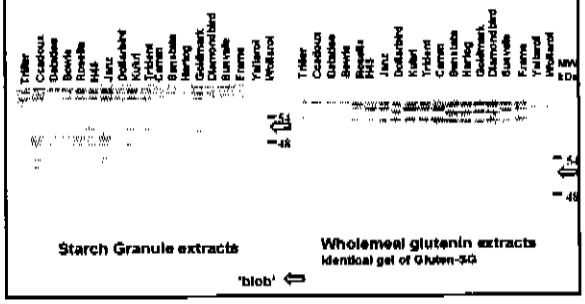
Characterisation of the 'blob'

Protocols for Glutenin Preparation



Characterisation of the 'blob'

SDS-PAGE Protein Profiles of SG and Gluten extracts from 19 Soft and Hard Wheat Varieties



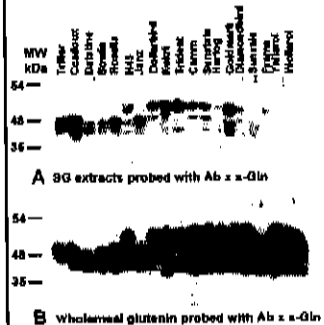
Characterisation of the 'blob'

Distribution of s-Gln and m-Gln by immunoblotting in 19 Soft and Hard Wheat Varieties

Probing antibodies:

- Mouse polyclonal antiserum against purified 50 kDa 'blob' LMW s-Gln
- Monoclonal #42327 against LMW m-Gln peptide - METSHIPGLERPS prepared by M. Sissons

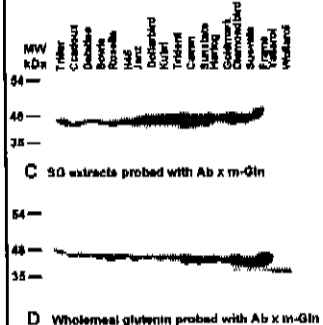
Distribution of s-Gln in Starch Granule and Wholemeal extracts from 19 Soft and Hard Wheat Varieties



Results:

- 'blob' is present in some hard varieties such as H45, Dollarbird Kukri, Trident, Camm, Sunstate, Hartog, Goldmark, Diamondbird, and in Yallaroi and Wollaroi
- 'blob' is absent in all soft varieties studied so far
- The 'blob' profiles are identical in both wholemeal glutenin fraction and starch granule protein extracts
- The s-Gln allele is associated only with either the A or B genome

Distribution of m-Gln in Starch Granule and Wholemeal extracts from 19 Soft and Hard Wheat Varieties



Results:

- LMW m-Gln is present in both hard and soft varieties
- Durum varieties Yallaroi and Wollaroi do not have m-Gln

The Sisson Ab can be used to differentiate between Durums (AB genome) and hexaploid wheats (ABD genome)

Summary - Distribution of s-Gln and m-Gln in 19 Soft and Hard Wheat Varieties

Variety	LMW s-Gln 66 kDa in endosperm	LMW s-Gln 50 kDa in starch	LMW m-Gln endosperm	LMW m-Gln starch	Hardness
Weaver	N	N	Y	Y	soft
Cadoux	N	N	Y	Y	soft
Deltaline	N	N	Y	Y	soft
Savoie	N	N	Y	Y	soft
Rosella	N	N	Y	Y	soft
H45	Y	Y	Y	Y	hard
Janz	N	N	Y	Y	hard
Buffalobird	Y	Y	Y	Y	hard
Kukri	Y	Y	Y	Y	hard
Trident	Y	Y	Y	Y	hard
camm	Y	Y	Y	Y	hard
Sunstate	Y	Y	Y	Y	hard
Herog	Y	Y	Y	Y	hard
Goldmark	Y	Y	Y	Y	hard
Dimondbird	Y	Y	Y	Y	hard
sunvale	N	N	Y	Y	hard
Frame	Y	Y	Y	Y	hard
Yallerol	Y	Y	N	N	hard
Wollerol	Y	Y	N	N	hard

Conclusions

- 50 kDa 'blob' is a glutenin peptide associated with starch granules
- The 'blob' exists in some hard wheat including durums, and is not present in all soft wheats we have studied so far
- The 'blob' is a LMW 's' Glutenin by amino acid sequencing data and database alignments
- The LMW s-Gln or the 'blob' is an excellent target antigen for generating Abs that can react with some hard wheat varieties
- Full-length blob protein and SHIPGLERPS-MAP immunised mice are scheduled for fusion and hybridoma production

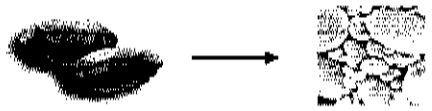
1.1.2 Antibody Diagnostics (EMAI)

Develop other immunisation strategies

Vaccinating with Starch Granules?

Starch Granule Immunisation

Starch Granule Purification



Grain is crushed and soaked overnight in Radiant Laundry Detergent (1:5 respectively)

↓

Homogenised and Filtered through Nylon mesh (65 µm)

↓

Filtrate centrifuged and pellet washed by resuspending in water x2 and ethanol x1

↓

Pellet is allowed to air dry

Immunisation Protocol: Starch Granules

The path to a multi-specific GBSS Mab?

Route	Subcut - 3 sites, 50 µL/site
Dose	50mg equivalent 50µg protein
Adjuvants	Emulsigen/Diluvac (only 1st injection)
Antigen:	Starch Granules purified from Triller and GBSS null lines

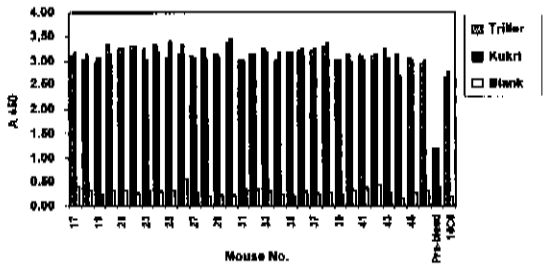
Time Week	Treatment
0	Pre-Bleed (B0)
1	1st Injection
6	2nd Injection
8	Bleed 1 (B1)
10	3rd Injection
12	Bleed 2 (B2)

SG source GBSS		Adjuvant	Mouse No.
EH9	-	Diluvac	17-19
EH9	-	Emulsigen	32-34
EQ6	7D	Diluvac	20-22
EQ6	7D	Emulsigen	35-37
EE14	7A	Diluvac	23-25
EE14	7A	Emulsigen	38-40
EF11	4A	Diluvac	26-28
EF11	4A	Emulsigen	41-43
Triller	4A, 7A, 7D	Diluvac	29-31
Triller	4A, 7A, 7D	Emulsigen	44-46

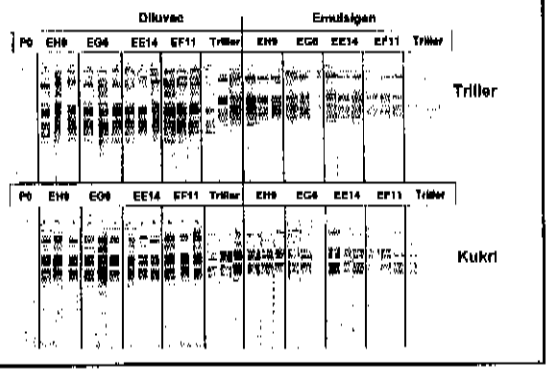
Screening Strategies
For SG Immunisation

- ELISA X SG protein extracts (8M Urea) of selected wheat cultivars and GBSS null lines
- Immunoblots of extracts from
  - SG proteins from selected wheat cultivars and GBSS null lines
  - Glutenins

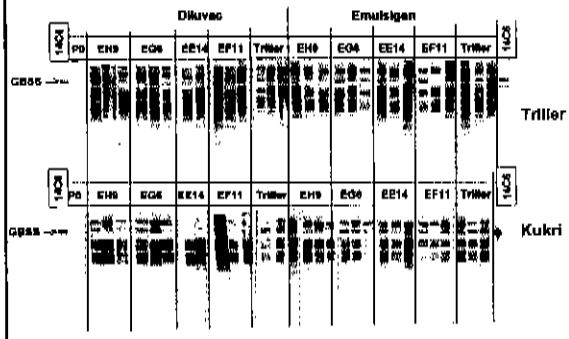
Assessment of Immune Response to Starch Granule Immunisation (Bleed 1) against 8M Urea of SG extracts by ELISA



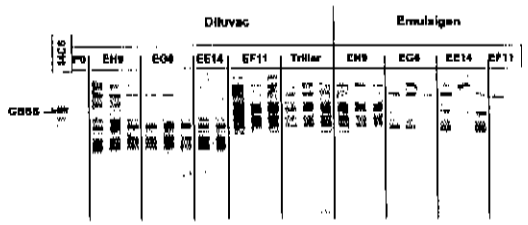
Specificity Assessment of Mouse sera (Bleed 1) on Western Blots of SG Extracts from Triller and Kukri



Specificity assessment of mouse sera (Bleed 2) on western blots of SG extracts from Triller and Kukri



Specificity Assessment of Mouse Sera (Bleed 2) on Western Blots of SG Extracts from the GBSS double null line TE (only 7A)



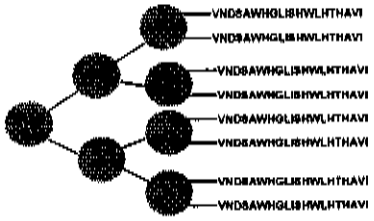
1.1.2 Antibody Diagnostics (EMAI)

Still more TARGETS?

Lipoxygenase

Preparation of Wheat Lipoxygenase Antigen for Immunisation

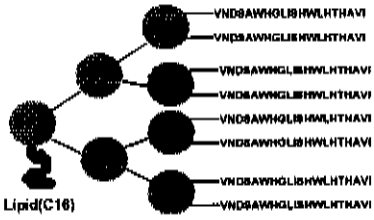
MAP synthesis



$(VNDSAWHGLISHWLHTHAVI)_8K_4K_2K$

Preparation of Wheat Lipoxygenase Antigen for Immunisation

Lipid-MAP synthesis



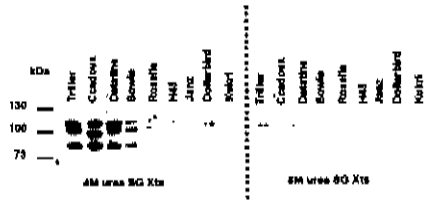
$(VNDSAWHGLISHWLHTHAVI)_8K_4K_2K$(palmatoyl)

1.1.2 Antibody Diagnostics (EMAI)

Another useful Mab?

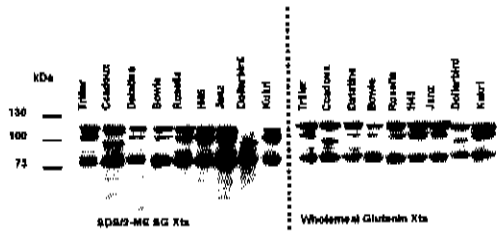
Mab 14B6 x HMW Gln

Western Blotting of HMW-GS in Sequential Urea Extracts of Starch Granule from Soft and Hard Wheat Varieties



Probing antibody:
HMW-GS Mab 1486/81 prepared by Dr. Gianach VAW-CRC

Western Blotting of HMW-GS In Starch Granule and Gluten From Soft and Hard Wheat Varieties



Probing antibody:
HMW-GS monoclonal Ab, 1484/81 prepared by Dr. Gianach VAW-CRC

Western Blotting of HMW-GS In Starch Granule and Gluten From Soft and Hard Wheat Varieties

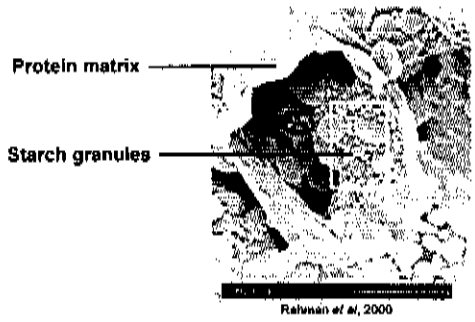
Conclusions:

- HMW-GS are present in SG of soft and hard wheat, but in striking different nature
- HMW-GS are weakly associated with SG of soft wheat (except Rosella). They can be extracted by 4M urea
- HMW-GS are strongly associated with SG of hard wheat by S-S bond. They can not be extracted by 4M or 8M urea, but can only be extracted with the aid of reducing reagent such as 2-ME.
- Thus, it is postulated that the association of HMW-GS with SG is directly related to grain hardness

1.1.2 Antibody Diagnostics (EMAI)

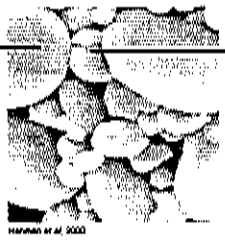
The Question of Surface vs Internal Proteins Associated with the Starch Granule

The Endosperm



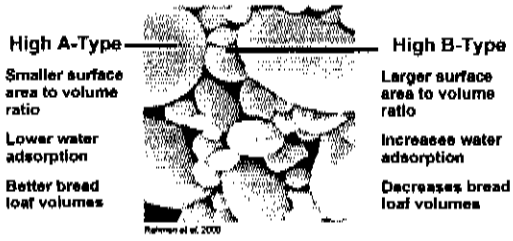
Starch Granules

Comprise 65-70% of dry grain weight



Generally, B-type outnumber A-type by 10:1

Starch Granules in Products



Starch Granule Protein

SG proteins affect end-product characteristics

SG surface protein significance

Influences SG interactions with other substances by virtue of location

↓

Influences characteristics of SG as a whole

↓

Influences characteristics of starch end-products

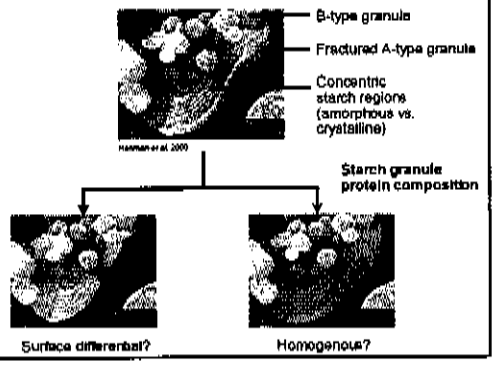
Starch Granule Protein

Common SG protein location classification

'Surface'	'Internal'
Low molecular weight (~5-30 kDa)	High molecular weight (~60-149 kDa)

- Basis for classification is ease of extractibility
- Extractibility may or may not depend solely upon location in starch granule
- Identity of true SG surface proteins is undefined

Starch Granule Protein



Characterisation of Surface SG Protein

Label surface SG protein *in situ* (biotinylation)

↓

Total SG protein extract

- SDS-PAGE
- electrotransfer to membrane
- detect biotinylated protein (enzyme conjugated streptavidin)
- identify select SG proteins via Western analysis

↓

Surface protein extractibility

- sequential urea extractions of biotinylated SG protein
- detect biotinylated protein as previously

↓

Localise biotinylated & select SG proteins on SG sections

- immunohistological characterisation under TEM

SG extraction

Crush wheat grains

↓

Steep grains in 4% Radiant laundry liquid (1:4 ratio)

↓

Gentle homogenisation, filter (65µm)

↓

Centrifugation to pellet SG

↓

Wash SG 2x in water, 1x in ethanol

↓

Air dry SG

SG Surface Protein Analysis

Biotinylation of SG surface protein *in situ*

(reactions at 4°C; 30 sec - 30 min labeling)

↓

Total protein extracted

↓

Protein separation (SDS-PAGE)

↓

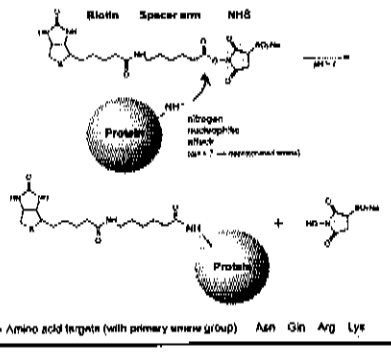
Stain gel protein | Blot protein

↓

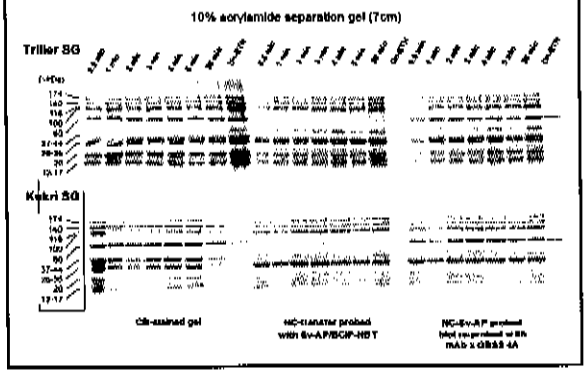
Detect biotinylated protein

(streptavidin-enzyme conjugate & anti-4A GBSS mAB)

Biotinylation of Protein

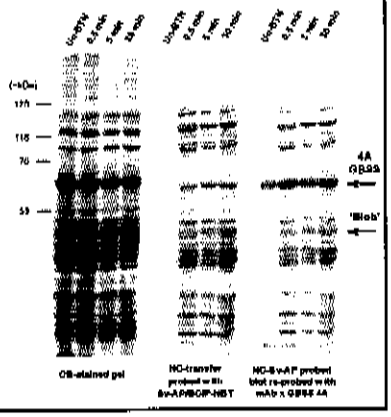


Analysis of Biotinylated Starch Granules



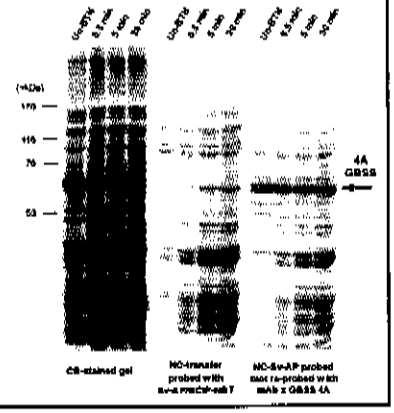
Analysis of Kukri Starch Granules

10% acrylamide separation gel (11cm)



Analysis of Triller Starch Granules

10% acrylamide separation gel (11cm)



SG Surface Protein Extractability

Biotinylation of SG surface protein
(reactions at 4°C; 30 sec - 30 min labelling)

↓

Sequential protein extractions in urea
(2M, 4M, 6M, 8M, 8M with 6% β-ME)

↓

Protein separation (SDS-PAGE)

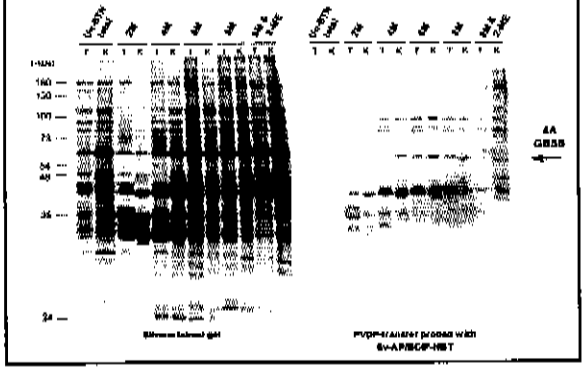
↓

Stain gel protein | Blot protein

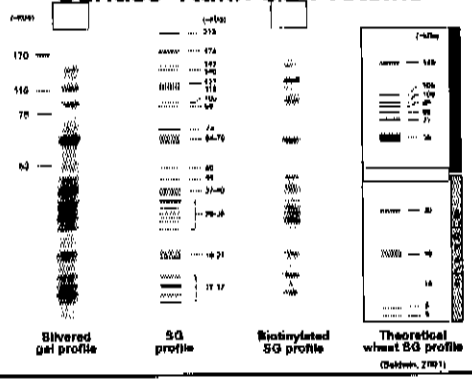
↓

Detect biotinylated protein
(streptavidin-enzyme conjugate & anti-4A GBSS mAB)

Sequential Urea Extracted Surface SG Protein



'Surface' Kukri SG Proteins



Progress

Label surface SG protein *in situ* (biotinylation)

↓

Total SG protein extract

- SDS-PAGE
- electrotransfer to membrane
- detect biotinylated protein (enzyme conjugated strepavidin)
- identify select SG proteins via Western analysis

↓

Surface protein extractibility

- sequential urea extractions of biotinylated SG protein
- detect biotinylated protein as previously

↓

Localise biotinylated & select SG proteins on SG sections

- immunohistological characterisation under TEM

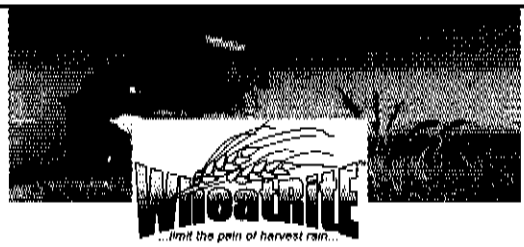
Project 1.2.3:
Diagnostics Delivery

Felice Driver

COMMERCIAL DEVELOPMENT of RAPID DIAGNOSTIC ASSAYS for the GRAINS INDUSTRY

- Core commercial participant
- Commercialise the OUTPUTS of programs 1 & 3
- Support project 1.2.3, "Diagnostics Delivery", by providing a commercially acceptable test delivery platform and associated instrumentation

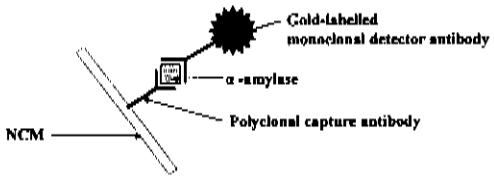
C-Qentec DIAGNOSTICS *In partnership with* and CRC



- Diagnostic tools for Australian agriculture
- Commercial license WheatRite® and ReadRite
- Quality Assurance tests for growers and the value added chain

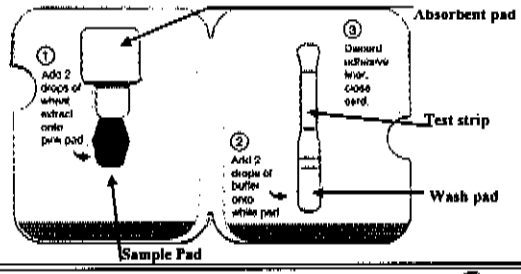
WheatRite® test principle

Double Antibody Sandwich Immunoassay



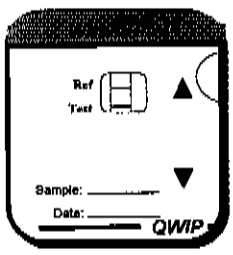
WheatRite

Patented Folding Card (Binax, USA)



TEST RESULT

- Test band intensity correlated with FN equivalents
- Speed, Reliability
- Monitor and STOP reaction
- Manufacture at AMRAD-ICT



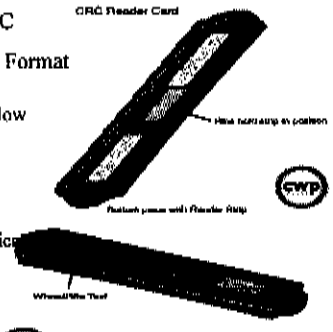
+ VE

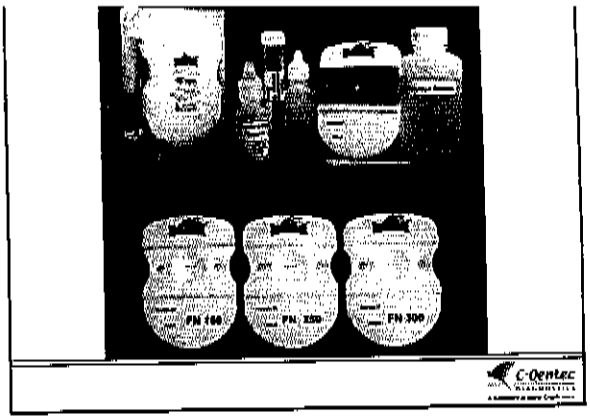
- VE

VAW-CRC

New "Dip Stick" Format

- Unidirectional Lateral Flow Device
- Molded plastic cassette
- Less operating steps
- Sample addition less critical
- Challenge stable colour development in 5 mins





Completing a WheatRite® Test:

- Add a measured volume of sample suspension to the green filter bowl.
- Add *one* drop of pink colour indicator.

- Use the sample dropperto mix the parts together.
- Leave in the filter for 30seconds.

- Press down on the green filter assembly to the final STOP position using even pressure.
- Allow the mixture to filter through completely (about 30seconds).

- Add *three* drops of wash buffer to the filter unit and allow to filter through completely

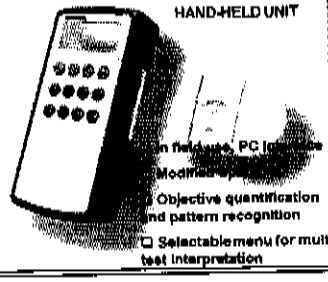
- remove the entire filter assembly from the cassette by lifting the unit off from underneath using the thumb holds at the sides.
- Add a further 1-2 drops of wash buffer to the test window of the cassette.

Interpreting Test Results:

- Results can be evaluated visually by comparison with a colour chart to estimate FN equivalents
- Alternatively placed the cassette in a ReadRite Instrument and read FN.
- SPROUTED GRAIN: two definite lines,C-control and 'T- test lines are visible in the viewing window
- Intensity of the test band indicates degree of weather damage.
- SOUND GRAIN:*one* definite line, C- control, is visible and 'T- test line is either absent or faint.

ReadRite: *Instrument manufacture for objective interpretation of test results*

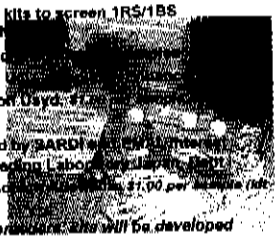
HAND-HELD UNIT



- Objective quantification and pattern recognition
- Selectablemenu for multi-test interpretation

BREEDERS' KITS: ELISA plate assay
1RS/1BS translocation and GBSS Null 4A

- SARDI assembled ELISA kits to screen 1RS/1BS translocation and GBSS
- Both kits can use cut and
- 1RS/1BS kits supplied to Program and Frank Ellison Lloyd *$230)*
- GBSS Null 4A developed by SARDI expressed by Wheat Breeding, Agriculture WA, Grain Bio cost, *$450)*
- *Next two quarters LMA*



C-Qentec

VARIETY MARKER KITS:
the COMMERCIAL FTIA –TEST FORMAT

- Panel of antibodies to be developed
- Use FTIA multi-target capability, compatible sample extraction procedures
- Rely on pattern recognition by the new generation Reader
- First target antigen will GBSS, Breeders' ELISA Kits available
- DAS-Immunoassay format using single monoclonal conjugated to colloidal gold with reactivities against GBSS 4A, 7A and 7D (IP CSIRO)
- Specific monoclonal capture antibodies striped to report 4A, 7A and (7D)separately
- "Blanks" of commercial test components available for in house use at SARDI and EMAI

C-Qentec

COMMERCIAL APPLICATIONS OF FTIA

- *WheatRite* for the detection of α-amylase in wheat, durum, barley
- Variety Identification and quality markers of wheat (GBSS 4A,7A & 7D)
- Pesticide residues in grain (GRDC/MLA)
- Mould testing Ergosterol as target analyte (GPWA)
- Mycotoxin contaminatants
- Foliar disease identification

- Suite of tests with familiar format targeted to crop production and Value Chain
- Rapid, in-field or "point of care" diagnostic tools
- Simultaneous multi-target tests
- Coordinated Reader development
- Objective interpretation and correlation with industry standards

C-Qentec

Program 3 Overview

Peter Sharp

Program 3 Genomics & Proteomics

Use advanced technologies to expand knowledge and identify reagents for use as tools

- in cultivar identification
- as markers for quality gene discovery, and in marker assisted selection
- new quality-related variants

3.1.1 Markers & Mapping Wheat Quality Traits

Developing SSR and DArT markers for wheat

Aim: Comprehensive set of high-throughput markers for wheat genetics and breeding

Matthew Hayden Plant Breeding Institute

Mona Akbari CAMBIA

3.1.2 Wheat Grain Proteomics & Bioinformatics

Identifying grain proteins of potential significance

Aim: Knowledge of grain proteins related to quality traits or of potential use in cultivar identity

Daniel Skylas Australian Proteomics Analysis Facility (APAF)

3.1.3 Targeted mutagenesis of wheat

Making specific new variation in wheat quality traits

Aim: Develop targeted mutagenesis for wheat as use to discover and make new variation

Chong-Mei Dong Plant Breeding Institute (Cibus Genetics, San Diego)

Project 3.1.1: Markers and Mapping
SSR marker development

Matt Hayden

Project 3.1.1

Markers and Mapping Wheat Quality Traits

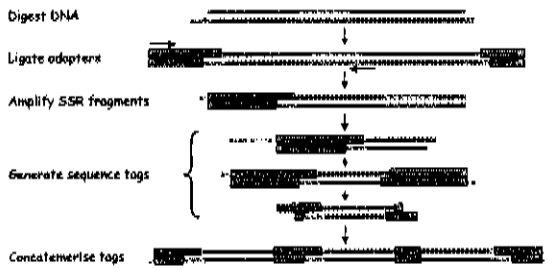
Introduction

- Molecular markers are widely used in agriculture
- Many different opinions about how markers can be best utilised
- General consensus is a need for marker technologies to enable the cost-efficient development of informative markers, which are amenable to high throughput assays

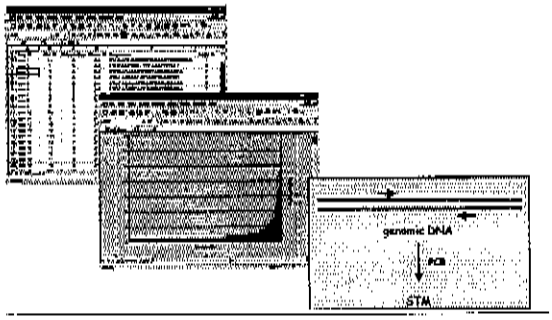
Objectives

1. Use sequence tag profiling (STP) technology to develop a large number of mapped SSR markers that can be assayed using high throughput methods
2. Adapt the STP technology to enable the discovery and high-throughput analysis of SNPs

Overview of STP procedure



Sequence tag analysis

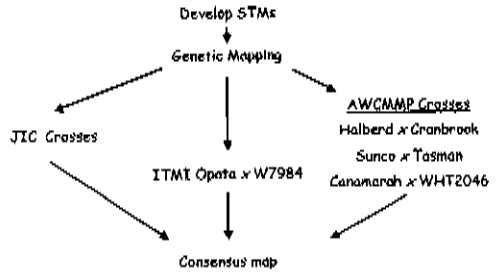


Development of a STM map in bread wheat

- Joint project between John Innes Center (JIC), AWCMMP and VAW-CRC
  - AWCMMP and JIC to each generate ~1000 mapped STMs

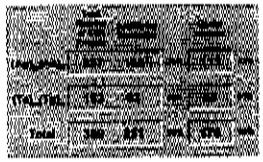
Outcome: A consensus SSR map of bread wheat, relevant to current Australian and European germplasm, and fully integrated with published genetic maps

Strategy

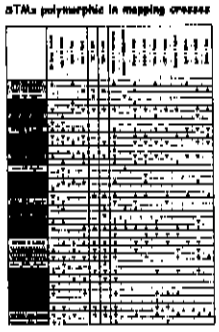


Progress - JIC Project

- Sequenced 6 tag libraries
- Tested primers for 2 libraries



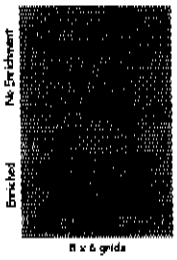
- ABI3700 platform using 5 dye system, multiplex PCR and multiloading



Progress - AWCMMP Project

- Constructed 8 tag libraries
- Partially sequenced 4 libraries
- Extracted DNA for mapping populations
- Optimised procedures for fluorescent-based analysis of STMs
- Commenced polymorphism screening and mapping using existing STM markers
- Developed procedure to enrich STMs for microsatellite repeat length

Dot blot hybridisation of STM fragments using a $(GT)_{20}$ oligonucleotide probe



Why STMs Are Useful Genetic Markers

- Reduced cost of marker development
  - ➢ high sequencing throughput
  - ➢ can generate "super" libraries
  - ➢ only one specific primer is required for marker amplification
- Cost efficient fluorescent-based detection of STMs
- Highly amenable to multiplex PCR
- Can convert STMs to conventional SSRs
- Can convert conventional SSRs to STMs

3-plex STM amplification in Sunco x Tasman population

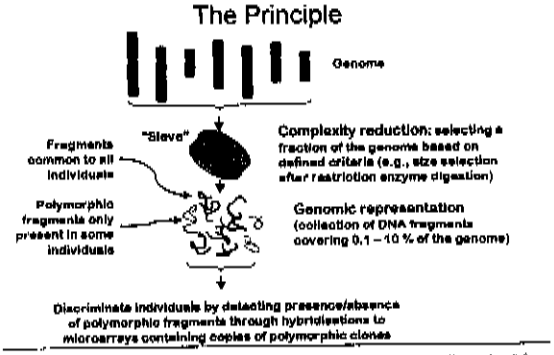
Project 3.1.1: Markers and Mapping
Diversity Array Technology

Andrzej Kilian

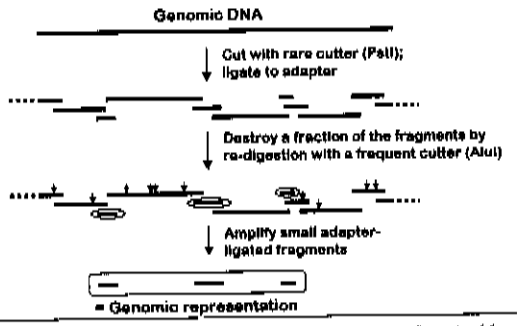
Development of Wheat DArT

Mona Akbari
Ilaria Catizone
Andrzej Kilian

The Principle



A Closer Look at Complexity Reduction



Wheat Libraries

T. ***Aestivum*** – Hexaploid - 13 cultivars (Australian)

T. ***Durum*** – Tetraploid – 32 cultivars (Italian)

- Pooled cultivars – digested with *PstI* and ligated *PstI* adaptors
- Redigested with *MseI* or *AluI*
- PCR amplified and cloned fragments into PCR2.1 TOPO vector

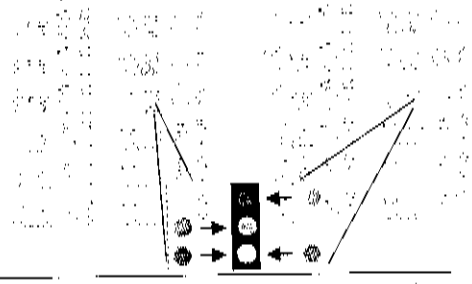
Tetraploid – *PstI/AluI* and *PstI/MseI* libraries - 1280 clones each

Hexaploid – *PstI/MseI* library - 1280 clones

Polymorphic Features: Green or Red

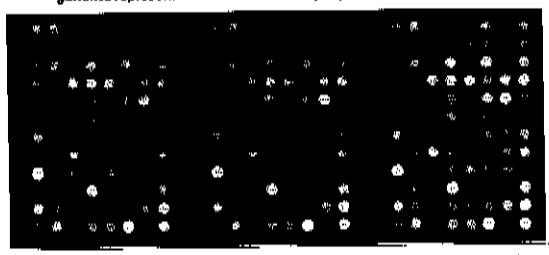
Green: Is fragment present in genomic representation of Clipper?

Red: Is fragment present in genomic representation of Sahara?



Detection of polymorphic DArT clones in hexaploid wheat

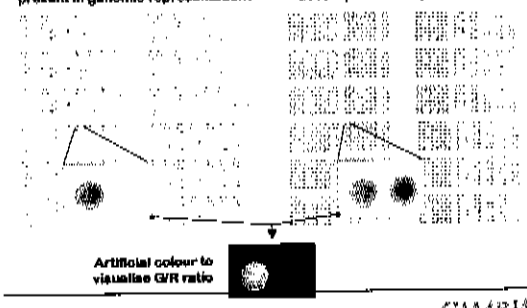
Three replicates of a panel of 96 random clones, hybridised with genomic representations from cv Grebe (red) and Westonia (green).



Scanned Images

Genotyping channel: Is fragment present in genomic representation?

Reference channel: How much DNA does a particular spot contain?

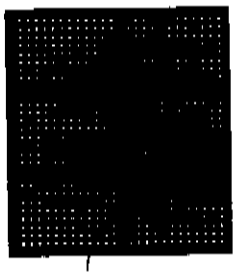


Artificial colour to visualise G/R ratio

Automatic Data Extraction

Algorithm

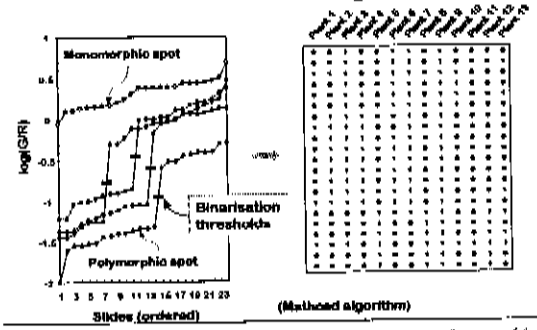
- Localises spots in scanned images
- Measures G and R intensities for all pixels in a spot
- Subtracts background G and R intensities
- Computes G/R ratio for all pixels in a spot
- Selects the most informative subset of pixels
- Provides an estimate for the G/R ratio of a spot
- Normalises the G/R ratio of all spots to compensate for gradients across the array



White areas: spots recognised by software

(Method file embedded in Excel)

Binarisation → Scoring Table

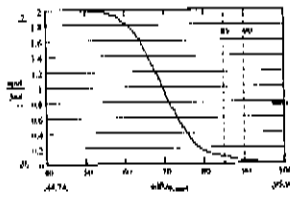


(Method algorithm)

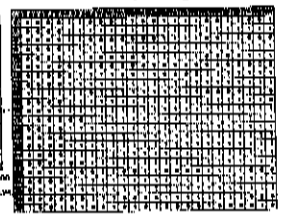
Spot Dimorphism Analysis

Hexaploid Wheat– Cultivar vs TOPO

Q Function plot

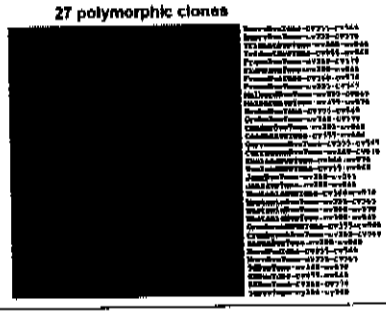


Binary scoring table

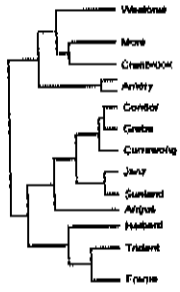


Cluster Analysis

27 polymorphic clones

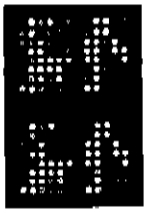


Dendogram from Cluster Analysis



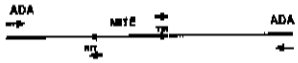
Analysis based on 16 polymorphic clone subset

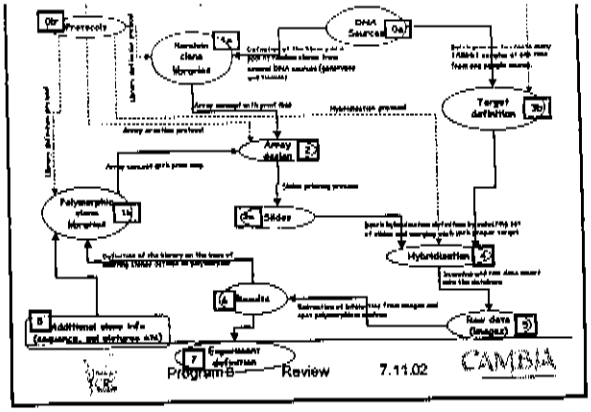
MITE display using DArT format



- Miniature Inverted Repeat Transposable Elements – superfamily of DNA type transposons,
- abundant in eukaryotic genomes (thousands of copies) – wheat including!
- No obvious clustering in the genome, good genome coverage
- Recognized source of genetic variation (insertion/deletion events)
- Restriction digestion/ligation and two step PCR using Terminal Inverted Repeat primer (round 1 & 2) and adapter primer (round 2)

Stowaway MITE family
Azucena versus IR64





Project 3.1.2:
Wheat Grain Proteomics

Daniel Skylas

3.1.2 WHEAT PROTEOMICS

(1) Proteome studies of soft wheats
- Bowie and Rosella wheat cultivars were used for the analysis
- Comparisons were made in terms of protein composition
  - wholemeal protein fractions
  - starch granule protein fractions
  - storage gliadin protein fractions
- Leading to the characterisation and identification of a number of cultivar-specific target proteins
- Project has been finalised and targets can now be provided to diagnostics

(2) Serine protease inhibitor (serpins) polymorphism
- Serpins were extracted from a number of Australian bread wheats
- Cultivars were screened to investigate the extent of polymorphism
- Serpin polymorphisms characterised

(3) Proteomics of wheat germ tissue (PhD student Yunxian Mak)
- Brief summary of work completed so far

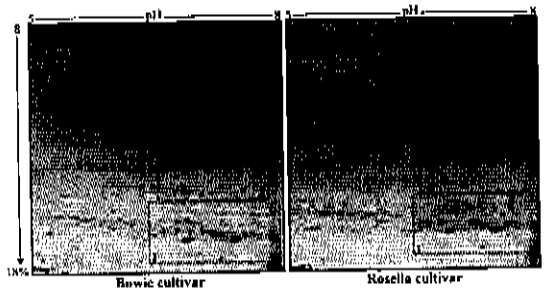
(1) Proteome studies of soft wheats

AIM: To investigate, characterise and identify cultivar-specific proteins existing between Bowie and Rosella, which could be used for cultivar discrimination purposes

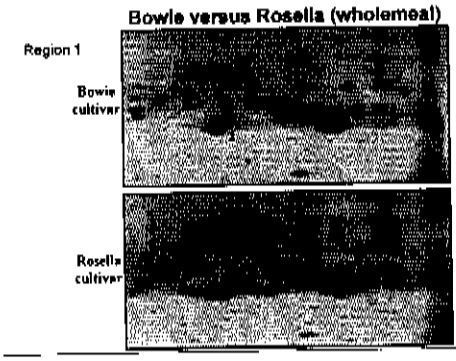
(1) Wholemeal protein fractions
- Flour samples were prepared for 2-DE
- Proteins were initially screened and separated using an array of IPGs; pH 3-10, 4-7, 5-8, 6-9 and 6-11
- As a result of the screening, the pH 5-8 range was chosen for analysis

- Cultivar-specific proteins were cut from the gel and characterised by peptide mass fingerprinting (PMF), tandem mass spectrometry (MS/MS) or *N*-terminal amino acid sequencing

Bowie versus Rosella (wholemeal)



Bowie versus Rosella (wholemeal)



Bowie versus Rosella (wholemeal)

In total, 8 cultivar-specific proteins selected for analysis

Characterisation of target proteins

PMF analysis:

Spot #	Matching protein	Peptide hits	Sequence coverage (%)	Organism	Accession No.	Function
1	Endogenous alpha-amylase/ subtilisin inhibitor	5	30.6	Wheat	P16347 (swiss-prot)	Inhibitor of endogenous alpha-amylase and subtilisin

Only spot # 1 could be matched using PMF

Reasons for this-
insufficient tryptic digestion (cleaves C-terminal side Lys/Arg residues)
insufficient extraction of peptides from gel
limited wheat protein sequence database

As a result of this, other methods were used to provide internal or *N*-terminal amino acid sequences

Tandem MS (or MS/MS) analysis:

Spot #	Experimental peptide mass and sequence (Da)	Database match	Matching protein sequence	Organism	Accession No.
6	Peptide 1 1203.78 VTYPIMADPDR	Peroxiredoxin	VTYPIMADPDR (residues 94-104)	Wheat	P52572
	Peptide 2 968.7 AVDSLLTAAK	Peroxiredoxin	AVDSLLTAAK (residues 158-167)	Wheat	P52572
	Peptide 3 1712.02 MFPQGFETADLP	Peroxiredoxin	MFPQGFETADLP (residues 195-207)	Wheat	P52572

Peroxiredoxin is an antioxidant
Tissue specificity: embryo
Developmental stage: expressed during late development in the aleurone and embryo

N-terminal amino acid sequencing:

Spot #	N-terminal sequence	Matching sequence	Gene product	Identity (%)	Organism	Accession No.
7	VRVPVPQL	VRVPVPQL	Alpha-gliadin	100% in 8 aa	Wheat	BAA12318
8	VRVPVPQL	VRVPVPQL	Alpha-gliadin	100% in 8 aa	Wheat	BAA12318

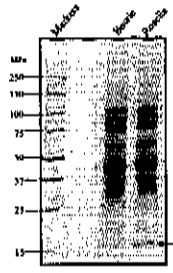
Protein spots 2, 3, 4 and 5 have not been identified after a number of attempts

(1) Proteome studies of soft wheats

AIM: To investigate, characterise and identify cultivar-specific proteins existing between Bowie and Rosella, which could be used for cultivar discrimination purposes

(2) Starch granule protein fractions
- Starch granules were isolated from Bowie and Rosella cultivars (EMAI; Ming, James and Thomas)
- Proteins were solubilised using SDS-reducing buffer
- Proteins extracts were fractionated by 1-D SDS-PAGE
- Proteins were also blotted to PVDF membrane and probed with antibody (mouse anti-puroindoline-A)

SDS-PAGE analysis of starch granule protein extracts

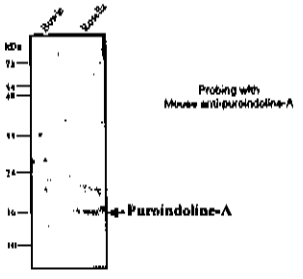


N-terminal sequence	Gene product	Identity (%)	Organism	Accession No.
EVNGGGAGQG	Puroindoline-A (precursor)	100% in 10 aa	Wheat	P33432

Comments on database match
N-terminal tag starts at residue 29 as the signal sequence of the precursor protein consists of residues 1-19 and there is a propeptide sequence, being residues 20-28

The mature chain of this protein consists of residues 29-148 Therefore, our sequence commences at the N-terminus of the mature chain-puroindoline-A

Western blot of starch granule protein extracts using anti-puroindoline-A antibody (conducted at EMAI)



(1) Proteome studies of soft wheats (Bowie and Rosella cultivars)

Summary

For Wholemeal samples
A total of 8 cultivar-specific proteins were analysed
- Alpha-amylase/subtilisin inhibitor
- Peroxiredoxin
- Two alpha-gliadins
- Remaining 4 spots not identified

For starch granule samples
Cultivars can be distinguished using anti-puroindoline-A antibody
Identity of band confirmed by N-terminal sequencing and immunoblotting

For storage protein samples (gliadins)
This work was not discussed here
Gliadin fractions (using 70% ethanol) were prepared and analysed by 2-DE
In total, 18 cultivar-specific gliadins were characterised and identified using N-terminal seq.
8 from Bowie and 10 from Rosella

(2) Investigation of serpin polymorphism in bread wheats

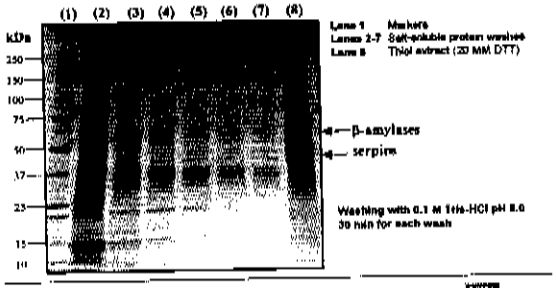
Collaboration with Dr Thomas Roberts (Biological Sciences, Macquarie Uni)

AIM: To characterise and identify serpin polymorphisms in Australian bread wheats

Background information
- Serine protease inhibitors (abbreviated as serpins)
- Are found throughout the plant kingdom
- They have been studied in a variety of plants-barley, wheat, rye, oat and pumpkin
- Structural and functional investigations of serpins in higher eukaryotes such as insects and humans number nearly 2000
- Six serpin forms have been identified in the grains of hexaploid wheats
- Five of these serpins have been cloned and purified from *E.coli*
- They are major albumins of wheat endosperm (~3-4 mg serpin/gram of grain)
- The mobility of specific cereal grain serpins using the native PAGE system is reproducible and anexcellent means of testing for polymorphisms in wheat

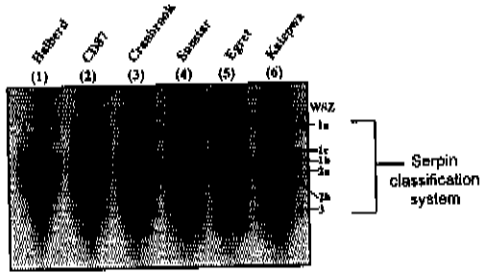
(2) Investigation of serpin polymorphism in bread wheats

Extraction of serpins from Cranbrook wheat cultivar
(method taken from Østergaard *et al.*, 2000)

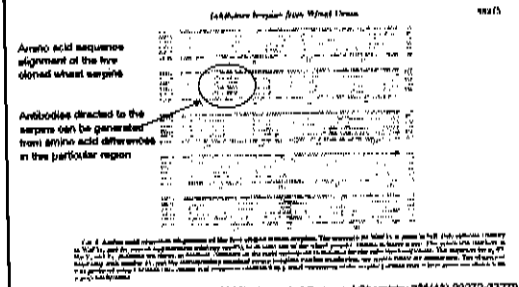


(2) Investigation of serpin polymorphism in bread wheats

Visualisation and screening of wheat serpins using native PAGE system



(2) Investigation of serpin polymorphism in bread wheats
Potential for diagnostics



Taken from Østergaard *et al.* (2000), *Journal of Biological Chemistry* 275(43) 33272-33279

(2) Investigation of serpin polymorphism in bread wheats

Summary of results so far:

Halberd x Cranbrook
- Do not contain polymorphism when compared to each other
- But are polymorphic for WRZ1a when compared to the rest of the cultivars which were screened

CD87 x Katepwa
- Do not contain polymorphism when compared to each other

Sunstar x Egret
- Are polymorphic for WRZ3 when compared to each other

Further investigations of serpin polymorphisms in:
Sunco x Tasman
Kukri x Janz
Kite x Bindawarra

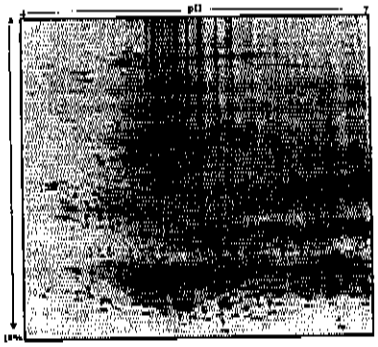
Hopefully, there will be polymorphisms in these varieties which could be mapped (this project is continuing)

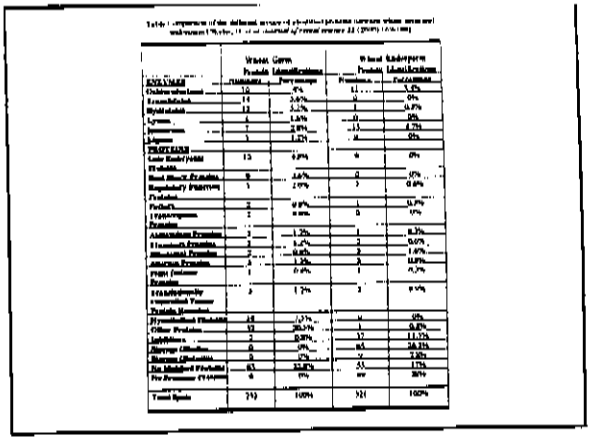
(3) Proteomics of wheat germ tissue
(PhD student Yunxian Mak)

Brief summary so far:

- Wheat germ samples were obtained for the Rosella wheat cultivar (provided by Di Miskelly)
- Sample preparation of germ was optimised (eg. removal of lipids with hexane)
- Proteins were fractionated by 2-DE
- Over 250 individual protein analyses were carried out by PMF in order to extensively characterise wheat germ protein composition
- Germ proteins were identified by interrogation of SWISS-PROT and trEMBL databases

Wheat germ tissue proteome (Rosella cultivar)





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Colin Wrigley
Peter Sharp
Neil Howes

Project 3.1.3:
Targeted Mutagenesis

Peter Sharp

3.1.3 Targeted mutagenesis of wheat

Aim: Develop targeted mutagenesis for wheat as use to discover and make new variation

Use chimeraplasty technology of Cibus Genetics

Chimeraplasts

- hybrid DNA/RNA oligonucleotides targeted to specific gene region
- on insertion to plant cells these cause specific mutations in the targeted gene region

3.1.3 Targeted mutagenesis of wheat

Project started recently

- project agreement with Cibus Genetics LLC
- appointment of Dr Chong-Mei Dong

3.1.3 Targeted mutagenesis of wheat

Initial work:

- tissue culture (haploid embryos, microspores)
- bioloistic delivery (model system - GFP)

Proof of concept:

- transient assay - mutant GFP + chimeraplast
- selectable system (herbicide tolerance) giving whole regenerated plant with mutation

3.1.3 Targeted mutagenesis of wheat

Generation of non-selectable mutations:

- waxy gene nulls (antibody/iodine staining)
- other genes of quality relevance, e.g.
  - HMW Glu nulls, new/deleted cysteines
  - starch branching enzymes
  - hardness locus (purindolines, etc)
- service for wheat functional genomics