VALUE ADDED WHEAT CRC PROJECT REPORT

Diagnostics for Variety and Quality-type Identification

Report of Workshops held
18th July and 27th August 2001

Compiled by:

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VAWCRC and Food Science Australia

Date: August 2001

VAWCRC Report No: 3
Copy No: 30

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DIAGNOSTICS FOR VARIETY AND QUALITY-TYPE IDENTIFICATION

Report of Workshops held 18th July and 27th August, 2001

The development of new diagnostic tests for wheat quality was identified as an industry need of high priority in preparing the renewal application of the Wheat Cooperative Research Centre during 2000/2001. Tests for varietal identity and for specific aspects of grain quality were the main requirements indicated in discussions with industry representatives. Accordingly, a Diagnostics Program was created in the new Wheat CRC, as the first of five programs. At the outset of planning the research directions, it was considered imperative to have further consultation with representatives of the Australian wheat industry to ensure that the new CRC's research in diagnostics was directed along the most worthwhile avenues.

The consultation process was undertaken in two stages. Firstly, a workshop was convened (on July 18th, 2001) to determine what types of diagnostics would be feasible, especially bearing in mind in advances made in the earlier research activities of the Quality Wheat CRC. This workshop brought together research scientists of the 'old' and 'new' Wheat CRCs, together with representatives of companies involved in the new Wheat CRC.

Secondly, a workshop was held (on August 27th, 2001), involving a wider range of wheat industry representatives, to obtain information about the types of tests needed by various branches of the industry, the likely requirements for speed and accuracy, and the volume of testing that would be expected.

This report provides a record of those two workshops, mainly in the form of print-outs of overhead- or computer-projector slides used in the presentations. These are supplemented by written summaries of comments during discussions.

At both workshops, participants were provided with copies of the following report, which provides information about a large number of Australian wheat varieties, assembling data from research scientists (see author list) and from the annual conference handbooks of the Cereal Chemistry Division of the Royal Australian Chemical Institute. This publication is entitled:
"Current Australian Wheat Varieties: Grain Quality Data." by Wrigley, C.W., Cracknell, R.L., Miskelly, D., Cornish,G.B., Sharp, P., and Mares, D. (2001). It is Report No 48 of the Quality Wheat CRC, North Ryde, NSW.

DIAGNOSTICS FOR VARIETY
AND QUALITY-TYPE IDENTIFICATION:
Workshop #1 SCIENTIFIC FEASIBILITIES
held 10 am, Wednesday, 18th July, 2001,
Food Science Australia, Delhi Road, North Ryde

PROGRAM:
Objectives and framework for discussions – Colin Wrigley

Discussion of industry expectations – Comments from Bob Cracknell, Ray Moss, Andrew Kennett, Felice Driver and Richard Daniel

Achievements of QW CRC diagnostics research. Part 1 - Kevin Gale

Achievements of QW CRC diagnostics research. Part 2 -Thomas Giersch

ICT-Lateral flow issues - Gill Mearns

How proteomics can contribute – Brad Walsh and Daniel Skylas

Plans to use proteomics for VAW-CRC immuno-diagnostics
– James Chin

Plans for VAW-CRC protein-analysis diagnostics – Ian Batey

Summary comments – Mike Perry, Neil Howes

Participants:
Kevin Gale, CSIRO Plant Industry
Thomas Giersch, CSIRO Plant Industry
James Chin, EMAI, NSW Agriculture
Bob Cracknell, AWB Ltd
Ray Moss, Goodman Fielder
Andrew Kennett, Arnotts
Mike Perry, GRDC
Brad Walsh, APAF
Daniel Skylas, VAW CRC and APAF
Felice Driver, C-Qentec
Richard Daniel, C-Qentec
Siri Siriamornpun, Uni NSW
Gill Mearns, VAW CRC
Alan Ellis, VAW CRC
Bill Rathmell, VAW CRC
Colin Wrigley, VAW CRC and FSA
Neil Howes, SARDI
Ian Batey, VAW CRC and FSA

Objectives and framework for discussions

– Colin Wrigley

Food Science Australia
and VAW CRC,
North Ryde

Objectives and framework for discussions – Colin Wrigley

Diagnostic tests needed for wheat quality ... [G x E]

➤ **Variety identification;** environmental factors are not indicated [G x E]
  ➤ **Grade suitability**
  ➤ **Royalty payments**
  ➤ **Ingredient wheats**

➤ **Quality-type identification [G x E], especially –**
  ➤ **Dough strength & extensibility**
  ➤ **Grain hardness**
  ➤ **Starch type**
  ➤ **Water absorption**
  ➤ **Milling yield**
  ➤ **'Ingredient wheats'**

Variety and quality-type testing needed along the utilisation chain

➤ **breeding**   - screen for quality; certainty of genotype
➤ **pure-seed production**   - certainty of variety
➤ **on-farm QA**   - verification of variety as seed
➤ **receival testing**   - verification of variety & grade type
➤ **buying**   - verification of variety & grade type
➤ **transport**   - verification of variety mix
➤ **marketing**   - verification of variety mix
➤ **processing**   - trouble shooting, id'n of variety mix

<u>Constraints imposed</u> by situations requiring diagnostics

➤ **For breeding, pure-seed, post receival, during transport, in processing**

  ➤ **Discriminating and objective**

  ➤ **Modest time constraints**

  ➤ **Plenty sample**

  ➤ **Homogeneity of sample?**

  ➤ **Cost per sample is critical**

  ➤ **So, multiple sample analysis**

  ➤ **<u>Central lab</u> appropriate**

➤ **At receival, on-farm, at export terminal**

  ➤ **Discriminating and objective**

  ➤ **Speed needed**

  ➤ **Simple test, with minimal facilities**

  ➤ **Small numbers of samples**

  ➤ **Cost per sample is critical**

  ➤ **<u>On-the-spot</u> tests needed**

Methodologies available

➢ **The 'grape vine' and 'stat. dec.'**

➢ **Grain morphology**   - On-the-spot

   ➢ **Image analysis, SKCS** - Central lab

➢ **Simple tests, e.g. phenol test, combined with...**

➢ **Protein composition**

   ➢ **Antibody-based kits** - On-the-spot & central lab

   ➢ **Gel electrophoresis** - Central lab

   ➢ **RP-HPLC**   - Central lab

   ➢ **Mass spectroscopy**   - Central lab

   ➢ **Capillary electro'sis** - Central lab [& on-the-spot]

   ➢ **DNA analysis**   - Central lab

   ➢ **Automatic interpretation** (e.g. PatMatch proram)

   ➢ **Specific marker proteins,** using any of the above methods

      ➢ Specific glutenin subunits, waxy proteins

REMEMBER ... Statistical evaluation of results needed,
            especially for heterogeneous samples

Scenarios:-

➢ **On-the-spot** (at silo, buyer)

Speed and portability

➢ **Regional lab** (back-up for silo, breeder, export terminal, miller)

Modest requirements for speed, expertise, equipment costs

➢ **Central lab** (contractor, post-harvest analysis of silo samples)

More elaborate equipment
Greater expertise
Large numbers of samples

Specific marker proteins

Quality trait	Marker proteins	Method
Genotype	Gliadins	A-PAGE, RP-HPLC, CE
Dough strength	HMW (LMW) glutenin subunits	SDS-PAGE, RP-HPLC, CE
Grain hardness	Purindoline a & b	Complex extract'n + SDS-PAGE
Null 4A (noodle)	GBSS 4A or not	Complex extract'n + SDS-PAGE
Waxy (ingredient)	No GBSS	Complex extract'n + SDS-PAGE
Low (Hi) %B starch		
High water absorp'n		

DISCUSSION OF INDUSTRY EXPECTATIONS

Comments by Bob Cracknell,
Senior Wheat Quality Specialist, AWB Ltd

There are two major applications for variety identification in the Australian wheat industry. Firstly, there is a need to check that varietal declarations at receival are indeed correct, as these are the cornerstone of the quality-classification system. Secondly, with the increasing use of Plant Breeders' Rights by breeders to secure a return on their investments in wheat breeding, there is a need to ensure that growers are not mis-declaring varieties in order to avoid paying end-point royalties.

It was also noted that Canada is giving serious consideration to dropping its dated and controversial Kernel Visual Distinguishability (KVD), the requirement for all but its top quality CWRS and Durum classes, and moving to an affidavit system similar to our own. This move could provide another substantial market for rapid on-site variety-identification methodology.

Traditionally, post-harvest laboratory-based electrophoretic methods have been used to check samples randomly drawn at harvest in order to monitor the accuracy of variety declarations. These have generally shown very low levels of false declaration, and when detected, these have often not been to the advantage of the farmer, suggesting that they were simple mistakes.

The introduction of variety-based pools for payment purposes under the recently introduced Golden Rewards payment for quality initiative has, to a certain extent, reduced the incentive for growers to mis-declare varieties. Now, by simply growing the right variety, the grower is rewarded regardless of where the delivery is ultimately binned. However situations still exist, and with the introduction of more specialty wheats, will continue to exist, where there are real incentives for growers to make false declarations. The most obvious one is the WA noodle segregation where premiums for modest protein levels of around 10.5% can be in the $20-30 per tonne range.

Variety declaration is the cornerstone of the wheat classification system, as together with simple physical quality tests and an NIR test for protein and moisture at receival, deliveries can be rapidly categorised. The major failing of this approach, however, is that it does not measure seasonal effects on quality, particularly on dough strength, which can vary considerably from year to year under Australia's volatile seasonal conditions.

Ultimately therefore, a "black box" approach is required, with an instrument capable of measuring at receival all of the key quality parameters, and particularly dough strength, which would then allow us to bin deliveries on their precise quality, rather than relying on the indirect method of variety declaration as we do now.

This then leaves the need to confirm variety declarations for end-point royalty collection purposes. Initially these were quite low in value, providing little incentive for growers to make false declarations. However, the industry is now seeing the development of higher performing varieties, some handled under contract-based marketing arrangements with end-point royalties of $5 per tonne being mooted. These create new challenges for the owners of these varieties wishing to ensure that growers are not making false declarations in order to avoid this significant charge, and real incentives for growers to falsely declare in order to participate with the wrong variety in a lucrative, but high-quality pool.

Clearly all of the foregoing demonstrates the need for rapid tests for variety identification that can be applied at the point of delivery.

DISCUSSION OF INDUSTRY EXPECTATIONS

<u>Comments presented by Ray Moss,</u>
<u>Goodman Fielder Milling</u>

Goodman Fielder hope that the work on variety / quality ID will underpin our own work on wheat quality. Currently, the company relies on variety ID for a selected range of products, mainly noodle and biscuit grists. Last year 7,000 tonnes of GF noodle wheat were found to be contaminated with non-noodle varieties, which indicates the potential value of such tests in screening. For breadmaking purposes, there may be more leeway as far as variety is concerned. Mixing time is still important but not necessarily short mixing time for all processes. As indicated by other speakers, quality ID is the ultimate goal as it takes into account the interaction of genotype with environment.

Variety / Quality ID is becoming ever more important to millers, due to more stringent customer specifications and other factors. Changes in grain-marketing practices, increased use of contract growers and agents other than AWB will also increase the need for quick, cheap variety ID kits. Previously, when purchasing wheat through AWB, there was the possibility of swapping within grades in order to optimise quality. The scope of AWB wheat segregations is also changing, and currently there is no soft segregation made by AWB. These sorts of changes have resulted in GF having to train our own silo operators as grain classifiers, and variety ID kits would be a useful tool in such classification.

The requirements for test kits are as outlined in the aims of Quality Wheat CRC Project 5.1.8, i.e. rapid [<10 min]; require minimal training; few steps, results readable by eye and inexpensive.

Mass Spectroscopy also appears to have potential, as indicated by Brad Walsh. GF have had preliminary discussions with Bio Test in WA concerning Mass Spectroscopy applications for wheat quality and wonder whether it is worthwhile for the CRC to contact them re possible involvement.

DISCUSSION OF INDUSTRY EXPECTATIONS

<u>Comments presented by Andrew Kennett, Arnotts</u>

Objectives
To develop technologies which will permit "on the spot" diagnosis of a range of wheat quality attributes for growers, buyers, handlers and processors of wheat, thereby improving classification, storage, handling and processing.

Specific Objectives
- Rapid diagnosis of wheat's suitability for specific processes, meeting the needs of breeders, grain handlers, millers and processors.
- Automated identification of varieties and ability to identify starch-type and quality-related proteins.
- Ability to identify varieties and quality type under field conditions by development of diagnostic kits, to meet the needs of growers and farm advisers.
- New, automated grain testing protocols for application to standard grades and product-based segregations, with protocols and calibration available for worldwide use.

Strategy
- Application of new methods (capillary electrophoresis) to protein analysis for routine quality assessment, targeting dough strength and extensibility, grain hardness, starch characteristics, water absorption and milling yield.
- Development of rapid diagnostic tools based on antibodies.
- Bar-code identification of varieties based on WheatRite technology.
- Integrated use of advanced methods for automated, objective grain grading.

Variety Test Structure
Variety Tests can be placed in three tiers.
Tier 1
Expensive, skill-intensive, highly-accurate slow methods used at a central laboratory. These methods are used to validate other methods and as research tools. Each year there may be 10s to 100s of tests. Likely methods are capillary electrophoresis and HPLC (already in use to some extent). Per test and equipment/training costs are likely to be high.
Tier 2
Methods suitable for a regional laboratory, these would need to be fairly rapid and may need substantial skill but can make use of expensive equipment. Each year they may be 100s of tests. Likely methods are capillary electrophoresis and HPLC (already in use to some extent) and antibody tests with a reader (e.g. ELISA). Per test costs should be low but equipment/training costs are likely to high.

Tier 3

Methods suitable as on-the-spot tests at receival stations (testing trucks) or on-farm need to be quick, cheap and easy to use. Each year there may be 10s to 100s of tests. The most likely method is an antibody test card like the WheatRite™. Per test and equipment/training costs should be low.

What this means for Arnotts

Arnotts is the only, or at least almost the only, flour/wheat user that buys on variety as well as quality grade. This program offers the opportunity to develop tests that could be used to identify the variety in a truckload of wheat and so ensure that Arnotts get what it wants (and is paying for). Currently the grower may be asked to sign a declaration but this is rarely crosschecked because of the lack of suitable methods. As the wheat value chain develops and farmers are charged for an end-point royalty for premium varieties and this cost (as well as the cost of the premium variety) flows onto the end user, then variety testing becomes even more important.

So the benefits will be:

- Improved ingredient quality by preventing unwanted wheat entering the supply chain.

- Improved flour supply as growers move to grow premium varieties that provide a premium price.

- Protection to Arnotts by ensuring that what Arnotts is paying for, Arnotts is getting.

Arnotts involvement

The two varieties that Arnotts already requires with high purity provide an ideal test of the methods. Arnotts also wants certain varieties excluded from the general Arnotts soft blend, so once again variety identification is important.

Proposed Development Plan

Arnotts is providing a range of flour samples (which GF have as wheat samples) to the program researchers. These samples cover all the soft varieties currently grown in southern NSW (the main area for soft-biscuit wheat). These samples are of farmer-declared varieties – the variety identity will be confirmed by Tier 1-type tests. Since many of these samples are of the one variety grown in a range of areas, under different agronomic conditions, they will allow an assessment to be made of how growing environment effects the test accuracy. Once the identities are confirmed the samples can be used to derive antigens for the antibody methods. The aim will be to have a range of tests (across the testing tiers) available for assessment during the 2002-2003 harvest.

DISCUSSION OF INDUSTRY EXPECTATIONS

<u>Comments from Felice Driver and Richard Daniel</u>
<u>C-Qentec Diagnostics Pty Ltd</u>

An array of alternate technologies exists offering the potential for identity preservation through on-farm testing, at centralised testing laboratories or for use in laboratories with limited facilities, such as grain handling and receival sites. The quality of the science and technology does not guarantee its uptake by end users; rather it is linked to robustness and utility of the technology, and perceived value by the end user. It is likely that technology that can accurately identify variety, as well as assess the impact of environmental factors that affect the expression of quality traits, may facilitate the uptake of such tests. Detecting quality traits as markers to assist breeders provides a desirable tool for monitoring genetic flaws as well as beneficial characters, but assessing the quality of grain due to both genetic and environmental factors would be of particular benefit to end-users such as growers, BHC, millers and bakers, etc. C-Qentec would consider the development of broadly applicable enabling technologies to underpin the potential creation of commercial testing opportunities - the most important goal at this stage. Potential test formats and delivery mechanisms should not be too narrowly defined or restricted at this stage. Final outcomes and commercialisation of tests will depend on consultation with industry and end users.

Achievements of QW CRC diagnostics research

– Kevin Gale

CSIRO Plant Industry,
Canberra

Project Staff

Kevin Gale/Jasjit Johal
Malcolm Blundell
Thomas Giersch
(Amanda Hill/John Skerritt)

QWCRC Project 5.1.8

Commenced April 1999

Aim: A rapid, field-based assay for varietal identification of
wheat based on ICT card format ('WheatRite')

Required for receival testing for the purposes of
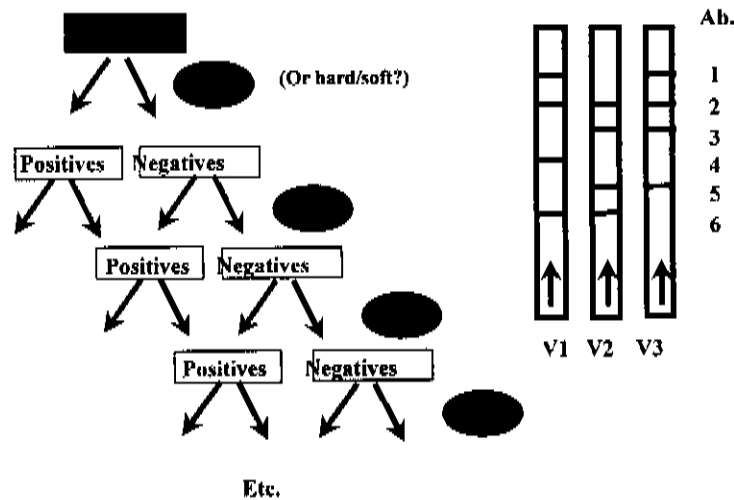-end-use segregation
-end-point royalty payments
-industry domestic buyers

Plus quality control for breeding programs & PBR registration

-Only diagnostic tools with sufficient specificity
and sensitivity currently available for rapid, field-
based diagnostic tests

-1BL.1RS, 1AL.1RS, -2B.2RS wheat-rye translocations
 (Sec-1, Sec-2, Gli-B1)
-α-amylase isoforms- PHS and LMAA
-Wcs-120 FTM cold-induced protein

-Feasibility proven using ELISA and ICT card formats
-Library of antibodies already available

QLD. VARIETIES, 82208 CAPTURE

TAG MAB

Legend: BATAVIA, CUNNINGHAM, HARTOG, KITE, PELSART, SUNDRI, SUNBROOK, SUNCO, SUNECA, SUNLIN, SUNSTATE, SUNVALE, AMERY, ARRINO, CASCADES, CURRAWONG, HALBERD, CRANBROOK
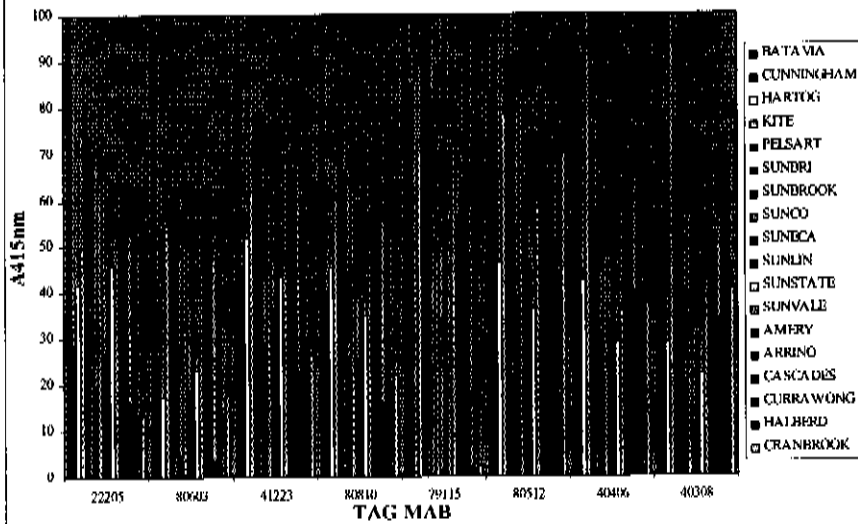
F3 X 8 TAGS X 4 LOCATIONS, 82208 CAPTURE (0.5 ug/mL)

TAG

Legend: ARIAH, NARRABRI, ROMA, WALPEUP

Effect of Heat Stress: c= control, hs= heat stressed
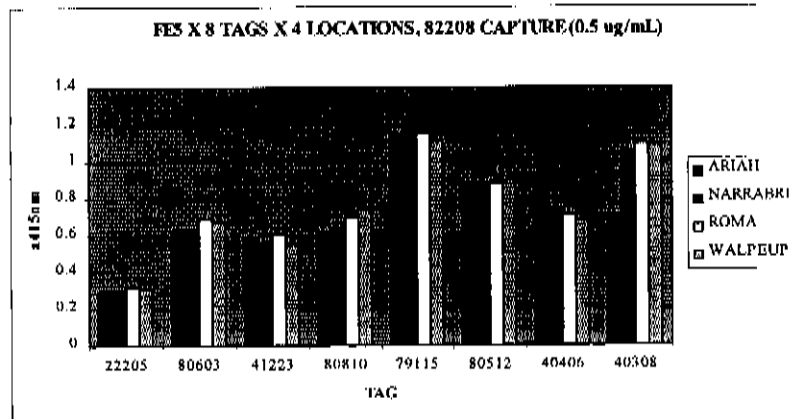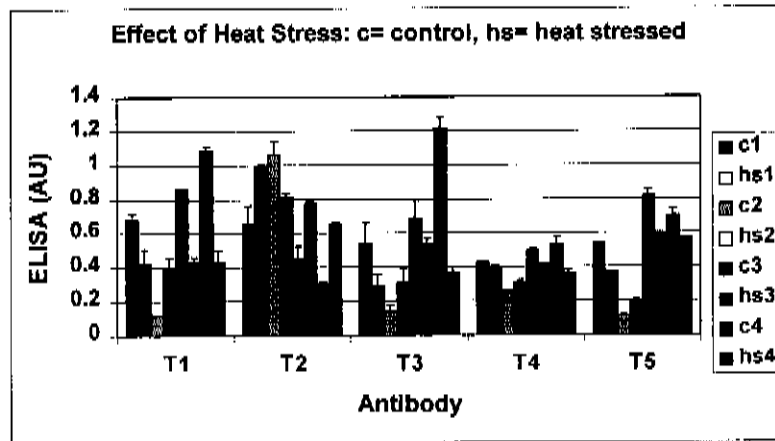
-MAb 79115 is specific for an antigen linked closely
with PinA on chr. 5DS

-Negative cultivars are hard , positive cultivars
 may me hard or soft

-ELISA does not work in indirect format, yet no capture
antibody is required

-Target antigen has high affinity for blocked polystyrene
well

MAb 79115

Nunc maxisorp plate
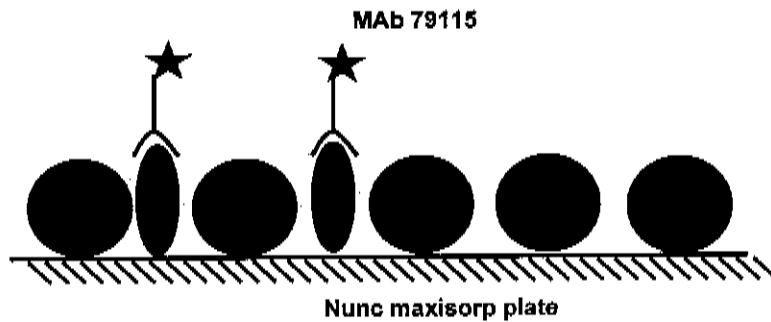
-For rapid format, need second antibody   -purify Ag
                                            -pinA??

GBSS is the major protein found within the starch
granule of mature grain

Approximately 50% of Australian varieties are null
for one of the three homeoalleles

The null phenotype is associated with enhanced
suitability for Udon noodle production

Protein sequences for the 3 homeoalleles show
approximately 97% identity in mature peptide

Figure 1 (CSIRO Plant Industry):

```
                    *          20              40            *        :A  55
GBSS2 :                                                      T—       :B  56
GBSS3 :                                                      Q        :D  55
GBSS1 :              SV          P    L    N    P     T      —
        MAALVTSQLATSGTVLgiTDRFRRaGFQGvRPRsPADAaLGMRTvGASAAPk  QSR

              60        *   ↓        80         *        100          *
GBSS2 :                                                               : 111
GBSS3 :                         A                                     : 112
GBSS1 :    P  FD                                              A       : 111
        KaIIRgtRRCLSMVVRATGSgGMNLVFVGAEMAPWSKTGGLGDVLGGLPpAMAANCH

              120           *      140            *         160       : 167
GBSS2 :                                  K                            : 167
GBSS3 :                             A  E                              : 168
GBSS1 :                      I      R                                 : 167
        RVMVISPRYDQYKDAWDTSVvSEIKVvD YERVRYFHCYKRGVDRVFVDHPCFLEK

          *          180          *        200          *        220
GBSS2 :                                          N D                  : 223
GBSS3 :                          L         A                          : 224
GBSS1 :                                                    H          : 223
        VRGKTKEKIYGPDAGTDYEDNQqRFSLLCQAALEvPRILdLnNNPyFSGPYGEDVV
```

Figure 2 (CSIRO Plant Industry):

Sequence: E A P R I L D L N N N P Y F

KD Hydrophilicity   5.0 / -5.0
Surface Prob.   10.0 / 0.0
Flexibility.   1.2 / 0.7
Jameson-Wolf(Al)   1.7 / -1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
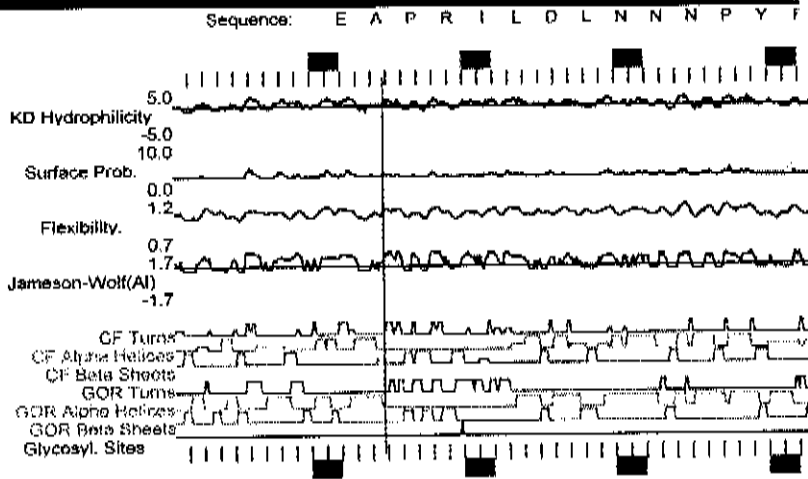
Position: 137 of   534

Sequence: E A P R I L D L N N N P Y F
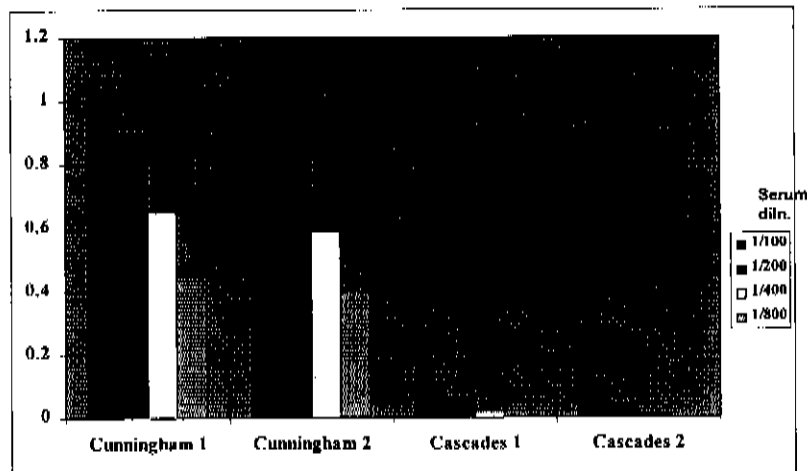
Slide 1
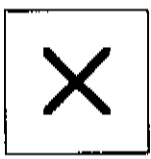
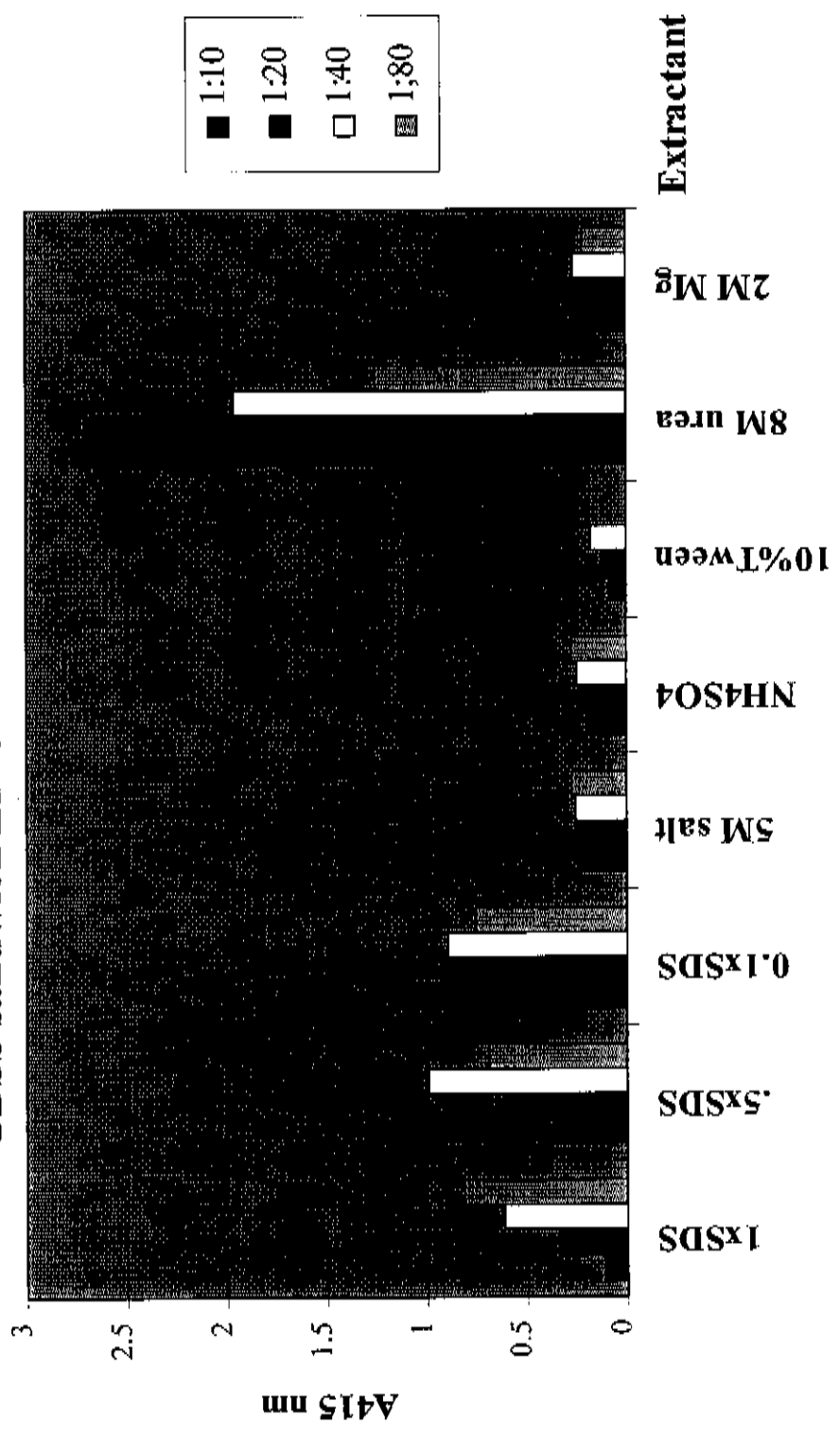| 201		240
'D' GENOME:	LEVPRILNLDNNPYFSGPYGEDVVFVCNDWHTGLLACYLK	
'B' GENOME:	LEAPRILDLNNNPYFSGPYGEDVVFVCNDWHTGLLACYLK	
'A' GENOME	LEVPRILDLNNNPHFSGPYGEDVVFVCNDWHTGLLACYLK	

EAPRILDLNNNPYF[GC]--

SYNTHETIC PEPTIDE

↓

IMMUNISE
RABBIT

↓

TEST SERUM
IN INDIRECT ELISA
AGAINST PURE SGP

→

Slide 2
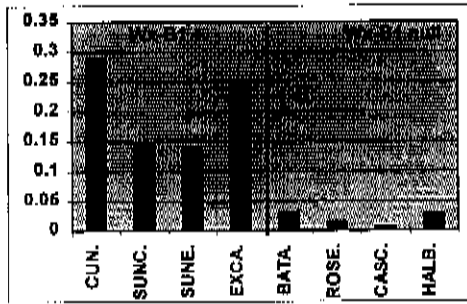
-No requirement for SG purification prior to SDS-boil

Serum
diln.

- 1/100
- 1/200
- 1/400
- 1/800

Cunningham 1   Cunningham 2   Cascades 1   Cascades 2

GBSS sandwich ELISA: Effect of extractant

PinB1:   KLH/OVA(CG)PTKWWKSG

PinB2:   KLH/OVA(CG) PTKWWKGG

PinBMAP1: WKSGCEHEVR(8K4K2KA)

PinBMAP2: WKGGCEHEVR(8K4K2KA)

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55 kDa —

S.star Spear Still. Suneca Suneig S.vale T.bird Tern T.currin Trident W.goyne Wyuna Hartog
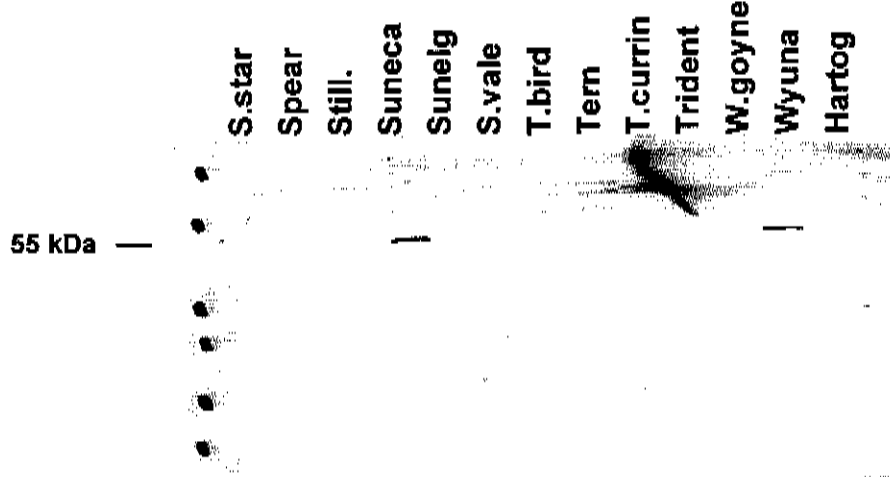
**Harrier also positive
NWMMP parents?**

A-gli4
Agli-3
A-gli1
A-gli-1

51
51
51
51

MRTFLIL LLAIVATTATTAVR PVPQLQPQNPSQQQPQLQVPLVQQQPFL

60   *   80   *   100

A-gli4
Agli-3
A-gli1
A-gli-1

102
102
102
102

GQQQPFPQQPIPQPQPFPSQ PILQLQPF QPQLPFSQPQPFRPQQPYPQ

*   120   *   140   *

A-gli4
Agli-3
A-gli1
A-gli-1

151
151
152
153

PQPQYSQPQQPIS QQQQQQQQQQQQQQQQ QQISQQILQQQLIPCMDVVL

F11 Primer site: SQQQPGE
                       SQQQPQG

F4 Primer site: QQPYPQP
                       QQPYPQL

Primer D site: QHNIAHGR
                       QHNIAHGS

Cross	Wx-B1 ELISA classificatio	1997		1998	
		Number of lines	Swelling power	Number of lines	Swelling power
Matong/Rosella	a	17	10.23 ± 0.35	18	11.51 ± 0.21
	b	38	11.86 ± 0.32	38	13.27 ± 0.14
	a,b	62	10.96 ± 0.30	61	12.37 ± 0.10
	7 a, 1 b	27	10.54 ± 0.33	28	11.49 ± 0.17
	7 b, 1 a	22	11.84 ± 0.34	22	13.16 ± 0.19
Matong/Gameny	a	48	11.01 ± 0.26	47	12.33 ± 0.13
	b	59	11.93 ± 0.25	59	13.27 ± 0.11
	a,b	131	11.31 ± 0.24	132	12.43 ± 0.07
	7 a, 1 b	30	11.11 ± 0.28	29	12.10 ± 0.16
	7 b, 1 a	26	11.29 ± 0.28	26	12.48 ± 0.17
Lillimur/Rosella	a	32	11.43 ± 0.21	27	12.25 ± 0.15
	b	38	12.15 ± 0.20	35	12.98 ± 0.13
	a,b	149	11.82 ± 0.16	138	12.56 ± 0.06
	7 a, 1 b	26	11.16 ± 0.23	25	11.97 ± 0.16
	7 b, 1 a	42	12.02 ± 0.20	36	13.05 ± 0.13
Lillimur/Gameny	a	17	11.10 ± 0.23	17	12.13 ± 0.24
	b	76	12.12 ± 0.14	74	13.41 ± 0.11
	a,b	180	11.55 ± 0.12	178	12.97 ± 0.07
	7 a, 1 b	26	10.87 ± 0.20	28	12.35 ± 0.18
	7 b, 1 a	47	11.72 ± 0.16	47	13.10 ± 0.14

Ha locus (5DS)

├— Pina ——▶ -null allele linked to hard phenotype

└— Pinb ——▶ -some mutations linked to hard phenotype
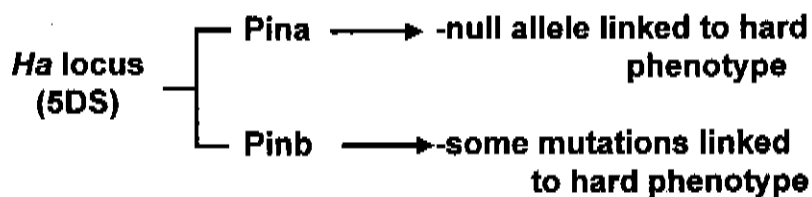
Pinb SNP's (Limello & Morris 2000)

Pinb-D1b   G ——▶ A Gly_{46} —— Ser

Pinb-D1c   T ——▶ C Leu_{60} —— Pro

Pinb-D1d   T ——▶ A Trp_{44} —— Arg

Pinb-D1b polymorphism present in approx. 50% Australian cv. by PCR

Three allele-specific antibodies identified
  -Wx-B1. MAb production underway
  -*Ha* Ab. Requires second antibody
  -55 kDa.Ag. Requires second antibody

Some antibodies useful as stand-alone breeding tools
All antigens extracted by 8M urea at R.T.

Target amino acid polymorphisms identified by database mining

Synthetic peptide approach: resource intensive, long lead time

Future screening of existing antibody library:
  -limited by availability of pure antibodies
  -should utilize microarray approach

Achievements of QW CRC diagnostics research

– Thomas Giersch

CSIRO Plant Industry, Canberra

→ There is a need in rapid and reliable methods to determine the allelic composition of HMW- GS in breeding programs and in industrial processing.

→ Immunochemical test systems such as ELISAs are easy to perform and have the advantage of high sample throughput, but need specific antibodies

Objective:

Development of monoclonal and recombinant antibodies to wheat HMWG subunits for quality and variety screening

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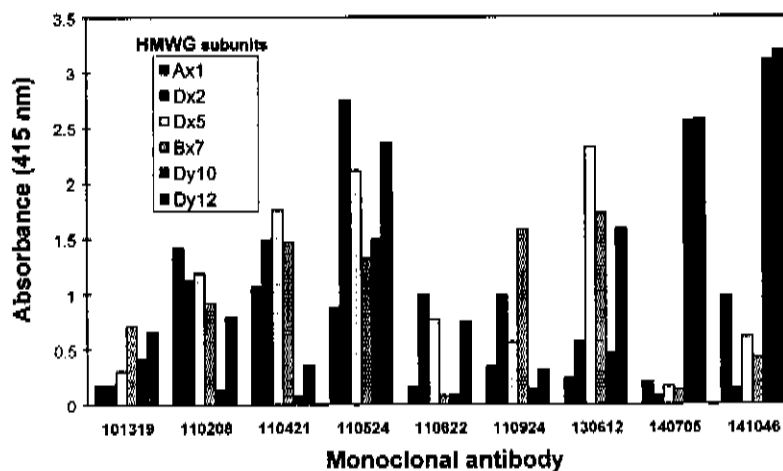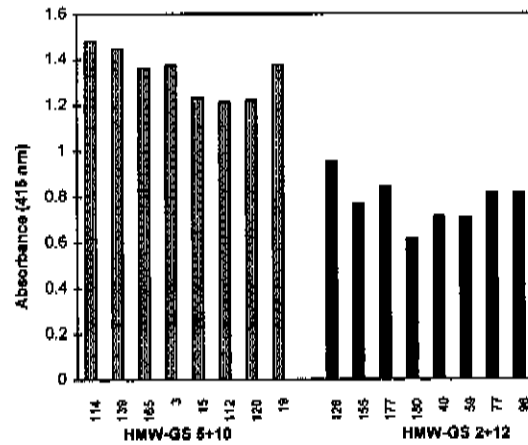
Why antibody engineering?

→ The availability of monoclonal antibodies with required specificities is still limited.

→ Phage display libraries enable screening of diverse pools of antibody genes to select single chain antibody fragments (scFv) with suitable specificities to HMW-GS.

→ Potential of further refinement (eg. mutagenesis)

Phage display libraries have been developed from RNA of spleen cells of mice immunised with individual HMW-GS subunits

Subunit	Library
1	TG1
2	TG3
5	TG8
7	TG14
10	TG17
12	TG6
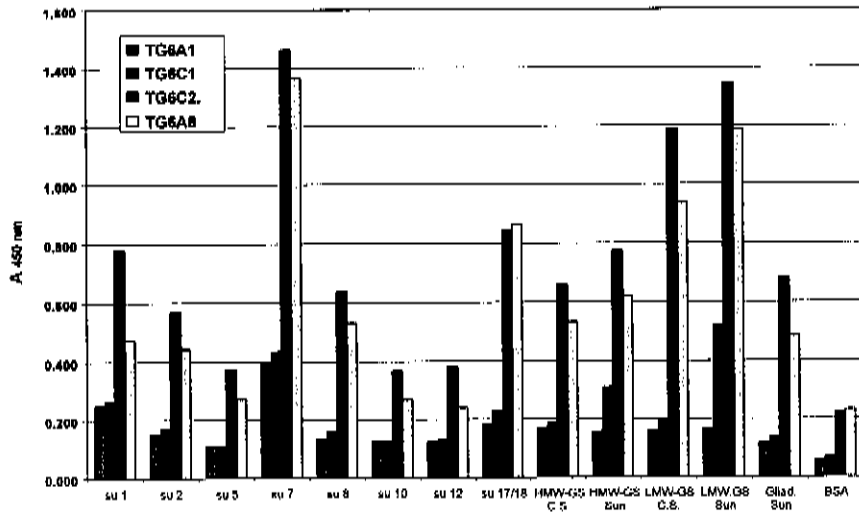
Specificity pattern of four scFv antibodies selected from a library derived from a mouse immunised with HMWG subunit 12
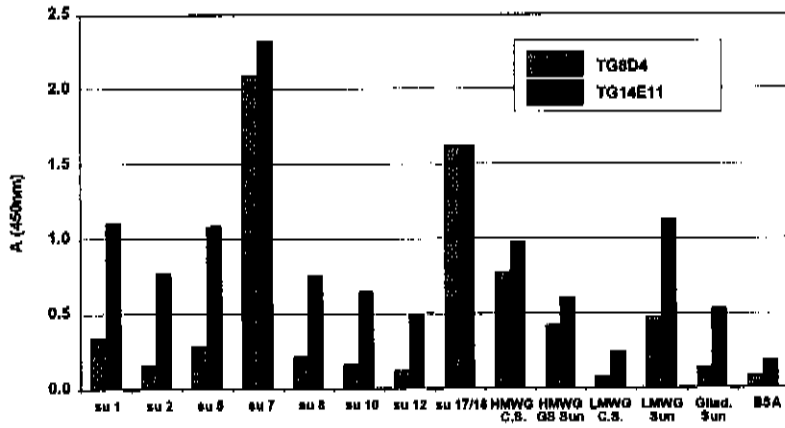
Specificity pattern of two scFv antibodies selected from libraries derived from mice immunised with HMWG subunits 5 (TG8D4) and 7 (TG14E11)
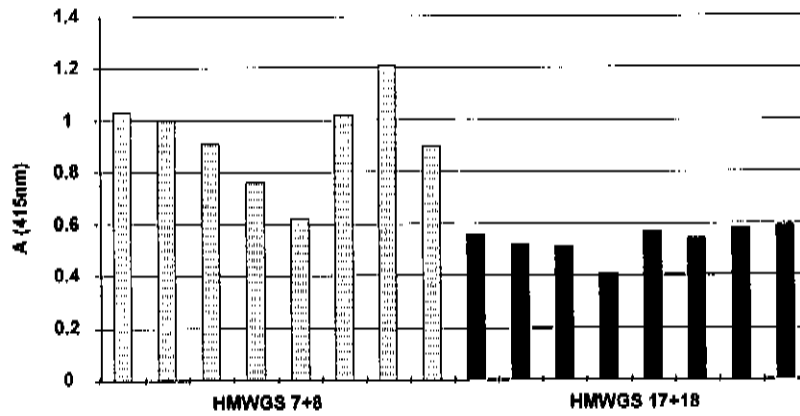
Application of TG8D4 scFv to wheat samples of cross-bred lines from a CD87 (HMW-GS 7+8) x Hartog (HMW-GS 17+18) cross

Application of TG8D4 scFv to samples of Australian wheat cultivars

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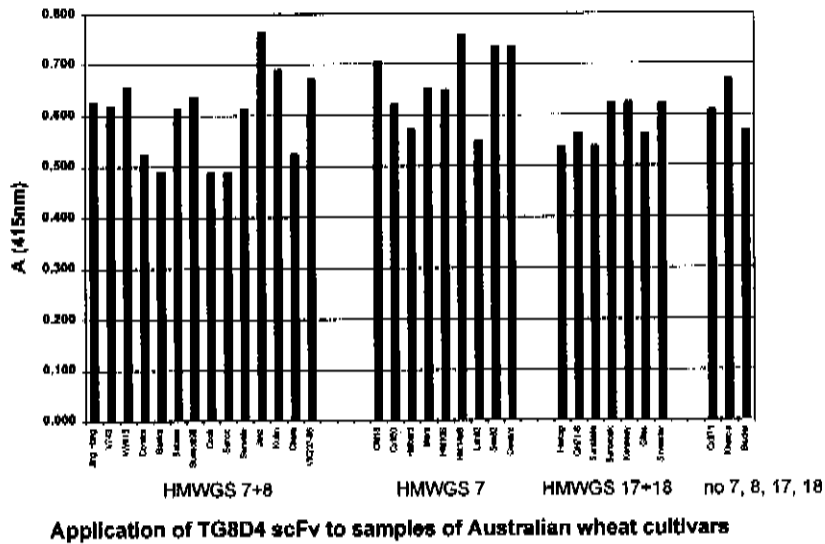
mabs and scFvs don't give a yes/no signal

Possible reasons:

→ Varying expression levels of different glutenins in different wheat cultivars

→ Cross reactivity with other protein components of the wheat grain (LMW-GS)

Antigen
Soybean lipoxygenase
bacterially expressed wheat LOX

A 450

soybean
bacterially expressed
durum wheat

Lipoxygenase
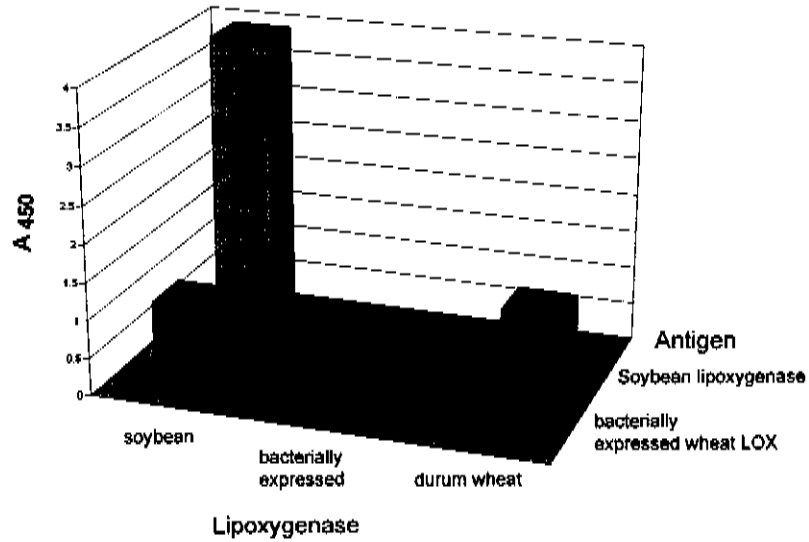
- Target proteins to discriminate closely related wheat varieties

- Antibodies which react qualitatively with the target protein

- The target proteins must be expressed in the same tissue at the same developmental stage (depends on user of the test)

- They should be extractable with the same extractant

  This should be considered early in the screening procedure
  and proteins/antibodies which do not meet these requirements
  should be discarded.

ICT-Lateral flow issues

- Gill Mearns

VAW CRC, North Ryde

Lateral Flow Tests

Lateral Flow Tests

- Membrane-based assays

- Provide a visual result

- Rapid (5-30 minutes)

- Inexpensive

- Foolproof

Components of Lateral Flow Tests

Porous materials:

- Membranes

- Conjugate pads

- Sample pads

- Absorbent pads

Components of Lateral Flow Tests

Reagents:
- Capture antibodies and/or antigens
- Conjugated antibody or antigen
- Detector particle (e.g., gold or latex)
- Blocking agents, detergents, surfactants, stabilisers, buffers, etc.

Membranes

- Nitrocellulose

- Nylon

- PES

- Others

Advantages of Nitrocellulose Membranes

- Good wicking characteristics

- Good protein binding

- Low non-specific background

- Well known product with published protocols

Nitrocellulose Membranes

- Several different manufacturers

- Different pore sizes and flow speeds

- Each treats the NCM with different surfactants during manufacture

- Treatment ensures re-wetting and makes the NCM suitable for lateral flow applications

Detector Reagents

- Colloidal metals –
              gold most popular

- Latex particles –
              dyed, magnetic and
              fluorescent types most
              commonly used.

Feature	Gold	Dyed Latex
Visibility	3*	3*
Sensitivity	3*	2*
Stability	3*	2*
Colours	1*	3*
Reproducibility	3*	1*
Scale-up	3*	1*
Multi-analyte detection	3*	2*
Low cost	3*	3*
Ease of use	3*	3*

Conjugate Pad Materials

- Glass Fibre

- Polyester

- Polypropylene

- Cellulose Acetate

Key Properties of Conjugate Pad Materials

- Allow conjugate to dry without damage
- Allow rapid sample penetration to all parts of the pad
- Allow rapid and complete release on sample application
- Show low binding or interference with the sample

Blocking of Conjugate Pads

Blocking agents improve the release of the conjugate

- Surfactants
- Hydrophilic polymers
- Sugars

Usually involves a combination of reagents

Antibodies

- Monoclonal antibodies

- Polyclonal antibodies

- Good affinity, avidity and specificity

- Need reproducibility lot to lot

Factors that can influence test sensitivity

- Affinity and mass of capture antibody
- Affinity and mass of conjugated antibody
- Flow rate of membrane
- Location of test line
- Bed volume and release attributes of conjugate pad
- Additives to conjugate and sample pads
- Pad overlaps and compression with the membrane

Optimisation

Maximum test performance is
achieved by careful selection
and optimisation of all
components and reagents

Lateral Flow Tests

- Most commonly used for simple Yes/No answer

- Can be quantitative or semi-quantitative

- Small differences in line intensity are hard to determine unless using a reader

Lateral Flow Tests

- Single analyte detection

- Multiple analyte detection

Each line striped onto the NCM represents a totally different assay

Requirements for Multiple Analyte Testing

- Highly specific pairs of capture and detector antibodies
- Same sample
- Same extraction protocol
- Compatible reagents (membranes, pads, buffers etc)
- Similar reaction times

Summary

- Antibody selection

- Component choice

- Optimisation

- Reproducibility

How proteomics can contribute

– Brad Walsh

Australian Proteome Analysis Facility, Macquarie University

WHEAT GRAIN PROTEOMICS
HOW CAN IT CONTRIBUTE TO DIAGNOSTIC APPLICATIONS ?

Daniel Skylas and Brad Walsh

Australian Proteome Analysis Facility (APAF)

• Located in Sydney

• At Macquarie University
• 800 square metres of space
• The first in the world !

Proteomics

- •Proteome is the PROTEins expressed by a genOME at any one time.

- •It may be as complex as a whole organism or tissue or as simple as a single cell type

- •Proteomes are dynamic - they reflect the state of biological systems at any one time
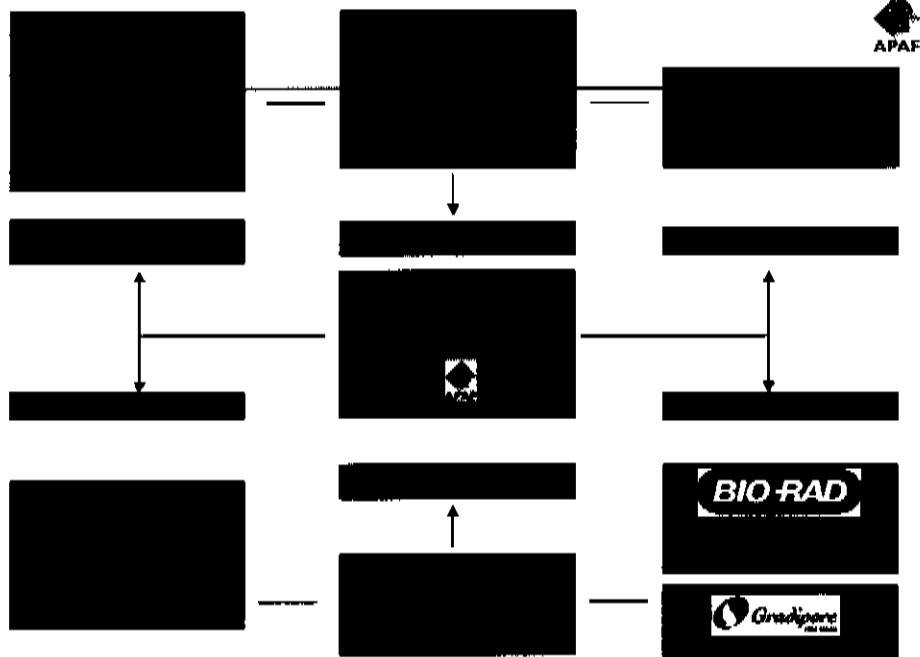
Overview of Proteomics Experiment

1. Sample Prep

2. Isoelectric Focusing
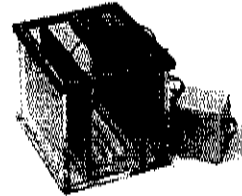
3. SDS-PAGE

4. Protein Detection and Image Acquisition

Post Separation Analysis
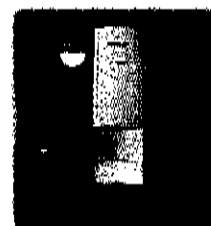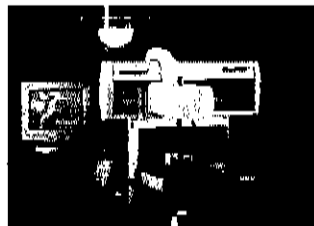
7. Protease Digestion and Mass Spectrometry to Identify Proteins

6. Image Analysis and Spot Excision

8. Post-translational modifications
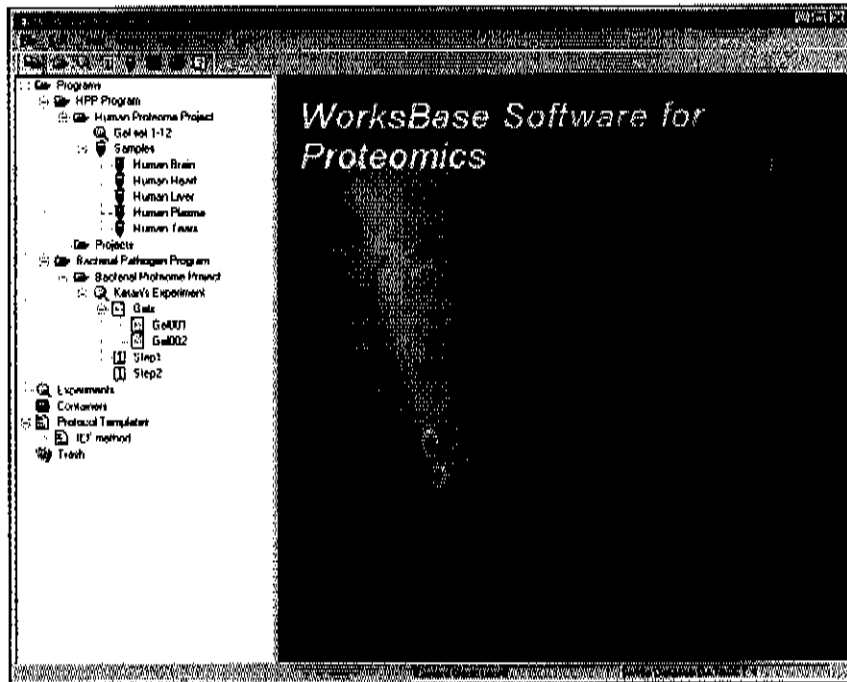
Experimental Design

- **Plants from cv. *Wyuna* and *Fang* grown at day/night temperatures of 24/18°C during development**
- **Plants subjected to heat shock at day/night temperatures of 40/25°C 15, 16 and 17 days post-anthesis**
- **Grain samples taken at 17 days post-anthesis**
- **Dough rheology indicates *Fang* is tolerant**

Markers of Heat Tolerance in Wheat

4 ——————————— pH ——————————— 11

230 Proteins characterised by Edman sequencing
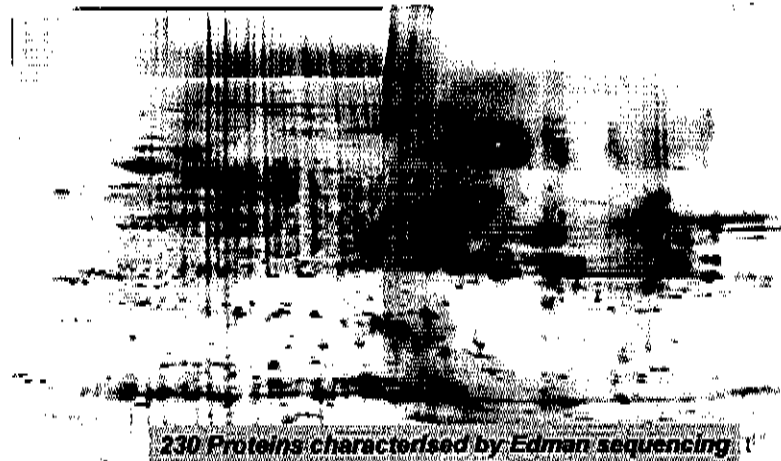
Susceptible Wheat cv. *Wyuna*

Control   Heat Stress
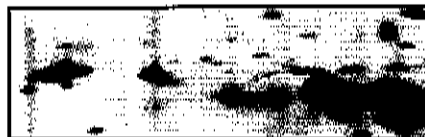
Tolerant Wheat cv. *Fang*
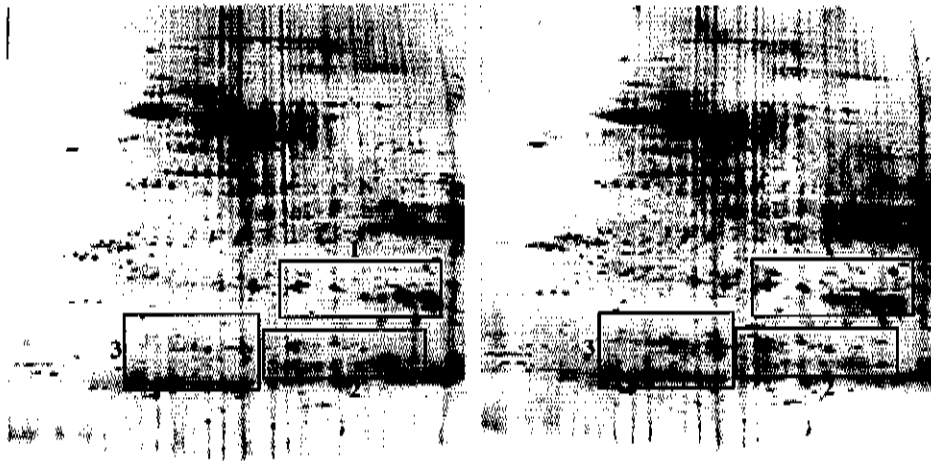
Control   Heat Stress

Region One – Little Difference
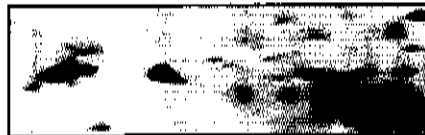
Wyuna
Control
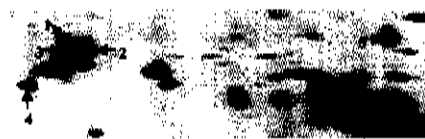
Wyuna
Heat shock

Fang
Control

Fang
Heat shock

Region Two: Possible Markers of Tolerance

(A)   Wyuna Control

(B)   Wyuna Heat shock

(C)   Fang Control

(D)   Fang Heat shock
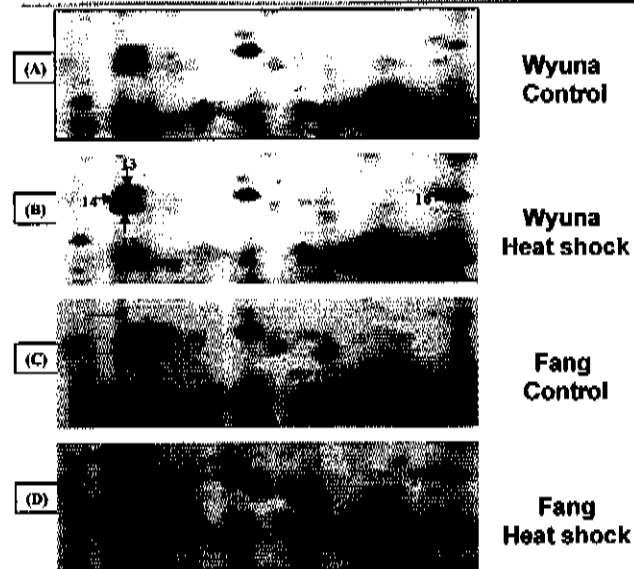
Region Three: Possible Markers of Tolerance

(A)   Wyuna Control

(B)   Wyuna Heat shock

(C)   Fang Control
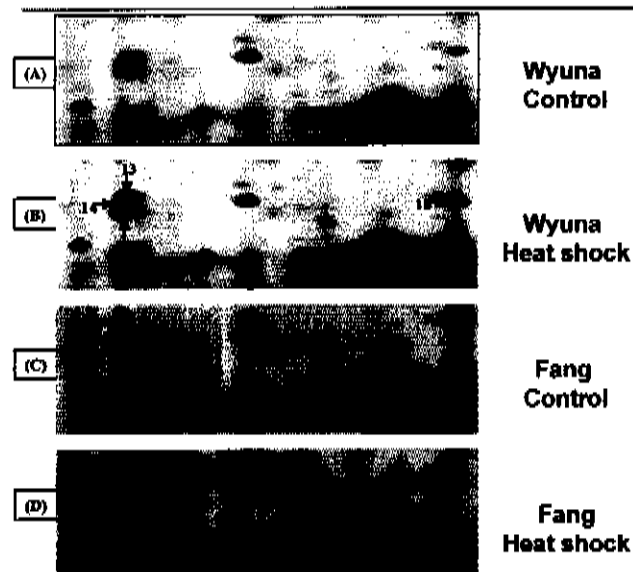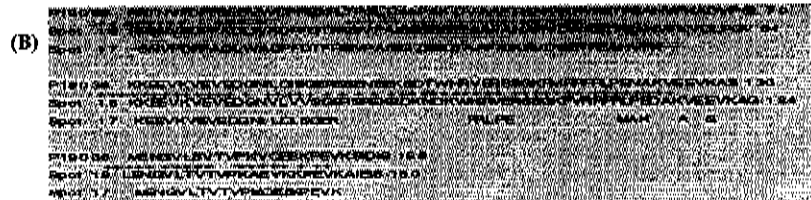
(D)   Fang Heat shock
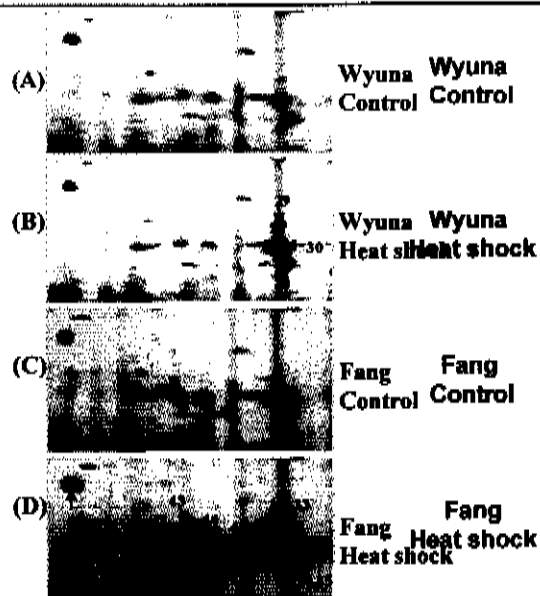
Summary of Identifications

- **17/48 protein spots analysed by PMF, were matched from the database**
- **3 protein spots matched 17.4 kDa HSP from *Arabidopsis thaliana***
- **3 protein spots matched the 17.6 kDa HSP from the same organism**
- **11 protein spots matched the 16.9 kDa HSP from wheat**
- **These proteins belong to the smHSP family and have a subcellular location within the cytoplasm**

Region Two: Possible Markers of Tolerance

(A) Wyuna Control
(B) Wyuna Heat shock
(C) Fang Control
(D) Fang Heat shock

Chromosomal variation in two genes expressing heat tolerance

(A) Spot # 17
Spot # 18

(B)
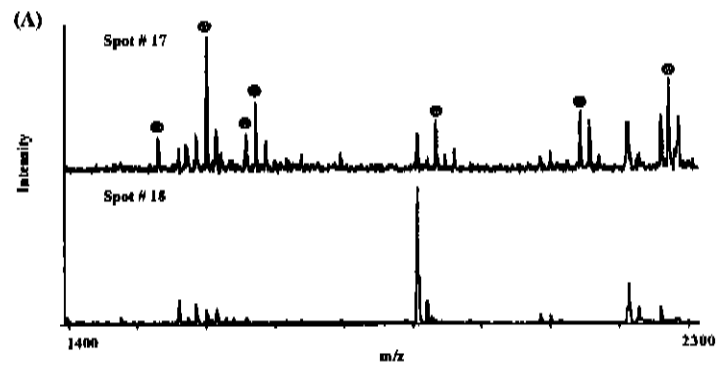
Conclusions

- **Host of proteins in the heat-tolerant Fang cultivar were rapidly identified as being possible markers**

- **Fang exhibited the strongest response to heat shock at the molecular level and in terms of dough-quality**

Conclusions

- HSP's (spot numbers 17 and 18) arise from separate genes. The gene responsible for the expression of protein spot 18 (16.9 kDa wheat HSP) is not active in the heat-susceptible Wyuna cultivar

- Identification of such protein markers provides breeders with means to more rapidly produce cultivars with desired characteristics and to produce cultivars with even greater potential for improved dough-quality

- This work has now been accepted to J. Cer. Sci. Work is now moving into variety identification

Mass Spectrometry - Variety Identification

- Canadian wheats extracted with 70% ethanol for gliadins

- Supernatant added to sinapinic acid and put on MALDI target for TOF analysis

- Whole mass of 30-40,000 daltons differentiated 16 varieties and showed common bands amongst wheat classes

Plans to use proteomics
for VAW-CRC immuno-diagnostics

– James Chin

Elizabeth McArthur Agricultural Institute,
NSW Agriculture, Camden

EMAI-
Centre of Excellence

- Developing Advanced Technologies for Producing Innovative Diagnostic Reagents
- Integrating Proteome, Genomic and Immunological Technologies
- A Joint Initiative between Animal and Plant Industries in NSW Agriculture

Animals

IMMUNOLOGY

- Diseases of sheep
  - Fleecerot (*Pseudomonas aeruginosa*)
  - Flystrike (*Lucilia cuprina*)
- Diseases of pigs
  - Polyarthritis (*Erysipelothrix rhusiopathiae*)
  - Pleuropneumonia (*A. pleuropneumoniae*)
  - Enzootic pneumonia (Mycoplasma)
- Vaccine development
  - New adjuvant for skin and mucosal immunization

Animals

IMMUNOLOGY

- CRC Beef and Meat Quality
  - Food safety
  - Alternatives to antibiotic growth promotants
- Nutraceuticals and Health conferring benefits
  - Dietary supplementation
  - Herbal medicine (mushrooms)
  - Medihoney
- Commercial alliances
  - International Animal Health
  - Intervet

Animals

COLLABORATIONS

- University of Sydney
  - Obesity and immunity
- University of NSW – Liverpool Hospital
  - Diet/tolerance and autoimmunity
- University of Wollongong
  - Development of a vaccine for *Streptococcus pyogenes* infections

Animals

COLLABORATIONS

- John Curtin School of Medical Research
  - cellular immunity
  - Immunological memory
- University of Sydney -Lidcombe
  - Antibiotic resistance
  - Molecular detection of mobile gene elements
- University of Western Sydney
  - Role of dairy probiotics in promoting intestinal health

Animals

APPLIED RESEARCH

- Bunge Meat Industries
  - Fecal microbial profiles for predicting pig heath and benefits from proper dietary supplementation
  - Promotion of neonate gut growth and health by conventionalization with healthy bacterial consortium
- Intervet
  - Vaccine evaluation
  - Vacccine breakdowns

Animals

Producing Diagnostic Reagents

- **Identifying and purifying a unique antigen**
- **Testing the antigen for immunogenicity**
  - ie. Ability to elicit an antibody response
- **Immortalizing the antibody response**
  - eg. hybridoma production
- **Specificity screening**
- **Scale up production of diagnostic antibody**
- **Quality assurance**
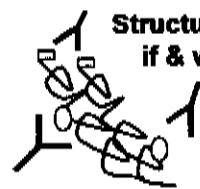
Structural epitope - no longer recognised by Ab if & when the 2 subunits are separated

| Specificities within Specificities |

Antigen composed of 2 identical subunits in red and black

↓

Antigen is denatured and separated and linearized

Part-conformational epitope recognised by Antibody

Linear amino acid epitope recognised by Antibody

Barriers to a Successful Outcome

- Failure of industry to identify problem
- Inability to find a unique antigen
- Unique antigen is not immunogenic
- Antibody does not bind strongly
- Assay not robust to withstand field conditions
- Quality assurance and cost of test expensive
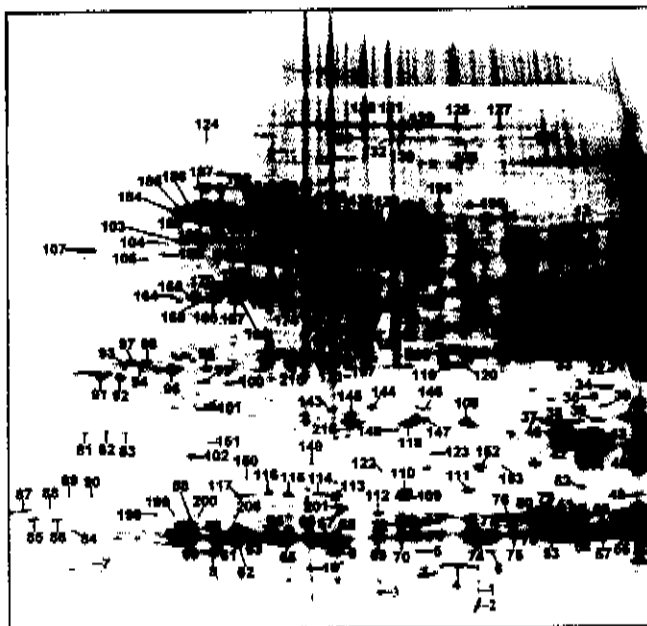
Schematic of Polypeptides Resolved by 2D PAGE

○ Unique spot found only in variety 1 (virulent) and not in varieties 2 and 3 (avirulent pathogens)
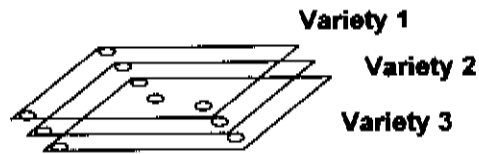
Variety 1

Variety 2

Variety 3

○ Spot same in relative location and intensity for all 3 varieties (shared common antigen)

○ Spot same in relative location and intensity for all 3 varieties (shared common antigen)

○ Spots same in relative location for all 3 varieties
○ but variety 2 has a more intense spot

Identification of Unique Polypeptide Spot

○ Computer is instructed to align unique spot and to remove sample (robotic technology)

Sample is subjected to tryptic digestion in-gel followed by mass spectrophotometric analysis

End terminal analysis to provide amino acid sequence

Redundancy in genetic code allows for DNA sequence information and subsequent search in data base for parent gene

Identification of Polypeptide Spots with Different Intensities

○
○ Computer is instructed to remove spots with different intensities from their respective gel locations

Samples are subjected to tryptic digestion in-gel followed by peptide mass fingerprint analysis

End terminal analysis to provide amino acid sequence

Redundancy in genetic code allows for DNA sequence information and subsequent search in data base for parent gene

Further amino acid sequencing if needed

Comparison of Peptide Mass Fingerprint (PMF) for two different heat responsive proteins in wheat endosperm
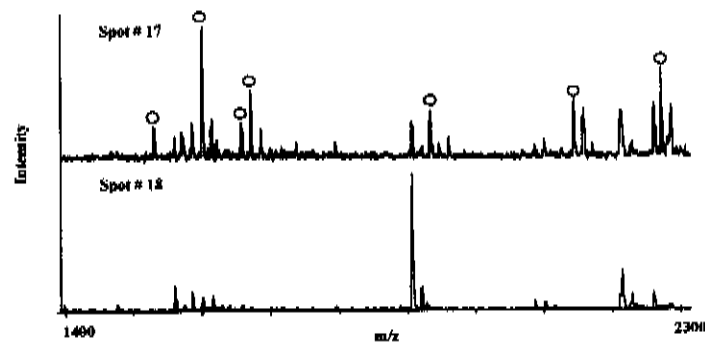
Spot # 17 matches a 17.4 kDa Class I heat shock protein from *Arabidopsis thaliana* (SWISS-PROT Accession # P19036).

Spot # 18 matches a 16.9 kDa Class I heat shock protein from wheat (SWISS-PROT Accession # P12810).

The green circles mark peptides which are unique for that protein

**Designing and Assessing Antigenicity
of Unique Polypeptide Spot**

○ Amino acid sequence of unique polypeptide spot is
modelled by 3D to assess structural configuration

Amino acid substitutions are introduced to model
conformational changes

Synthetic peptides (SPs) are generated

SPs are separately conjugated to carrier proteins

Mice are vaccinated with SPs and antisera assessed
by immunoblot screening against 2D gels

Antisera are also screened against antigens extracted
from each wheat variety (or pathogen)

ENABLING TECHNOLOGIES

- Novel immunisation strategies
  - Targeting conformational and linear
    epitopes
  - Subunit antigens (glycosylated or non-
    glycosylated)
  - Carbohydrate antigens (eg. Bacterial cell
    wall derived peptidoglycans)
- T and B cell immunisation
- *In vitro* enrichment

Animals

ENABLING TECHNOLOGIES

- Multiscreening techniques for subunit antigens
- Hybridoma production
- Phage peptide displays
- Animal facilities - Polyclonal antibodies
- Cell culture facilities

Animals

Producing the Desired Outcome

Antibodies react specifically against ○ in 2D gel blots

Antibodies react specifically against proteins extracted from Variety 1 wheat grains only (or virulent pathogen)

Produce monoclonal antibodies with equivalent specificity

Validate varietal-specific antibody (VSA) under field conditions

Package a panel of different VSAs for multi-variety diagnosis