Project Report

Integrating quality trait development with large scale gene expression analysis in potato

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1. Summary

Potato consumption faces significant competition from other staple sources of carbohydrates such as rice and pasta. Strategically, therefore, the development of well differentiated potato products is an important goal. Tuber quality traits such as flavour, texture and nutritional value are assuming a greater importance in R&D since consumers are becoming increasingly demanding with respect to convenience, quality, novelty, eating experience, nutritional value and safety. Thus the challenges are clear for UK PLC to develop market advantage by producing cultivars and processed products that exhibit distinctive and desirable characteristics. However, as with many food crops, quality and nutritional/developmental traits are probably driven by multi-factorial parameters and hence are difficult to assess in breeding programmes. Given the advances made in modern genetic research, approaches will facilitate the discovery of the genes which control crop traits. This project has provided an opportunity to advance our understanding of potato tuber quality and hence to provide options for contemporary, knowledge-based potato breeding.

As its starting material, the project has used tubers from field grown *Solanum tuberosum* (cv. Montrose and Pentland Dell) and *Solanum phureja* (cv. Mayan Gold and Inca Sun) as these are known to have different quality traits. For example, Mayan Gold has a distinctive flavour and texture (cooking properties) whereas Montrose stores very well (with regard to its sugar content). Tubers were harvested during development, maturation and post-storage to determine which genes change in expression with time, but also to determine which genes differ between the cultivars. Importantly, the work on genes funded by Potato Council has proceeded in parallel with RERAD funded work at the SCRI on the chemical composition and quality attributes of the cultivars so that we can begin to correlate specific genes with trait differences. This complementary funding has added significant value and novelty to the research.

The project has generated vast amounts of data which will take some time to analyse to its full potential. However, we are already seeing that specific genes are not expressed in the same way in *S. phureja* compared with *S. tuberosum* and that some of these genes can be linked, using prior knowledge, to the ways in which starch is made in the tuber and the ways in which cell walls are held together. Both starch and cell wall components are believed to be involved in defining tuber texture, so these are important findings. Similarly, we have found some genes which can help to explain differences in storage sugar accumulation between Pentland Dell and Montrose, for example.

The SCRI now plans to complete its detailed comparison of the chemistry of the cultivars used and to correlate other aspect of quality with the genes discovered. This will put us on track to provide breeders with appropriate lists of genes that can be used to accelerate the breeding of improved cultivars by tracking the best genes in their crossing programmes.

2. Experimental Section

Introduction

Potato consumption faces significant competition from other staple sources of carbohydrates such as rice and pasta. Strategically, therefore the development of well differentiated potato products is an important goal. Tuber quality traits such as flavour, texture and nutritional value are assuming a greater importance in R&D since consumers are becoming increasingly demanding with respect to convenience, quality, novelty, eating experience, nutritional value and safety. On top of this the storage and processing industries still require better markers for crop maturity, control of tuber dormancy and texture. Thus the challenges are clear for UK PLC to develop market advantage by producing cultivars and processed products that exhibit distinctive and desirable characteristics. However, as with many food crops, quality and nutritional/developmental traits are probably driven by multi-factorial parameters and hence are difficult to assess in breeding programmes.

Developments in genomics research have opened up the opportunity to use contemporary tools to determine which genes may be controlling economically important traits. This will provide the industry with a sound scientific basis to move forward with the market introduction of novel cultivars rather than relying on empirical approaches to solve complex problems. This *ca*. 9 month project has used state of the art DNA microarrays to complete a detailed analysis of gene expression in potato tubers during development and storage. Importantly, the study is linked to phenotyping studies being carried out as part of RERAD's commissioned research programme at SCRI. In this way we can begin to link specific genes with target traits. This is where real added value and novelty arises. Importantly, the project included comparisons of gene expression and quality parameters in two tetraploid and two diploid cultivars which are well differentiated in terms of quality traits such as flavour and texture. The study used field grown material and to our knowledge this is the most comprehensive study yet. The major objectives were:

i. To provide a comprehensive analysis of gene expression (covering *ca*. 75% of all potato genes) during important stages of the tuber life cycle.
ii. Compare gene expression profiles across four genotypes which are differentiated in specific quality traits.
iii. To assess correlations between temporal and genotype specific changes in gene expression with corresponding changes in important quality parameters. The primary focus is on the control of flavour and texture but the samples developed will also address the potential to discover novel markers for crop maturity (defined here as a prediction of sugar accumulation in storage). However, it is important to note that, as all samples will also be analysed using metabolomics, there are significant opportunities to address linkages between gene expression and the accumulation of a wide range of metabolites e.g. phenolics, amino acids (including asparagine implicated in acrylamide formation), organic acids and glycoalkaloids, which can impinge on quality or nutritional value. The quality parameters in the four genotypes were measured in the RERAD funded work programme at the SCRI and included:

- **Flavour:** tuber volatiles (boiled potato) and matrix components associated with flavour including Umami compounds.
- **Texture:** texture analysis (physical texture analyser) of raw, boiled and crisped potato which will be linked to analyses of cell wall and starch properties. The

latter includes starch pasting profiles and amylose:amylopectin ratios (the latter can impinge on glycaemic index).

- **Processing Potential**: sugar analysis.
- **Metabolomics:** as a broad brush analytical tool to assess potential metabolite markers for maturity.

From the inception of the project we were aware that vast amounts of data would be produced on gene expression and that within the short time available we could only begin to address relationships of genes with quality traits. This report provides some examples of the approaches that can be used to make full use of the datasets, particularly as more data becomes available from the RERAD funded research that can be linked with the gene expression study.

Material and methods

Experimental Design and Field Layout

Four cultivars (Pentland Dell, Montrose, Inca Sun and Mayan Gold) were sampled over six time-points (T1 – T5B - see Table 1). The field trial was laid out in four complete blocks of four cultivars each, designed as a Latin square. Each of the 16 plots consisted of 100 plants in four drills of 25 plants each. At time-points T1, T2 and T3 32, 12 and 32 plants respectively were harvested from each block.

Time Point (T)	Temp (^{o}C)	Stage of Development
T1	n/a	Small, developing tubers, *ca*. 4cm diameter (40-50g fresh weight)
T2	n/a	Larger, developing tubers, *ca*. 7cm diameter (75-100g fresh weight)
T3	n/a	Fully mature tubers, harvested at senescence. (150-200g fresh weight).
T4	10	Fully mature tubers stored at 10^{o}C for 5 weeks (leading on from T3).
T5A	4	Fully mature tubers, stored at 4^{o}C for a further 4 weeks (leading on from T4)
T5B	10	Fully mature tubers, stored at 10^{o}C for a further 4 weeks (leading on from T4)

TABLE 1. DESCRIPTION OF DEVELOPMENTAL STAGES HARVESTED.

Harvesting and Sampling:

T1: For each block all small, developing tubers (*ca*. 5cm diameter) were mixed thoroughly and 3 replicate ('rep') samples of *ca*. 200g (5-7 tubers) were chopped, mixed and frozen in liquid nitrogen (LN_2) and freeze dried for one week prior to milling using a Retsch mill (Tecator Udy) with a 1mm sieve. Freeze-dried and milled potato powders were stored in re-sealable bags at -20^{o}C (in the dark) until required for analysis. Storage in this way has no impact on metabolite balance (as revealed by metabolomics) for at least 12 months (unpublished data).

T2: For each block all larger developing tubers (*ca*. 7cm diameter) from the 12 harvested plants were mixed thoroughly and three replicate ('rep') samples of *ca*. 500g (4-6 tubers) combined fresh weight were removed. As tubers develop in size metabolite gradients occur

within the tuber. In order to minimise this effect, two opposite eighths were removed from each tuber within a replicate (Griffiths and Dale, 2001), bulked together, frozen in LN_2, freeze dried and milled as described above.

T3: For each block all fully mature, undamaged tubers of similar size (150-200g fresh weight) from the 32 harvested plants harvested were mixed thoroughly and three replicate samples of *ca*. 800g (3-5 tubers) combined fresh weight were removed. For each rep two opposite eighths were removed from each tuber within a replicate, bulked together, frozen, dried and milled as described above.

T4: The remaining tubers after sampling T3 were pooled with the remaining tubers of the same cultivar at T3 from the other 3 blocks to provide one large bulk sample per cultivar. These tubers were placed at 10^oC for 5 weeks prior to sampling. After 5 weeks storage three replicate samples of *ca*. 800g (3-5T) combined fresh weight per rep were removed. For each rep two opposite 8^{ths} were removed from each tuber within a replicate, bulked together, frozen, dried and milled as described above.

T5A and T5B: Tubers remaining after sampling T4 were stored at either 4^oC or 10^oC for four weeks then three replicate samples freeze dried and milled as described above.

Total RNA Extraction from Freeze-dried Potato Tubers

Approximately 1.5 g of freeze-dried tuber tissue was extracted with 14 ml of hot (80°C) extraction buffer (50 mM Tris-HCl (pH 8.0), 50 mM LiCl, 5 mM EDTA, 0.5% SDS, 50% (v/v) phenol). 10 ml sterile distilled water was added and the samples were vortexed for 2 minutes. The samples were placed on ice and 16 ml of chloroform:isoamyl alcohol (24:1) was added and then vortexed as before. Following centrifugation at 4°C at 14,000 g for 20 minutes, the upper aqueous layer was removed to a fresh, sterile 50 ml Sorval tube, containing an equal volume (16 ml) of 4 M LiCl. The samples were shaken and incubated overnight at –80°C. The samples were centrifuged at 4°C at 14,000 g for 40 minutes. The supernatant was discarded and the pellet resuspended in 5 ml sterile distilled water. 0.1 volume of 3 M NaOAc (pH 5.2) and 3 volumes of 100% ethanol were added and the samples were incubated at –80°C for at least 1 hour.

The precipitated RNA was pelleted by centrifugation at 4°C at 14,000 g for 40 minutes, washed with 10 ml of ice-cold 70 % (v/v) ethanol, and centrifuged as in the previous step. The ethanol was removed and the RNA pellet allowed to air-dry prior to resuspension in 500 µl sterile distilled water. RNA samples (100 µg) were further purified and genomic DNA contamination was removed using QIAGEN RNeasy columns and DNaseI according to the manufacturer's protocol. RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (www.nanodrop.com) and quality tested using an RNA 6000 nano chip on an Agilent 2100 Bioanalyzer (www.chem.agilent.com). RNA samples were aliquoted in 20 µg (1 µg/µl) batches and stored at –80°C.

Microarray Processing

Total RNA was labelled by indirect incorporation of fluorescent dyes following cDNA synthesis. Reverse transcription was performed using 10 µg of total RNA in a 45 µl reaction containing 50 ng/µl oligo d(T)18, 0.5 mM each dATP, dCTP, dGTP, 0.2 mM dTTP, 0.3 mM aa-dUTP, 10 mM DTT, and 400 U Superscript II (Invitrogen) in 1 x reaction buffer. Primers and RNA were initially heated to 70°C for 10 min followed by cooling on ice, and the entire

reaction incubated for 16 h at 42°C. To denature the remaining RNA, 15 µl of 1 M NaOH and 15 µl of 0.5 M EDTA (pH 8.0) were added and incubated for 10 min at 65°C. The reaction was neutralized with 15 µl of 1 M HCl. Purification of cDNA was performed using MinElute columns as recommended (QIAgen), substituting phosphate wash buffer (4.75 mM K_2HPO_4, 0.25 mM KH_2PO_4, 84% EtOH) for PB and phosphate elution buffer (3.8 mM K_2HPO_4, 0.2 mM KH_2PO_4) for EB. Cy-dye esters were added to 10 µl of cDNA in a total volume of 13 µl, containing 150 mM sodium carbonate and 1.0 µl of the appropriate Cy-dye (GE Healthcare) suspended in DMSO (1/10 supplied aliquot), and incubated for 1 h at room temperature in the dark. To the labelled cDNA, 750 mM hydroxylamine hydrochloride was added and incubated for a further 30 min in the dark. Labelled targets for each array were combined and diluted with 24 µl sterile water and 500 µl of PB buffer (QIAgen) prior to MinElute purification and elution with 10 µl of elution buffer. Labelling efficiency was estimated using spectrophotometry.

Labelled cDNA was made up to a volume of 55 µl with sterile water and denatured by heating at 98°C for 3 min. The denatured cDNA was mixed with 55 µl of 2 x GEx Hybridization Buffer Hi-RPM (Agilent Technologies) and hybridization was carried out for 17 h at 65°C as recommended. After hybridization, the arrays were washed in the dark for 1 minute each with GE Wash Buffer 1 at room temperature, followed by GE Wash Buffer 2 at 37°C, and dried by centrifugation.

Microarrays were scanned with an Agilent G2565B Scanner (Agilent Technologies) at default laser settings for Cy3 and Cy5 at 5 µm resolution, generating TIFF images. Data were acquired from images using Feature Extraction software (v 9.5.3, Agilent Technologies), and signal intensities were determined for the Cy3 and Cy5 channels for each spot on the microarrays. Intensity values were imported into GeneSpring (v.7.3.1, Agilent Technologies) and data were normalized using default settings, followed by removal of unreliable data with consistently low signal (<50). Normalized and filtered data were subsequently exported for statistical analysis.

Statistical Analysis

Principal component analysis (PCA) based on the variance-covariance matrix of all spots remaining after the filtering described above was used to reduce the data set to a manageable number of dimensions. These were used to identify and summarise the main sources of variation in the data.

Analysis of variance (ANOVA) was used to identify those spots for which the two 'treatments', namely cultivar and time (and their interaction) generated significantly different expression levels.

Quality Trait Analysis

Starch Rheology (Cooking Properties)

At each time-point (and for each cultivar) three replicate samples of tubers of desired size (see Table 1) were removed to a combined fresh weight of 200g per Rep. The tubers were washed in cold water and blotted dry. The tuber(s) were diced into cubes (*ca.* 2.5 cm^3) and processed in a blender with 400 ml distilled water and 100 mg of sodium metabisulphite. The blended mixture was passed through a 1-2 mm sieve into a 1 l beaker. Any material retained in the blender (or sieve) was washed with another 400 ml of water and sieved. Any remaining solid was discarded

at this point. The sieved starch was allowed to sediment for 1 h at room temperature, and the supernatant carefully poured off. The sedimented starch granules were washed in 100 ml water and passed through one layer of cheesecloth, taking care to wash through any retained starch with small quantities of water. The starch was allowed to sediment for a further 30 min, and this process repeated until no cell wall debris or potato skin was visible under the microscope in the sedimented starch. Once satisfied that the starch was sufficiently separated from the other cellular components, it was dried in an uncovered beaker at 37°C overnight, after which any remaining clumps were broken up. This was repeated twice daily for a further 48 h. Starch was sub-sampled and its moisture content determined by oven drying at 100°C for 24 h. Drying at 37°C was continued until the moisture content reached 14±1% (w/w). The starch was then stored in sealed plastic containers at room temperature.

Viscosity determinations of starches prepared from tubers of each of the four cultivars harvested the time -points 1-5B (see above) were analysed using the Rapid Visco Analyser (RVA, Newport Scientific). This provides a measurement of the physico-chemical properties of starch in terms of its gelatinisation profile.

Air-dried tuber starch (1.2 g) was accurately weighed into an RVA aluminium canister which contained exactly 25.00 g of sterile distilled water. The contents were mixed vigorously with the plastic paddle accompanying the canister until no clumps of starch were visible. The paddle was placed into the canister, and the top of the paddle/canister clipped into the apparatus. The measurement cycle was initiated by depressing the motor tower of the instrument. The RVA apparatus was run according to manufacturer's instructions. The idle temperature of the RVA was 50 ± 1°C. The temperature/speed ramping profile used for each sample was as follows: 50°C and 960 rpm at 0 min; 50°C and 160 rpm at 10 sec; 50°C and 160 rpm at 1 min; 95°C and 160 rpm at 4.45 min; 95°C and 160 rpm at 7.15 min; 50°C and 160 rpm at 11.0 min, and 50°C and 0 rpm at 13 min.

Tuber Texture Analysis

Texture measurements were carried out on a QTS 25 Texture Analyser (CNS Farnell) with potato samples cut to a 20 mm cube from the centre of the potato. Measurements of peak force and work done to penetrate to a depth of 10 mm were assessed with a Perspex wedge (Vincent *et al*, 1991).

From each cultivar (two from each field block) eight potatoes were assessed for raw texture and eight tubers (two from each field block) assessed for cooked texture (at time points 1-3 only). Samples were cooked for timed periods in boiling water, two cubes only at a time to ensure that the temperature remained the same during boiling and then measured after a timed cooling period.

Metabolomics: Analysis of Tuber Metabolites by Gas Chromatography-Time-of-Flight (TOF)-Mass Spectrometry

Freeze dried powders (100 mg) prepared as described above (see Materials and methods), for each of the cultivars at each time-point, were analysed by GC-MS (polar and non-polar metabolites) using a Thermo-Finnigan Tempus GC-(TOF)-MS system as previously described (Shepherd *et al*., 2006).

Results

Starch Rheology (Cooking Properties)

Gelatinisation is a term used to describe the irreversible changes which accompany the disruption of the starch granule structure (Zobel, 1984). Differences in gelatinisation between starches are a sensitive indicator of differences in structure which can contribute to textural differences. They reflect, for example, differences in the numbers and lengths of double helices of amylopectin –the major component of potato starch (Cooke & Gidley, 1992; Moates *et al*., 1997).

When starch granules are heated in excess water, the starch granules gelatinise and swell. This is primarily a property of the amylopectin fraction (Tester and Morrison, 1990a). As the double helices unwind, and the hydrogen bonds break, the sugar hydroxyl groups are free to hydrogen bond with water. For granules to be able to expand to their maximum volume, water must be non-limiting. Under these conditions, this onset of granular swelling is associated with the onset of gelatinisation (Tester, 1997a). As temperature increases, there is a progressive linear swelling region, followed (for most starches) by a plateau region (peak viscosity). At the plateau, swollen volume does not increase markedly with temperature; however, a temperature is reached where granules begin to disintegrate.

Viscosity determinations, traditionally carried out with the Brabender Visko-Amylograph, are time-consuming (*ca*. 2 h) and require relatively large quantities of starch sample (Haase *et al*., 1995). The Rapid Visco Analyser (RVA, Newport Scientific) offers considerable advantages in both its short analysis time and small sample size required. RVA allows the analysis of starch pasting profiles. A granular starch suspension is subjected to a defined heating (linear ramping) and cooling programme (see Materials and methods), and the viscosity recorded as a function of temperature and time.

Each starch sample has its own characteristic pasting curve which measures peak time and peak viscosity (peak), pasting temperature, trough viscosity (hold), breakdown (peak viscosity – trough viscosity), final viscosity (final) and total setback (final viscosity – trough viscosity). Viscosity was measured in centipoise (cP).

Viscosity determinations of starches prepared from tubers of each of the 4 cultivars harvested at the time-points 1-5B (see Table 1 above) were analysed using the Rapid Visco Analyser (RVA, Newport Scientific). At each time-point and for each block all three starch reps were analysed. Analysing all three reps from a single cultivar within a single block revealed no significant differences between reps within a block. For this reason only rep 1 for any given cultivar, from block 1, will be shown.

Figures 1 and 2 show the general differences in viscosity profile of the starches derived from Montrose & Mayan Gold tubers harvested during development and following storage. The most marked differences in both cultivars during tuber development are changes in the peak viscosities and the time take to attain peak viscosity. Figure 3 illustrates the differences in pasting profiles between all 4 cultivars harvested at a single time-point (T3). *S. tuberosum* lines clearly have higher peak and breakdown viscosities compared with *S. phureja*, whilst the pasting temperature and peak viscosity times were lower for *S. tubersoum*. Within *S. tuberosum* cv. Pentland Dell had a significantly lower peak and trough viscosity, breakdown and final viscosity compared with Montrose

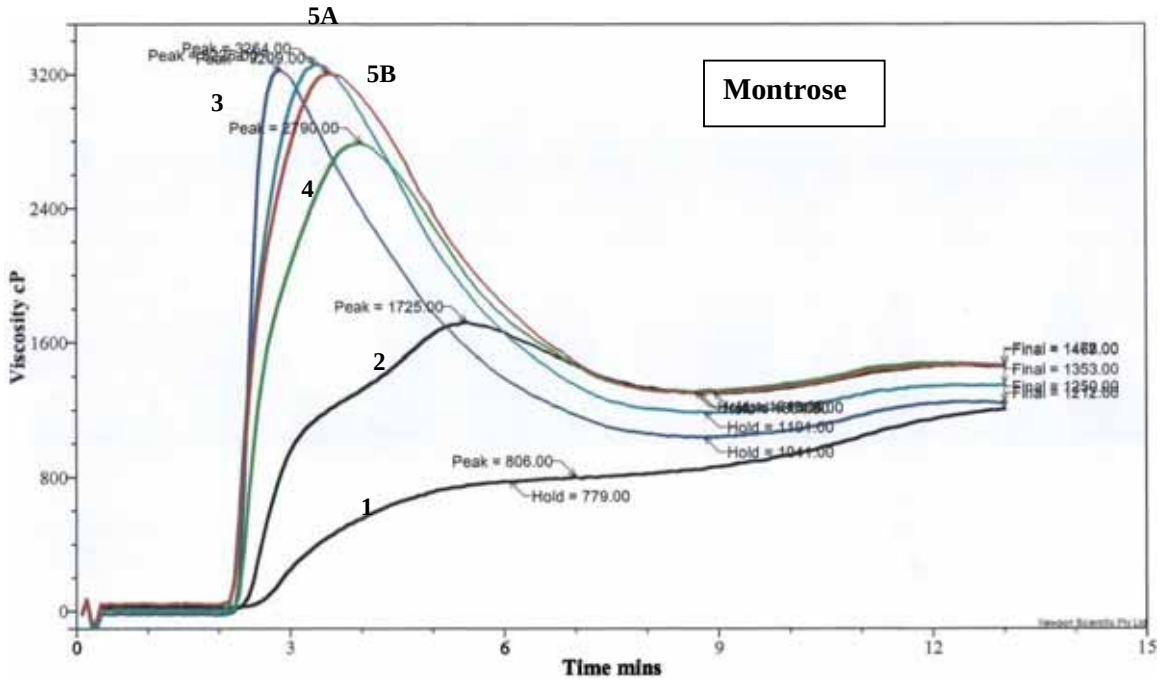


FIGURE 1. RAPID VISCOMETRIC ANALYSIS OF MONTROSE REP 1 STARCHES DERIVED FROM TUBERS HARVESTED AT TIME-POINTS 1-5B FROM FIELD BLOCK 1.

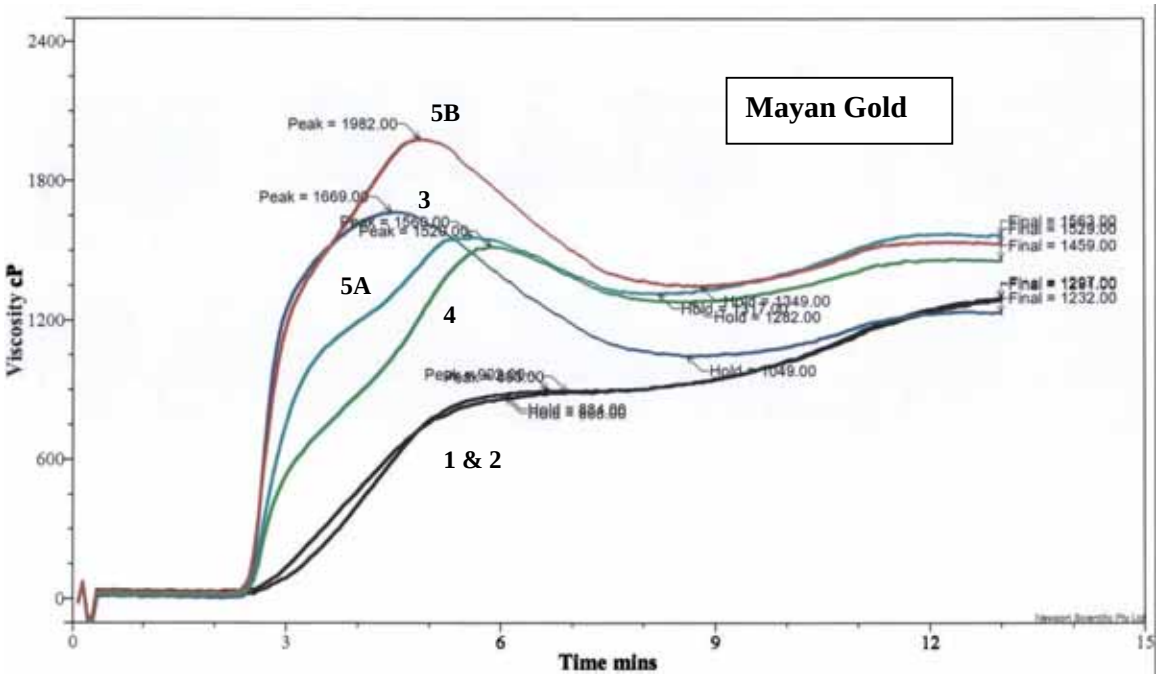


FIGURE 2. RAPID VISCOMETRIC ANALYSIS OF MAYAN GOLD REP 1 STARCHES DERIVED FROM TUBERS HARVESTED AT TIME-POINTS 1-5B FROM FIELD BLOCK 1.

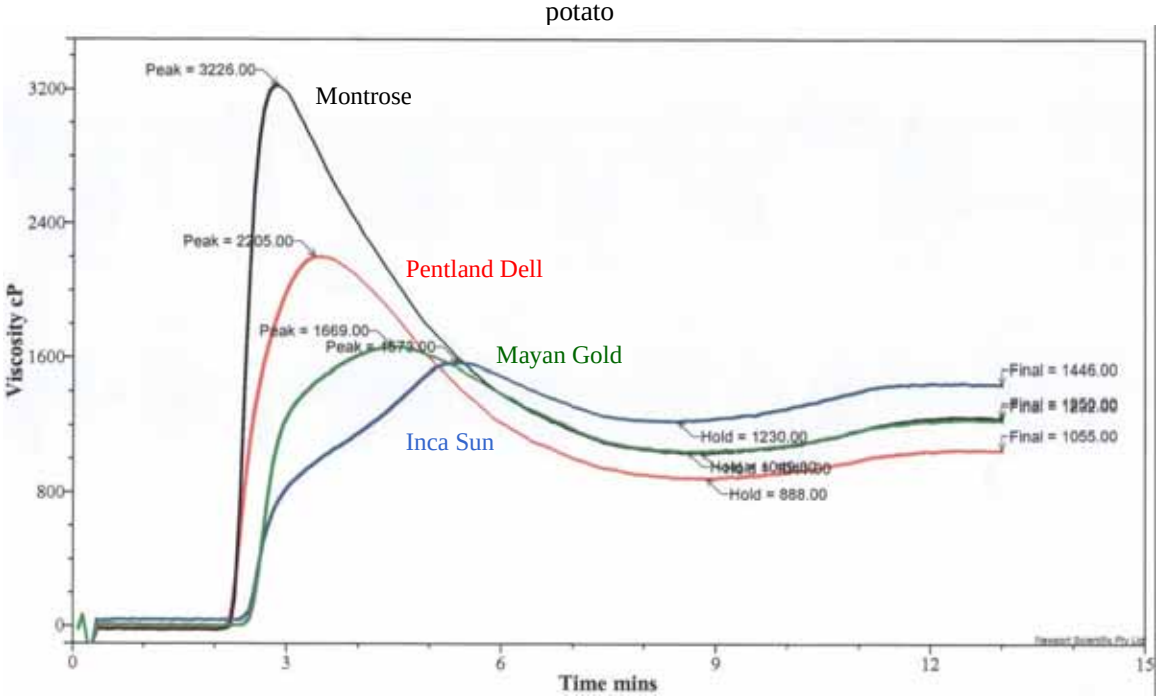


FIGURE 3. RAPID VISCOMETRIC ANALYSIS OF REP 1 STARCHES DERIVED FROM TUBERS OF ALL 4 CULTIVARS HARVESTED AT TIME-POINT 3 (SENESCENCE) FROM FIELD BLOCK 1.

Detailed quantitative data on starch gelatinisation characteristics for the four cultivars are summarised in Table 2 and for each time-point analysed. The data highlighted in yellow signify the gelatinisation characteristics of starches derived from tubers of all four cultivars harvested at T3 (senescence) – see Figure 3 for examples of pasting profiles of all four cultivars at this time-point.

The data clearly indicate that *S. phureja* and *S. tuberosum* starches are very different, and this may underpin differences in cooking times and texture changes post-cooking (see results in next section on tuber texture analysis). Several starch attributes are known to affect gelatinisation properties such as amylose:amylopectin ratios, phosphate contents and granule size and distribution. For example, higher amylopectin potato starches tend to have higher gelatinisation temperatures. These starch parameters are still under investigation but will provide important insights into genotypic differences in texture and cooking properties. This data will be correlated with expression profiles for genes involved in starch biosynthesis.

					Centipoise (cP)					Peak Time (min)	Pasting Temp (°C)
ID	Cultivar	T	Block	Rep	Peak Viscosity	Trough Viscosity	Breakdown	Final Viscosity	Total Setback		
1.1	P. Dell	1	1	1	1293.00	1195.00	98.00	1786.00	591.00	7.00	68.60
1.2	P. Dell	1	1	2	1306.00	1208.00	98.00	1794.00	586.00	7.00	68.65
1.3	P. Dell	1	1	3	1230.00	1142.00	88.00	1774.00	632.00	7.00	69.40
2.1	Montrose	1	1	1	806.00	779.00	27.00	1212.00	433.00	7.00	68.65
2.2	Montrose	1	1	2	875.00	838.00	37.00	1388.00	550.00	7.00	68.70
2.3	Montrose	1	1	3	901.00	867.00	34.00	1393.00	526.00	6.93	68.70
3.1	Inca Sun	1	1	1	777.00	743.00	34.00	1134.00	391.00	7.00	84.10
3.2	Inca Sun	1	1	2	825.00	788.00	37.00	1211.00	423.00	6.93	83.25
3.3	Inca Sun	1	1	3	840.00	802.00	38.00	1239.00	437.00	6.93	82.45
4.1	M. Gold	1	1	1	893.00	868.00	25.00	1291.00	423.00	6.93	72.65
4.2	M. Gold	1	1	2	984.00	954.00	30.00	1457.00	503.00	7.00	70.25
4.3	M. Gold	1	1	3	862.00	837.00	25.00	1243.00	406.00	7.00	70.20
17.2	P. Dell	2	1	1	1292.00	1126.00	166.00	1308.00	182.00	5.87	66.15
17.2	P. Dell	2	1	2	1705.00	1172.00	533.00	1296.00	124.00	5.13	65.45
17.3	P. Dell	2	1	3	1589.00	1173.00	416.00	1327.00	154.00	5.13	65.25
18.1	Montrose	2	1	1	1725.00	1308.00	417.00	1465.00	157.00	5.47	65.40
18.2	Montrose	2	1	2	2091.00	1421.00	670.00	1565.00	144.00	5.07	66.30
18.3	Montrose	2	1	3	1819.00	1366.00	453.00	1557.00	191.00	5.33	66.30
19.1	Inca Sun	2	1	1	982.00	934.00	48.00	1501.00	567.00	7.00	69.45
19.2	Inca Sun	2	1	2	1065.00	994.00	71.00	1635.00	641.00	7.00	71.05
19.3	Inca Sun	2	1	3	976.00	937.00	39.00	1508.00	571.00	7.00	70.20
20.1	M. Gold	2	1	1	902.00	884.00	18.00	1297.00	413.00	6.67	75.80
20.2	M. Gold	2	1	2	891.00	881.00	10.00	1290.00	409.00	7.00	74.25
20.3	M. Gold	2	1	3	904.00	847.00	57.00	1399.00	552.00	7.00	77.60
33.1	P. Dell	3	1	1	2205.00	888.00	1317.00	1055.00	167.00	3.40	63.80
33.2	P. Dell	3	1	2	2449.00	861.00	1588.00	1014.00	153.00	3.13	63.75
33.4	P. Dell	3	1	3	2510.00	911.00	1599.00	1086.00	175.00	3.07	63.75
34.1	Montrose	3	1	1	3226.00	1041.00	2185.00	1250.00	209.00	2.87	64.65
34.2	Montrose	3	1	2	2929.00	1032.00	1897.00	1225.00	193.00	3.00	63.60
34.3	Montrose	3	1	3	3315.00	1057.00	2258.00	1260.00	203.00	2.87	63.80
35.1	Inca Sun	3	1	1	1573.00	1230.00	343.00	1446.00	216.00	5.40	67.05
35.2	Inca Sun	3	1	2	1531.00	1211.00	320.00	1402.00	191.00	5.33	67.05
35.3	Inca Sun	3	1	3	1515.00	1256.00	259.00	1441.00	185.00	5.73	67.00
36.1	M. Gold	3	1	1	1669.00	1049.00	620.00	1232.00	183.00	4.47	67.05
36.2	M. Gold	3	1	2	1719.00	1092.00	627.00	1273.00	181.00	4.60	66.85
36.3	M. Gold	3	1	3	1800.00	1080.00	720.00	1286.00	206.00	4.20	67.00

TABLE 2. GELATINISATION CHARACTERISTICS, AS DETERMINED BY RVA, OF STARCHES EXTRACTED FROM POTATO TUBERS OF ALL FOUR CULTIVARS (REPS 1-3) FROM EACH FIELD BLOCK AT EACH TIME-POINT. WHERE T = TIME-POINT.

					Centipoise (cP)					Peak Time (min)	Pasting Temp (°C)
ID	Cultivar	T	Block	Rep	Peak Viscosity	Trough Viscosity	Breakdown	Final Viscosity	Total Setback		
49.1	P. Dell	4	1	1	2178.00	1163.00	1015.00	1348.00	185.00	4.47	63.75
49.2	P. Dell	4	1	2	2084.00	1162.00	922.00	1306.00	144.00	4.67	63.80
49.3	P. Dell	4	1	3	2528.00	1228.00	1300.00	1408.00	180.00	4.07	63.75
50.1	Montrose	4	1	1	2790.00	1313.00	1477.00	1467.00	154.00	4.00	64.65
50.2	Montrose	4	1	2	2561.00	1272.00	1289.00	1498.00	226.00	4.20	63.80
50.3	Montrose	4	1	3	2697.00	1306.00	1391.00	1461.00	155.00	4.13	63.60
51.1	Inca Sun	4	1	1	1526.00	1341.00	185.00	1527.00	186.00	5.67	67.05
51.2	Inca Sun	4	1	2	1830.00	1343.00	487.00	1535.00	192.00	5.13	67.05
51.3	Inca Sun	4	1	3	1771.00	1385.00	386.00	1583.00	198.00	5.27	67.05
52.1	M. Gold	4	1	1	1520.00	1282.00	238.00	1459.00	177.00	5.87	67.00
52.2	M. Gold	4	1	2	1490.00	1342.00	148.00	1572.00	230.00	6.13	67.05
52.3	M. Gold	4	1	3	1769.00	1468.00	301.00	1671.00	203.00	5.80	67.00
53.1	P. Dell	5A	1	1	2352.00	1130.00	1222.00	1288.00	158.00	4.20	62.95
53.2	P. Dell	5A	1	2	2501.00	1102.00	1399.00	1251.00	149.00	3.93	63.00
53.3	P. Dell	5A	1	3	2460.00	1095.00	1365.00	1224.00	129.00	4.07	63.85
54.1	Montrose	5A	1	1	3264.00	1191.00	2073.00	1353.00	162.00	3.33	62.95
54.2	Montrose	5A	1	2	3140.00	1213.00	1927.00	1390.00	177.00	3.53	63.00
54.3	Montrose	5A	1	3	3634.00	1260.00	2374.00	1404.00	144.00	3.20	63.00
55.1	Inca Sun	5A	1	1	1597.00	1418.00	179.00	1715.00	297.00	5.93	66.25
55.2	Inca Sun	5A	1	2	1615.00	1543.00	72.00	1865.00	322.00	6.60	67.75
55.3	Inca Sun	5A	1	3	1691.00	1540.00	151.00	1855.00	315.00	6.00	67.10
56.1	M. Gold	5A	1	1	1560.00	1317.00	243.00	1563.00	246.00	5.60	67.05
56.2	M. Gold	5A	1	2	1659.00	1361.00	298.00	1593.00	232.00	5.47	67.20
56.3	M. Gold	5A	1	3	1691.00	1389.00	302.00	1594.00	205.00	5.53	67.05
57.1	P. Dell	5B	1	1	2179.00	1201.00	978.00	1434.00	233.00	4.47	63.75
57.2	P. Dell	5B	1	2	2444.00	1196.00	1248.00	1383.00	187.00	4.07	63.70
57.3	P. Dell	5B	1	3	2357.00	1104.00	1253.00	1240.00	136.00	3.93	62.95
58.1	Montrose	5B	1	1	3209.00	1304.00	1905.00	1472.00	168.00	3.53	63.75
58.2	Montrose	5B	1	2	3142.00	1521.00	1621.00	1735.00	214.00	3.87	63.60
58.3	Montrose	5B	1	3	3010.00	1289.00	1721.00	1457.00	168.00	3.67	63.05
59.1	Inca Sun	5B	1	1	1610.00	1393.00	217.00	1597.00	204.00	6.00	66.25
59.2	Inca Sun	5B	1	2	1587.00	1401.00	186.00	1634.00	233.00	6.13	67.05
59.3	Inca Sun	5B	1	3	1671.00	1435.00	236.00	1658.00	223.00	5.87	67.05
60.1	M. Gold	5B	1	1	1982.00	1349.00	633.00	1529.00	180.00	4.87	67.05
60.2	M. Gold	5B	1	2	1906.00	1480.00	426.00	1728.00	248.00	5.33	67.05
60.3	M. Gold	5B	1	3	2124.00	1380.00	744.00	1546.00	166.00	4.80	67.10

TABLE 2 (CONT). GELATINISATION CHARACTERISTICS, AS DETERMINED BY RVA, OF STARCHES EXTRACTED FROM POTATO TUBERS OF ALL FOUR CULTIVARS (REPS 1-3) FROM EACH FIELD BLOCK AT EACH TIME-POINT. WHERE T = TIME-POINT.

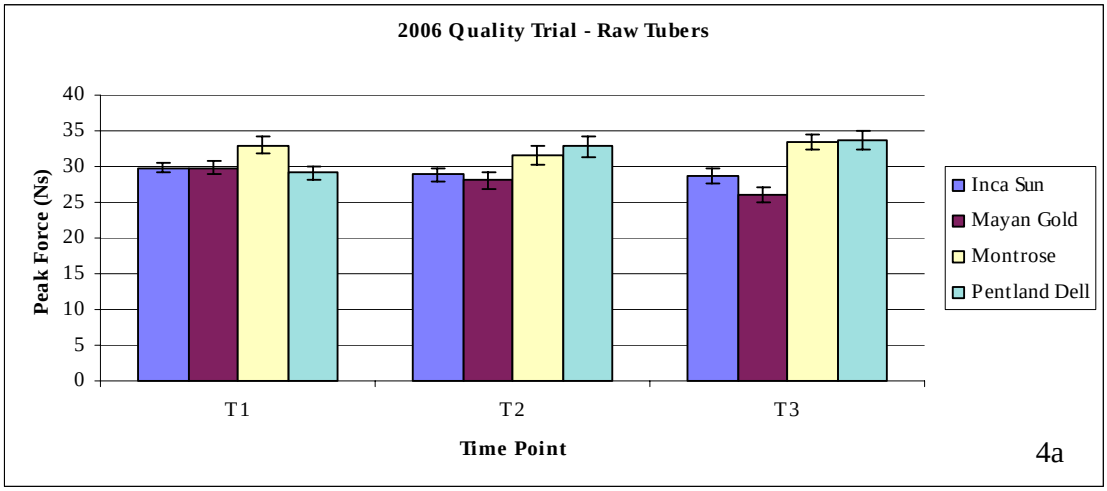
Tuber Texture Analysis

Eight potatoes from each cultivar (two from each field block) were assessed for both raw and cooked (boiled for 5 min) texture at time-points 1-3 only.

Figure 4a show the results of peak force measured (Newtons [Ns]) in raw tubers of Pentland Dell, Montrose, Inca Sun and Mayan Gold harvested at time-points 1-3. Figure 4b show the results of work done to penetrate to a depth of 10 mm (Jm^{-2}). Figure 5a show the results of peak force measured (Ns) for boiled tubers (5 min) and Figure 5b the results of work done to penetrate to a depth of 10 mm (Jm^{-2}) in boiled tubers (5 min).

Analysis of variance was carried out on the peak force and work done to penetrate to a depth of 10mm for both raw and cooked tubers for each time point. Results for both parameters measured in raw tubers showed both *S. phureja* cultivars generated significantly lower values (5% level) than the *S. tuberosum* cultivars indicating a weaker texture. However, there was no significant difference between the developmental time-points assayed.

For cooked samples (5 min boiling) the values generated were again lower for *S. phureja* compared with *S. tuberosum* cultivars, denoting a weaker texture in *S. phureja*. Differences between *S. phureja* and *S. tuberosum* were more pronounced after cooking particularly at time-point 1, primarily due to the fact that after cooking texture became weaker in the *S. tuberosum* cultivars after time-point 1. Firmness of cooked *S. phureja* was relatively stable during development. It should also be noted that when potato cubes were cooked in boiling water over a period of up to 20 min the results showed that the *S. phureja* lines took a much shorter time to reach optimal cooking stage (data not shown). This could be related to the lower peak viscosities found in starch gelatinisation profiles but changes in cell wall adhesion must also be considered.



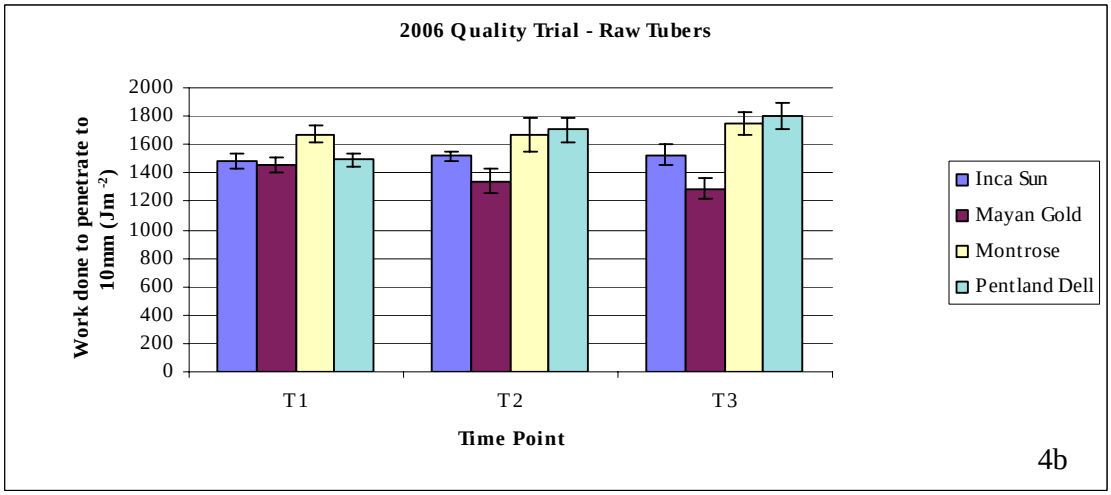
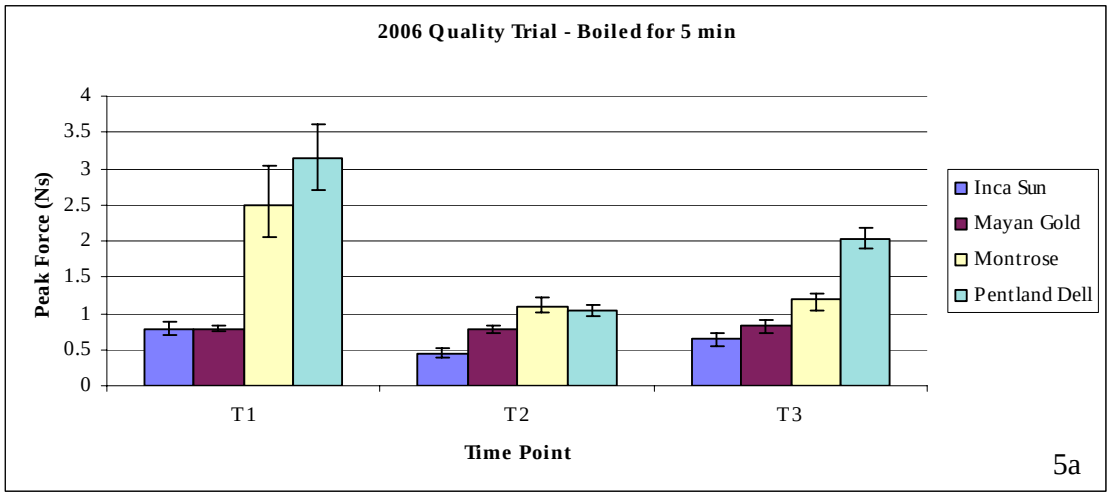


FIGURE 4. HISTOGRAM OF RAW TUBERS FROM 4 CULTIVARS AT 3 TIME-POINTS, T1-T3.
Each bar represents the mean of 8 tubers. Error bars represent standard error of mean.
a. Measurement of peak force (Ns). b. Measurement of work done to penetrate to a depth of 10 mm (Jm-2).



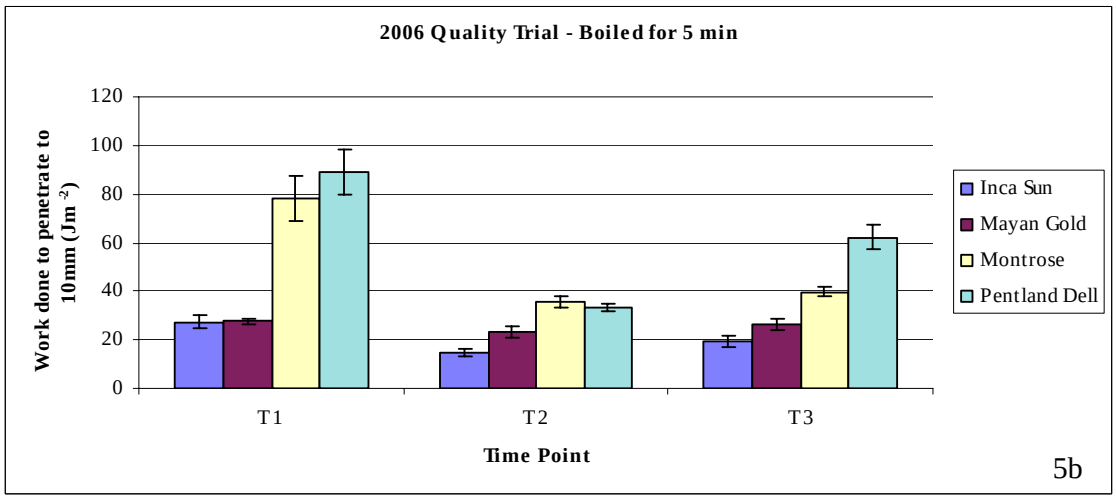
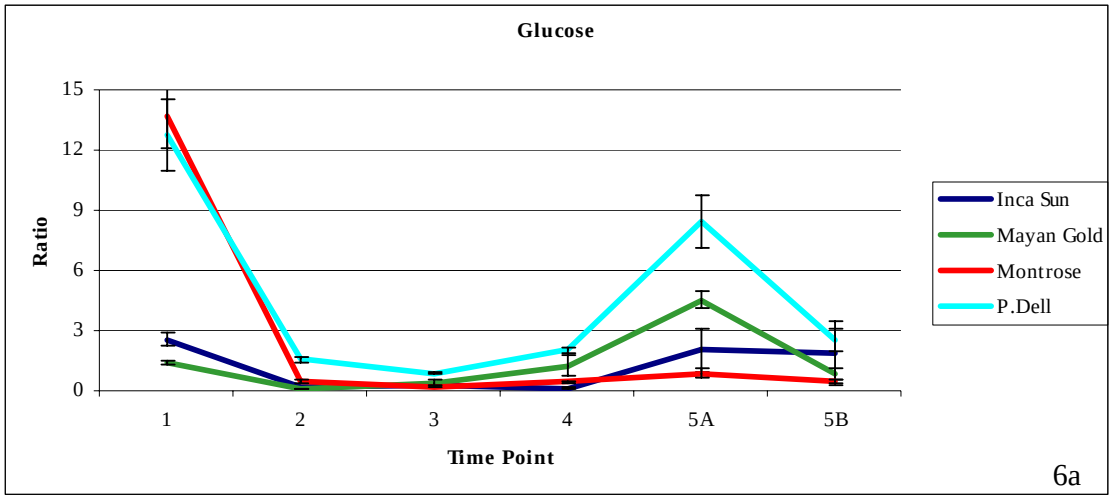


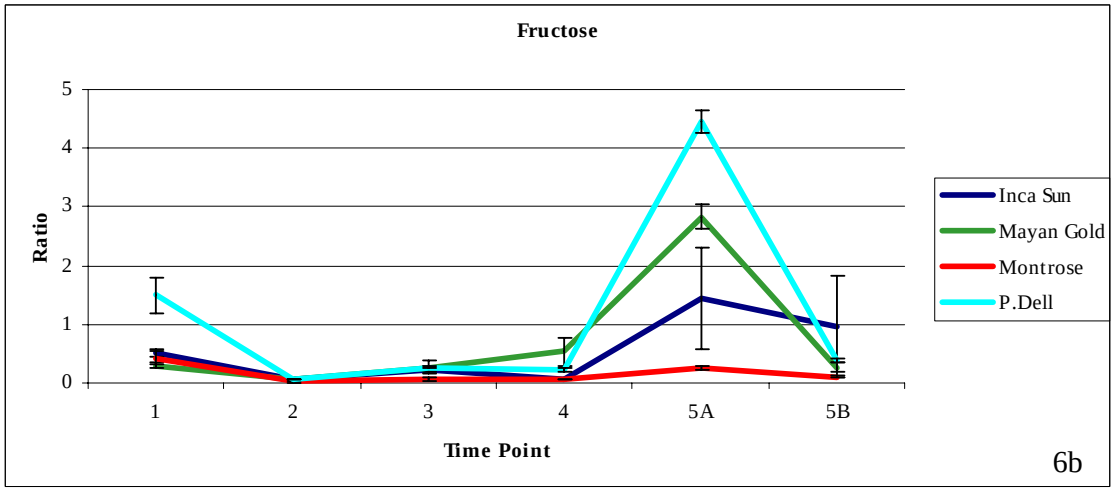
FIGURE 5. HISTOGRAM OF BOILED TUBERS (5 MIN) FROM 4 CULTIVARS AT 3 TIME-POINTS, T1-T3.
Each bar represents the mean of 8 tubers. Error bars represent standard error of mean.
a. Measurement of peak force (Ns). b. Measurement of work done to penetrate to a depth of 10 mm (Jm-2).

Gas Chromatography-Time-of-Flight (TOF)-Mass Spectrometry

As with many of the other biochemical analysis being carried out on the *S. phureja* and *S. tuberosum* material not all of the data has been processed at this point in time. However, from the GC-MS analysis to date we have mined the data for reducing sugars (glucose + fructose) and asparagine as these are determinant of acrylamide forming potential and so represent topical metabolites of interest. We will, however, have data for *ca*. 100 metabolites once all of our data are processed. This can then be more fully correlated with the gene expression profiles.

Figures 6a-c shows profiles of glucose, fructose and asparagine respectively in Pentland Dell, Montrose, Inca Sun and M. Gold at time-points 1-5B, where Ratio = peak area of compound/peak area of Ribitol (internal standard).





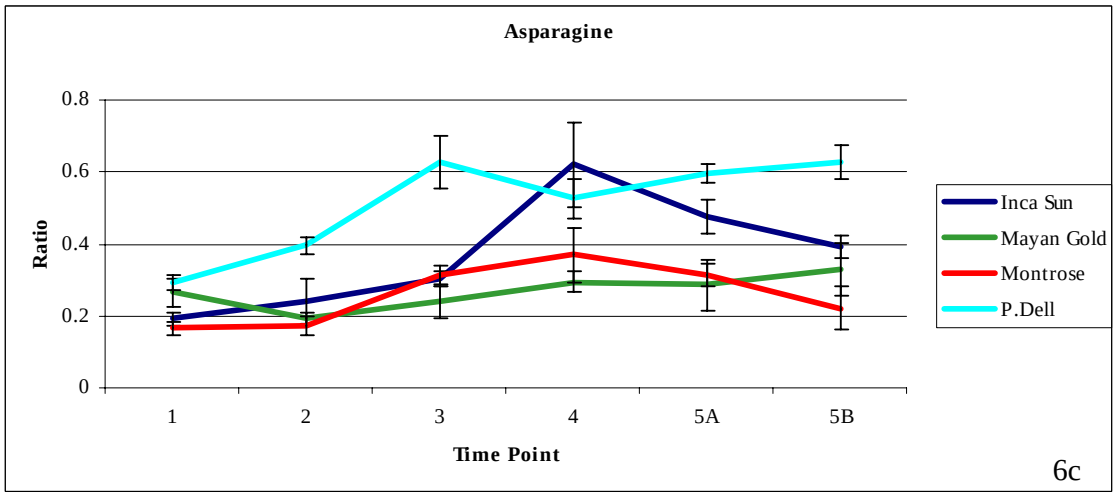


FIGURE 6. PROFILES OF SELECTED COMPOUNDS IDENTIFIED IN GC-MS (POLAR) FROM 4 CULTIVARS AT TIME-POINTS T1-T5B.
Ratio = peak area of compound/peak area of Ribitol). For T1-T3: each value represents the mean of 4 values. For T4-T5B: each value represents the mean of 3 values. Error bars represent standard error of mean.
a. Ratio of Glucose in each of the four cultivars from time-points 1-5B b. Ratio of Fructose in each of the four cultivars from time-points 1-5B. c. Ratio of Asparagine in each of the four cultivars from time-points 1-5B.

There are clearly major differences in storage sugar levels between all for cultivars, particularly following cold storage (stage 5A) but cultivars could also be differentiated in reducing sugar levels very early on in development. Montrose in particular was very low in post-storage sugar levels. There were also clear cultivar differences in asparagine levels with Pentland Dell having consistently higher levels than other cultivars. One would therefore predict Montrose would have low acrylamide content in crisps/French fries (this is being evaluated).

Gene Expression Analysis: Use of the POCI Potato Microarray

The microarray used in this project originated from an international collaboration (Potato Oligo Chip Initiative: POCI) which exploited the entire set of publicly available potato sequences, and therefore represents the most extensive transcriptomics resource available for this species (Kloosterman *et al*., 2008 submitted). Over 40,000 unique potato genes are represented on the POCI array, which is manufactured on the Agilent platform that utilises state-of-the-art, highly sensitive and reproducible microarray technology. SCRI have invested heavily in both instrumentation and expertise over the past few years to allow exploitation of such high-throughput transcriptional profiling.

Microarray Processing and Data Analysis

Purified RNAs from selected and replicated harvest dates were labelled with fluorescent dyes (see Materials and methods) and applied to POCI microarrays using an experimental design provided through consultation with BioSS, our statistical partners. Following array processing using standard operating procedures established at SCRI, data were extracted and analysed using dedicated microarray software. Data analysis included normalisation, to remove any technical bias, and filtering out of consistently low-expressed genes. The resultant high-quality dataset (~35,000 genes) was subjected to box-plot visualisation and principle components analysis (PCA), which ensured that no outlying samples were present and that the data were not influenced by artefactual components. It was clear at this stage that the most important component separating the data was potato genotype (i.e. *S. phureja* and *S. tuberosum*). ANOVA was applied to this dataset using strict statistical parameters (p-value<0.05, Bonferroni multiple testing correction) and identified around 7,000 genes with statistically significant changes in expression at the potato genotype level. However, a more manageable number of genes (230) exhibited interaction between potato type and time of harvest. Within this gene list, there are genes with clear homology to those previously implicated in metabolic processes associated with flavour and texture traits.

Genes that change significantly in expression in this experiment were compared with that of a previous SCRI study, which utilised different *S. phureja* and *S. tuberosum* genotypes. This comparison identified some 330 commonly regulated genes at the potato type level.

Within the data-sets obtained there are several options for analysis. For example, we can challenge the data to identify differences in gene expression between specific developmental or storage phases, or for any time point we can challenge the data to identify specific genotypic differences. We can also challenge the datasets for specific information on the expression of genes we believe could be important in controlling specific traits. The permutations are extensive and comprehensive analysis is beyond the scope of a 9 month project, particularly with the amount of phenotypic data that will also be needed to be cross correlated with gene expression. Therefore, below we provide examples of how the gene expression data has been exploited to date.

Genes Implicated in Starch, Sugar and Amino Acid Metabolism

Of the genes identified as significantly changing between potato genotype, specific homologues were selected as being relevant to cell wall and starch metabolism (texture) and sugar and amino-acid metabolism (processing quality, acrylamide forming potential). These are listed in Table 3. Simple clustering into two groups (those that are up-regulated or those that are down-regulated in *S. phureja* compared to *S. tuberosum*), determined major differences in gene expression profiles of some of these genes were induced in magnitude specifically in either *S. tuberosum* or *S. phureja*. The genes can therefore be implicated as potential determinants of key traits related to these metabolic processes.

Gene Homologue	Higher Expression In:
Starch Metabolism	
1,4-alpha-glucan branching enzyme (Starch branching enzyme)	Phureja
adenylate kinase isoenzyme 6	Phureja
alpha-1,4 glucan phosphorylase L-1 isozyme (Starch phosphorylase L-1)	Phureja
ADP-glucose pyrophosphorylase small subunit	Phureja
disproportionating enzyme	Phureja
glycogen (starch) synthase-like	Phureja
granule-bound starch synthase	Phureja
soluble-starch-synthase	Phureja
ADP-glucose pyrophosphorylase large subunit	Tuberosum
alpha glucosidase-like protein	Tuberosum
disproportionating enzyme	Tuberosum
Sugar Metabolism	
alpha-amylase	Phureja
invertase inhibitor homologue	Phureja
neutral/alkaline invertase	Phureja
sucrose-phosphate synthase (UDP-glucose-fructose-phosphate glucosyltransferase) isoform C	Phureja
acid invertase	Tuberosum
alpha-amylase	Tuberosum
cell wall apoplastic invertase	Tuberosum
neutral invertase	Tuberosum
sucrose-phosphate synthase (UDP-glucose-fructose-phosphate glucosyltransferase) isoform B	Tuberosum
Amino Acid Metabolism	
asparagine synthetase	Phureja
asparaginase	n/a
Texture	
pectin methylesterase	Phureja

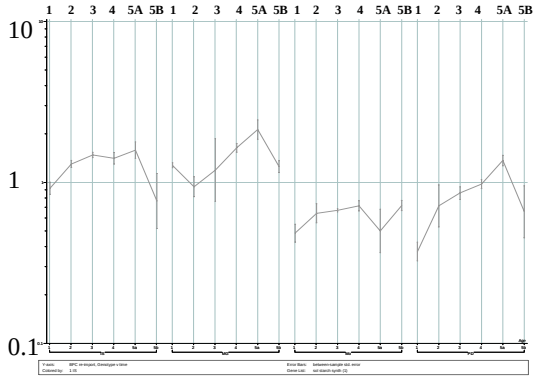
TABLE 3. SELECTED GENES RELATED TO STARCH, SUGAR AND AMINO ACID METABOLISM WITH SIGNIFICANT DIFFERENCE IN MAGNITUDE OF EXPRESSION PROFILES BETWEEN POTATO TYPES.

Detailed expression profiles of the genes in Table 3 are shown in Figures 7-9. Expression profiles are provided for all four cultivars and for all time-points in development and storage. Some key differential gene expression is evident. For example, comparing *S. tuberosum* and *S. phureja* in Figure 7 we note:

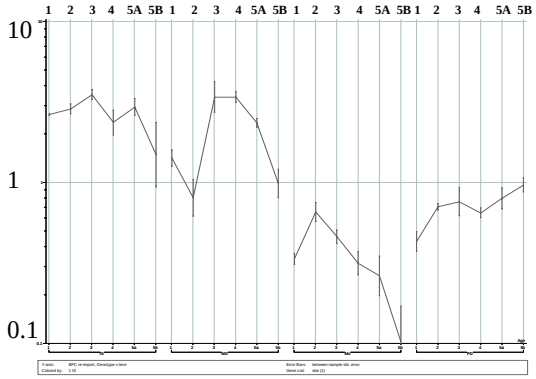
a. Soluble starch synthase and 1,4-alpha-glucan branching enzyme gene expression is higher in *S. phureja*.
b. The expression of two amylase genes differs.

c. The expression of ADP-glucose pyrophosphorylase (small and large subunits) differs.
d. Granule bound starch synthase gene expression differs.

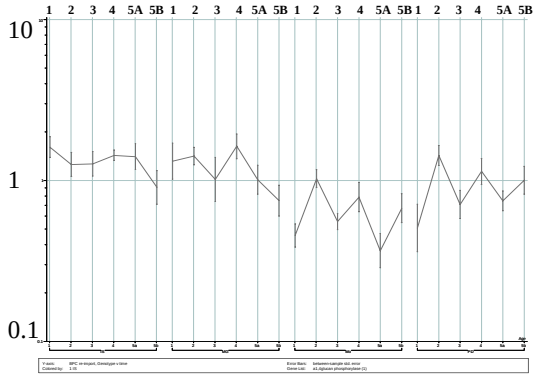
Notably, within *S. tuberosum* the expression of the 1,4-alpha-glucan branching enzyme gene and the adenylate kinase gene differs in Montrose compared with Pentland Dell. Also note differences between *S. phureja* and *S. tuberosum* in terms of genes involved in sugar metabolism (Figure 8, invertase and invertase inhibitor gene expression) and also genes defining cell wall properties (Figure 9, pectin methyl esterase).



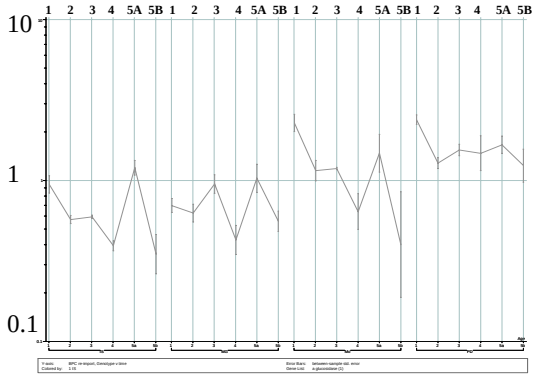
Soluble starch synthase



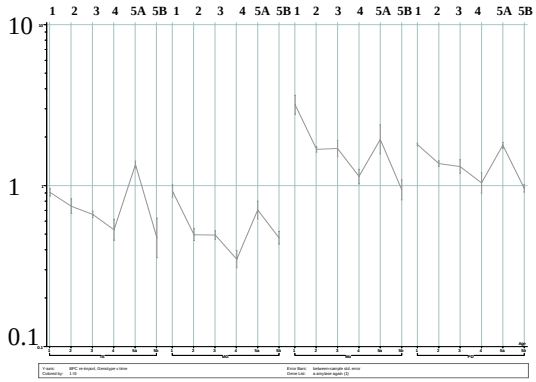
1,4-alpha-glucan branching enzyme



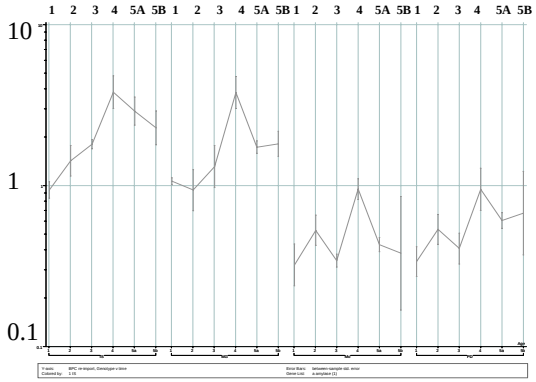
Starch phosphorylase



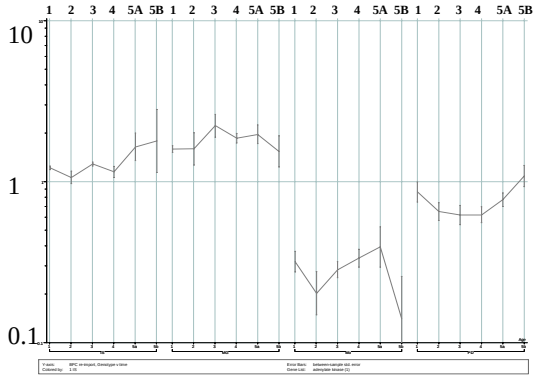
Alpha glucosidase



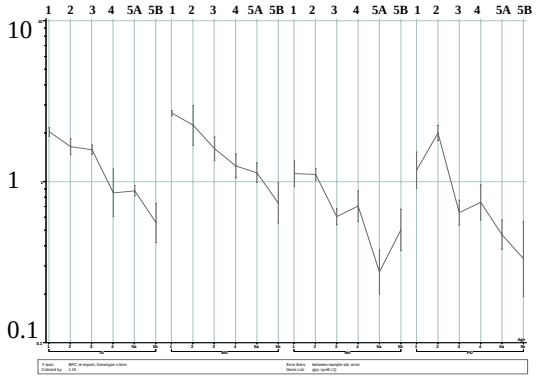
Alpha amylase



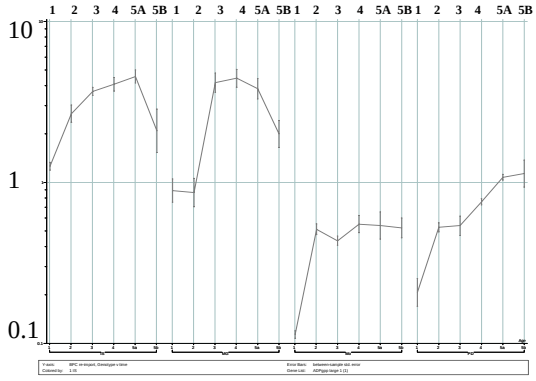
Alpha Amylase



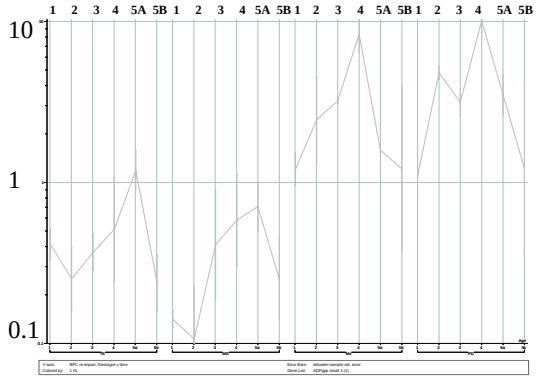
Adenylate kinase



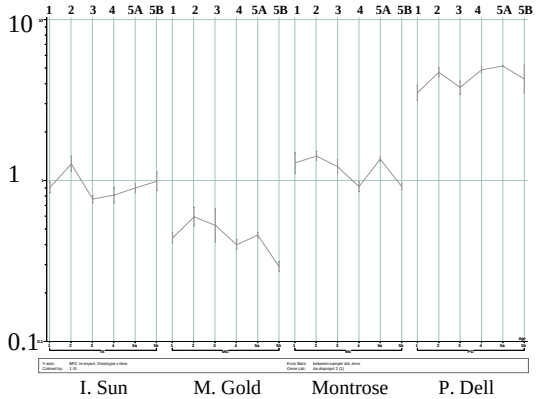
Glycogen synthase



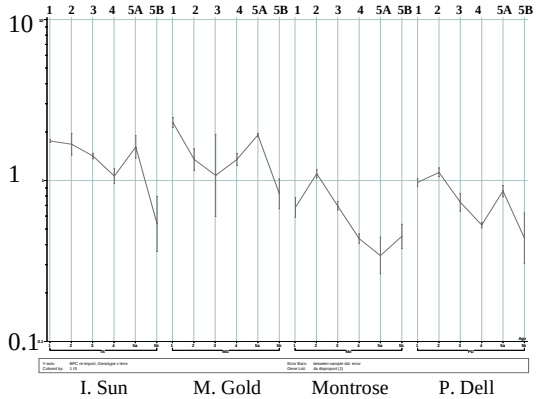
ADP-glucose pyrophosphorylase (large)



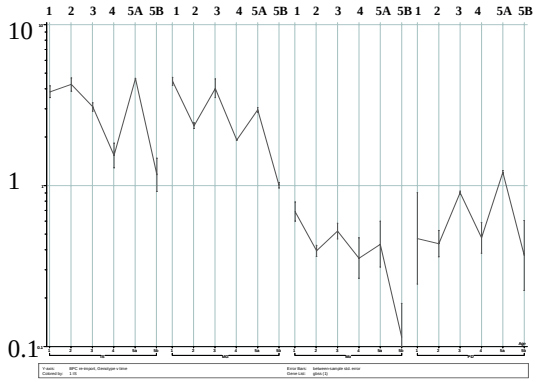
ADP-glucose pyrophosphorylase (small)



Disproportionating enzyme



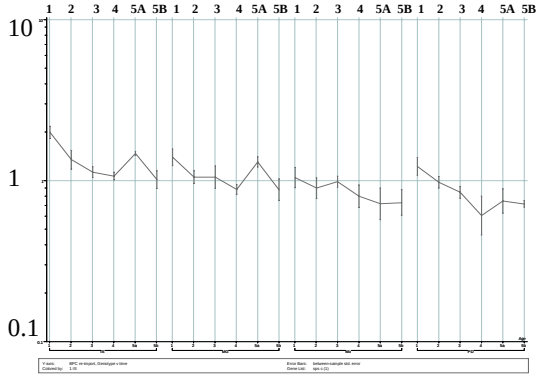
Disproportionating enzyme



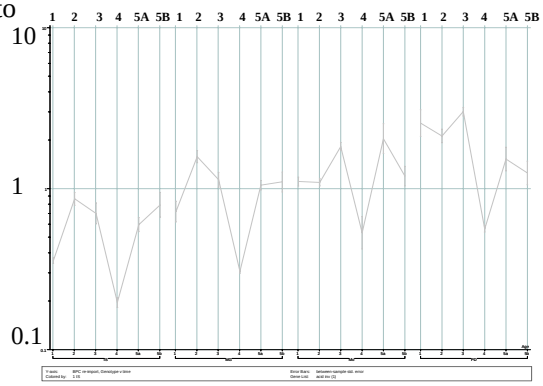
Granule bound starch synthase

FIGURE 7. EXPRESSION PROFILES OF SELECTED SIGNIFICANTLY CHANGING GENES WITH RELEVANCE TO STARCH METABOLISM.

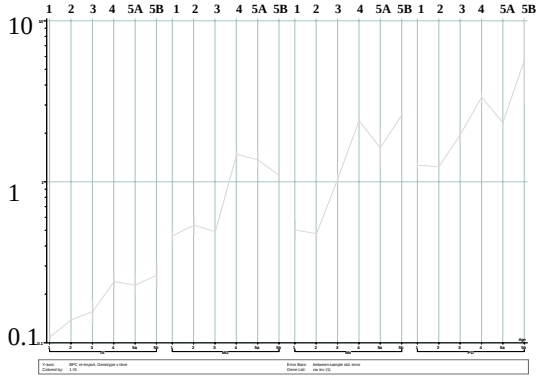
Horizontal axis indicates time-point of harvest for each potato cultivar and the vertical axis (log scale) shows fold change compared to median values across all samples. Error bars represent standard error.



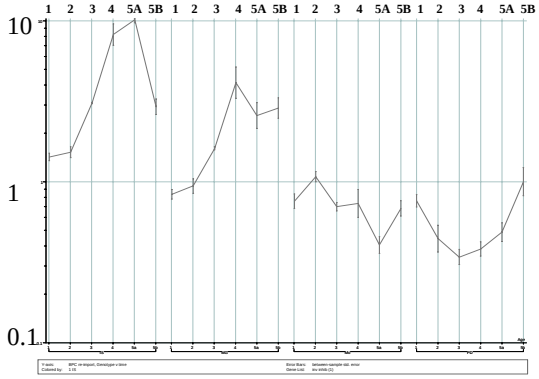
Sucrose-phosphate synthase C



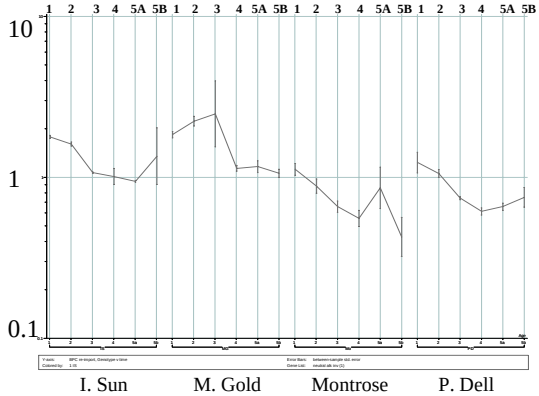
Acid invertase



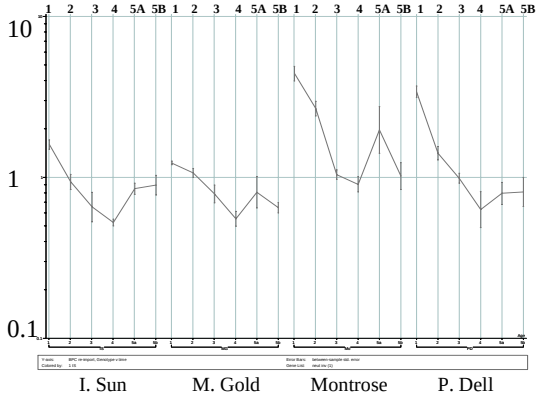
Cell wall invertase



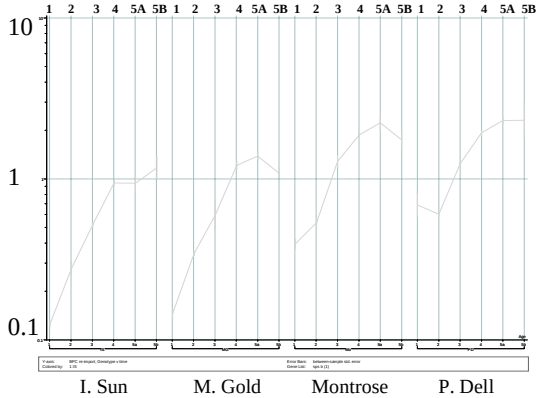
Invertase inhibitor



Neutral/alkaline invertase



Neutral invertase



Sucrose-phosphate synthase B

FIGURE 8. EXPRESSION PROFILES OF SELECTED SIGNIFICANTLY CHANGING GENES WITH RELEVANCE TO SUGAR METABOLISM.

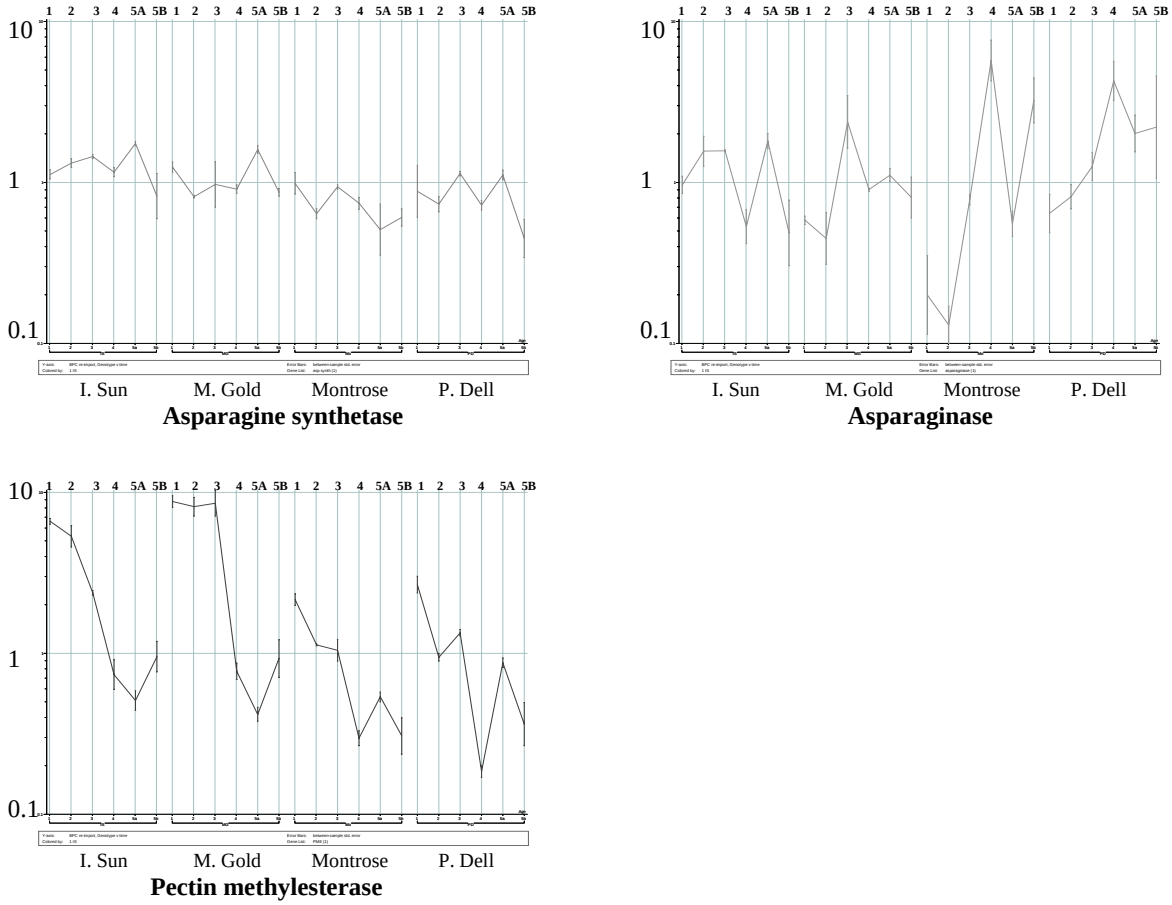


FIGURE 9. EXPRESSION PROFILES OF SELECTED SIGNIFICANTLY CHANGING GENES WITH RELEVANCE TO AMINO ACID METABOLISM AND PECTIN ESTERIFICATION.
Horizontal axis indicates time-point of harvest for each potato cultivar and the vertical axis (log scale) shows fold change compared to median values across all samples. Error bars represent standard error.

Discussion

Previous reports have demonstrated that *S. tuberosum* group Phureja are differentiated from *S. tuberosum* group Tuberosum on the basis of a number of important tuber quality traits such as flavour, texture, colour and reduced tuber dormancy (Morris *et al*., 2007, De Maine *et al*., 1993, De Maine *et al*., 1998, Dobson *et al*., 2004, Morris *et al*., 2004, Ghislain *et al*., 2006). It is therefore likely that the key genetic factors responsible for such differences are numerous. This study provides a unique and comprehensive examination of the differences in gene expression that exists between these groups. The POCI 44,000 feature microarray is the best platform currently available to analyse global gene expression in potato (described in Kloosterman *et al*., 2008 submitted). The POCI chip is based on 42,034 unigene sequences enabling a much more complete analysis of gene expression than has hitherto been achievable. Most potato microarray experiments performed to date have used the widely accessible spotted cDNA array produced by TIGR which contains around 12,000 cDNA clones (http://www.tigr.org/tdb/potato/microarray_desc.shtml). It has been estimated that 35,000 genes are expressed in tomato (Van der Hoeven *et al*., 2002) and a similar number are probably expressed in potato. Therefore the TIGR array probably only contains *ca*. 35% of the transcriptome, which is a serious limitation for global expression studies. Additionally, with spotted cDNA arrays it is inherently difficult to achieve a high degree of discrimination between similar sequences such as members of multigene families. Of the sequences on the POCI array, around 50% were not present on the TIGR array (Kloosterman *et al*., 2008 submitted).

On the basis of their differential expression patterns between *S. phureja* and *S. tuberosum* tubers genes involved in cell wall synthesis are candidates for those genes that may help to account for tuber texture differences observed in this study. A clear-cut example is the gene family encoding pectin methylesterase. Orthologous genes have been shown to impact on the texture of fruit from many species (reviewed in Fischer and Bennet 1991). However a role in potato tuber texture has not previously been considered. Indeed in the study of Pilling *et al* (2000) it was not possible to detect significant transcription of PME in the tuber. This is clearly different to the results presented here. As pectin is a major component of the cell wall and the middle lamella, its structure could be an important factor in texture in potato tubers as well as other plant tissues (Fischer and Bennet, 1991). However in our this study, the higher expression level of the specific PME gene identified in *S. phureja* tubers which may lead to a higher degree of Ca^{2+} cross-linking clearly does not correspond with the softer texture of cooked *S. phureja* vs. *S. tuberosum* tubers. However, we know from other studies at SCRI that other PME genes are more highly expressed in *S. tuberosum* compared with *S. phureja* (Taylor *et al*, unpublished data). Thus the expression of different genes in a gene family can vary as also evidenced by data on other genes identified in this Potato Council- funded project. This clearly indicates that selection of gene targets e.g. for marker-assisted breeding needs to take into account these issues.

In addition to a role for cell wall cohesion in defining texture, the literature also points to an important role for starch (Taylor *et al*., 2007 for review). It is clear from the rheology data presented in this report that the gelatinisation profiles of *S. phureja* starch are very different from *S. tuberosum*. However, it is also clear that in the early stages of tuber development *S. tuberosum* starch behaves like starch from mature *S. phureja*. We are still obtaining more detailed information on the chemical structure of the starches so the chemical reasons for these differences are not yet clear. However, there are clear differences between *S. phureja* and *S. tuberosum* with regard to the expression of genes known to be involved in starch biosynthesis. These become candidate genes for explaining the cooking properties of *S. phureja* starch which are likely to be caused by differences in granule sizes or amylose/amylopectin properties.

In our analyses to date we have a clear indication that cultivar Montrose stores extremely well at low temperature with regard to low sugar accumulation. Of the genes examined so far the expression of adenylate kinase stands out as being much lower in Montrose than the other three cultivars used in this study. Oliver *et al* (2008) have shown using transgenic approaches that that decreased expression of plastidial adenylate kinase in growing potato tubers leads to increased rates of respiratory oxygen consumption and increased carbon fluxes into starch. Thus, in Montrose the lower level of adenylate kinase in mature tubers may stimulate the recycling of starch breakdown products back into starch, maintaining low sugar levels. In Pentland Dell, which has high storage reducing sugars, its high invertase gene expression coupled with low invertase inhibitor expression, high expression of adenylate kinase and disproportionating enzyme may direct carbon flow more into reducing sugar accumulation.

Conclusions

This short term project has successfully generated substantial data sets on the expression of potato tuber genes during development and storage, using cultivars known to differ in specific quality traits. Thanks to a combination of RERAD and Potato Council funding we have been able to chemically and biochemically analyse the materials for metabolites, polymers and physical parameters which will facilitate wide correlation analysis with the gene expression data. This will require some time to complete as potential interactions will be significant in number. However, taking two of three examples of phenotypic differences together with data mining for specific gene expression profiles we can already present hypotheses for the genetic control of quality parameters. These will obviously require further testing. Further research and development needs include:

a. Completion of biochemical phenotyping analysis.
b. Correlation analysis: gene expression, tuber biochemistry, phenotype.
c. Fully quantitative gene expression analysis (selected genes/alleles) using real time PCR.
d. Functional testing of candidate genes e.g. using transgenics and mapping populations.
e. Genomic analysis to define optimum gene/allele combinations for breeding.
f. Integration with the potato genome sequencing initiative.

We still envisage the project leading to the development of gene specific markers for breeding programmes and variety development for the market place. Should GM approaches become more commercially acceptable the outcomes of this study will place us in a strong position to deliver novelty for the industry.

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