

## MICROARRAY TECHNOLOGY AS A NOVEL APPROACH TO GENE DISCOVERY IN FACIAL ECZEMA RESISTANCE OF SHEEP

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### INTRODUCTION

Facial eczema (FE) is a liver disease caused by the mycotoxin sporidesmin. The disease affects sheep, cattle and deer, and costs the New Zealand livestock industries NZ\$63-126M annually. Investigations into the genetic bases of FE resistance in sheep have included a genome QTL scan (Phua *et al.*, 1998a) and candidate genes approach (Phua *et al.*, 1998b). This report describes an alternative, but complementary, method using microarray technology to examine gene expression profiles in livers of FE resistant- and susceptible-line sheep in response to sporidesmin challenge.

### MATERIALS AND METHODS

**Microarray and probes.** A joint bovine EST project by Genesis company and AgResearch generated about 5500 liver cDNA clones, of which 1500 were selected, PCR amplified and arrayed onto polylysine-coated slides. One susceptible- and two resistant-line sheep were challenged with sporidesmin (0.15 and 0.4 mg/kg liveweight, respectively) and their livers collected 24 hours post dosing. In probe preparation, total RNA was extracted from a liver using TriZol reagent (Life Technologies), and further purified using RNeasy columns (Qiagen). Fluorescent labelling (with either Cy3- or Cy5-dCTP) of the RNA sample was performed as in Hedge *et al.* (2000).

**Slide hybridisation.** Probes from a resistant and a susceptible animals were pooled and hybridised to the arrayed slides. Following hybridisation and washing, the slides were scanned with a ScanArray 5000 (Packard) scanner and analysed with QuantArray software (Packard).

**Microarray analysis.** The method of Callow *et al.* (2000) was used for statistical analysis. Data was log<sub>2</sub> transformed and normalised using the housekeeping gene,  $\beta$ -2 microglobulin. Each gene was then considered a univariate testing problem and student t-statistics were calculated; multiple testing was corrected for using adjusted p-values.

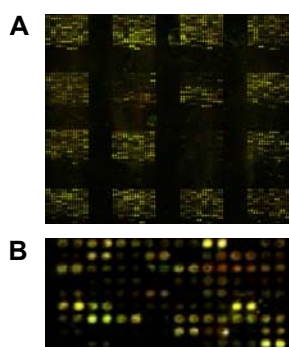
**Cluster analysis.** Expression data was clustered using two iterations of the J-Express clustering algorithm (<http://www.iu.uib.no/~bjarted/jexpress/>). The first used principal components analysis (PCA) to determine the major axes of variation within the data. The ESTs within each axis were then subjected to hierarchical clustering. Genes in each cluster were examined for trends in gene function and regulation.

**Quantitative RT-PCR.** The semi-quantitative method of Hoen *et al.* (2000) was used. It involved PCR amplification of the cDNA of interest multiplexed with 18S rRNA with [<sup>33</sup>P] end-labelled primers. The template cDNAs were generated from reverse transcription of total RNA with random hexamer primers. The linear phase of PCR amplification for both 18S rRNA and fibrinogen- $\gamma$  were first determined, and different samples were then tested at a cycle which fell within the determined linear amplification range.

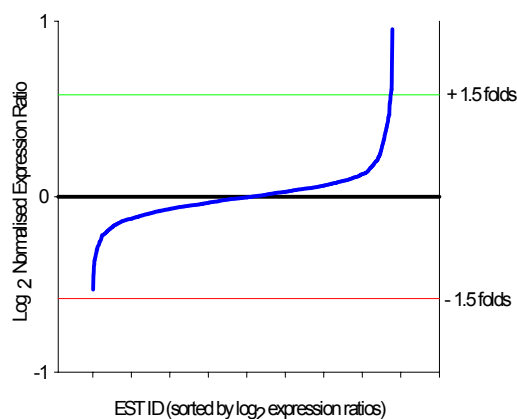
## RESULTS AND DISCUSSION

**Microarray result.** Four replicate experiments, against the same susceptible sheep reference, were performed: three were with one resistant sheep and the fourth was with a different resistant animal. A scanned slide with pseudo-coloured images is shown in Fig. 1A. Fig. 1B is an enlarged section showing differential expression of some genes: the red spots indicate over-expression in resistant animal, the green spots were over-expressed genes in susceptible animal, and the yellow dots indicate equal expression in both animal types.

The distribution plot of  $\log_2$  expression ratios against ordered EST ID is shown in Fig. 2: this gives an overall view of trends in the expression profiles. There are a number of genes in the resistant animals which have more than 1.5-fold higher expression than the susceptible animal.



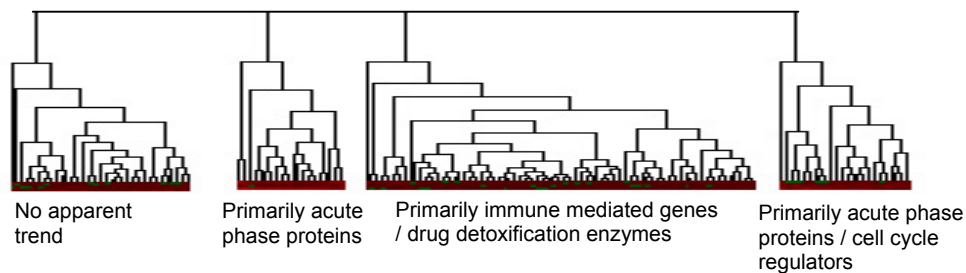
**Figure 1. A: A microarray image generated by QuantArray. B: An image enlargement showing differentially expressed genes**



**Figure 2. Distribution plot of global expression patterns across all four microarray experiments. Normalisation is to  $\beta$ -2 microglobulin**

The microarray data indicated differential expression of 133 genes between resistant and susceptible sheep livers. Twenty-four of these were differentially expressed at the 99% confidence interval, thirty-one at the 97.5% and seventy-eight at the 95% confidence level. Of the 133 differentially expressed genes, 24% were of unknown function: that is the ESTs have no hit to either known protein or nucleotide sequences. This highlights an advantage of using microarray technology as it allows rapid screening of genes with unknown function for involvement in the disease trait.

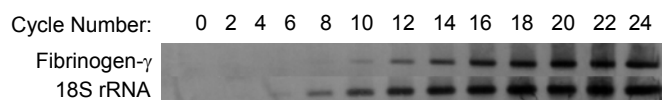
**Cluster analysis.** Cluster analysis was used in an attempt to place the observed differential expression patterns into a biological context; the underlying assumption of this analysis was that genes with similar expression levels share commonality in regulation. The J-Express clustering algorithms using principal components analysis indicated four major axes of variation in the microarray data. The four axes were further analysed using the hierarchical clustering algorithm, which gave ten sub-clusters of genes (Fig. 3). Examination of the clustered data revealed weak trends as shown in Fig. 3. The general theme across all clusters indicates the differential expression of several immune mediated and cell cycle regulated genes. This is the first time that these genes have been implicated in sheep resistance to FE disease.



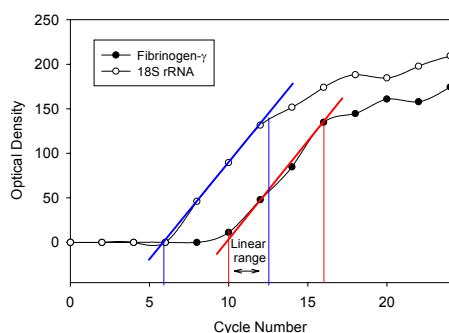
**Figure 3. Summary of clustering result. The four main clusters were defined by PCA analysis, the smaller clusters by hierarchical clustering**

Several functionally important classes of genes can be identified. One of these classes was of antioxidant enzymes, including catalase. Interestingly, the catalase gene has been previously found to be associated with FE resistance in sheep (Phua *et al.*, 1999).

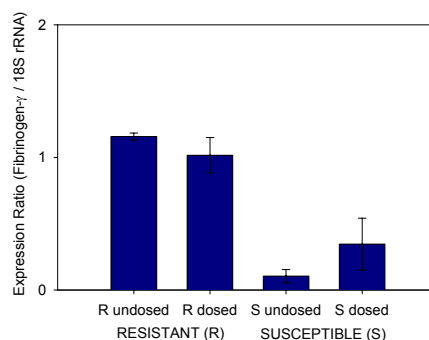
**Quantitative RT-PCR.** Confirmation of the microarray results is essential prior to candidate gene selection. Fig. 4 shows the multiplexed PCR results used to determine the linear amplification phases of fibrinogen- $\gamma$  and 18S rRNA genes, and the densitometry graph is plotted in Fig. 5. The exponential phases of the two genes overlap at cycle 12; thus 12 cycles of PCR was used for the measurement of fibrinogen- $\gamma$  in all further liver samples. Fig. 6 shows that fibrinogen- $\gamma$  is significantly over expressed in FE resistant animals irrespective of exposure to sporidesmin.



**Figure 4. Autoradiograph of multiplexed PCR products**



**Figure 5. Densitometry data of autoradiograph showing amplification curves of fibrinogen- $\gamma$  and 18S rRNA**



**Figure 6. Expression result for fibrinogen- $\gamma$  in livers of sporidesmin dosed and undosed FE resistant and susceptible sheep**

## CONCLUSION

Using microarray technology, at least twenty-four genes were detected to be differentially expressed between livers of FE resistant and susceptible sheep in response to sporidesmin challenge. Fibrinogen- $\gamma$  gene was independently validated, using a semi-quantitative RT-PCR technique, to be over-expressed in both dosed and undosed FE-resistant sheep.

## REFERENCES

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