A putative resistant DNA marker for wool yellowing susceptibility in sheep

M.V. Benavides, S. Damak and A.P. Maher

Abstract

An Australian Merino flock was screened for low (resistant) and high (susceptible) yellow predictive colour (YPC) breeding values in order to compare extreme individuals using the differential display of mRNA technique. One differentially expressed cDNA band was visualised only in the resistant group. This band showed no identity with the DNA sequences of public databases; however, they showed short homologies with three database sequences related to transmembrane signalling functions. The use of these candidate genes as DNA markers needs to be confirmed against sheep with a wide range of susceptibility to wool yellowing to verify the results.

INTRODUCTION

Clean wool colour is an important economic trait in the sheep industry. Yellow colour wools are penalised because it affects industrial versatility as far as dyeing is concerned. Clean wool colour expression is known to be genetically and, more importantly, environmentally affected, thus results from direct selection would cause variable responses. Direct selection against clean wool colour would be particularly difficult at low-challenging environmental conditions where the identification of resistant sheep could be biased with false-positive phenotypes, causing susceptible sheep to be selected along with resistant sheep, making selection against colour ineffective.

To overcome environmental influences on the expression of clean wool colour, the potential of molecular markers was investigated in order to allow genotypic identification of resistant and susceptible sheep to wool yellowing.

The aim of this experiment was to investigate the existence of a DNA marker for susceptibility to wool yellowing by screening groups of resistant and susceptible sheep by means of the differential display technique.

MATERIAL AND METHODS

Animals

Australian Merino sheep data were collected from a private property (Omarama Station, Omarama, Central Otago, New Zealand). Records on 668 offspring from 22 sires were collected over a 4-year period. In consecutive years the total numbers of sires and offspring were 6 and 137, 6 and 153, 5 and 186, and 9 and 192, respectively, with an average of 25 progeny per sire.

Midside skin and wool samples (accepted under ethical approval) were taken at shearing time. Yellow predictive colour (YPC) score was determined according to Wilkinson (1981). Greasy fleece weight (GFW), clean fleece weight (CFW), scouring yield percentage (Yield), and mean fibre diameter (MFD) were also recorded at shearing.

Statistical analysis

Data were analysed using the general linear models (GLM) of SAS package V6 (SAS, 1990) to determine which were the significant fixed effects. Year of birth and sex were used as fixed effects and sire was included as a random effect.

The model fitted to each variable was:

$$Y_{ijkl} = \mu + yob_i + x_j + s_k + e_{ijkl}$$

where $$Y_{ijkl}$$ = record of the $$l$$th individual; $$\mu$$ = population mean; $$yob_i$$ = year of birth; $$x_j$$ = sex; $$s_k$$ = sire, and $$e_{ijkl}$$ = error of the $$l$$th individual.

Heritability of YPC was estimated from variance components calculated using restricted maximum likelihood (REML) procedures with the average information restricted maximum likelihood (AIREML) statistical package (Johnson and Thompson, 1995) being used for the analyses.

Selection of individuals

The strategy used to select the animals for the differential display (DD) experiment was the same as that used by Lander and Botstein (1989), which was based on selecting individuals from the two YPC score extremes within the population. Ten unrelated sheep were selected based on YPC score’s breeding values (BV) out of the 668 Merino sheep. The resistant (lowest YPC score’s BVs) and susceptible (highest YPC score’s BVs) groups contained each 5 sheep.

The YPC score’s breeding values corrected for the significant ($$P < 0.01$$) fixed effects of sex and year of birth were calculated using the following formulae:
\[ BV_{ij} = (YPC_{ij} - YPC_{c}) * h^2 \]

where \( BV_{ij} \) is the breeding value of the sheep \( i \) in the year \( j \); \( YPC_{ij} \) is YPC score of the sheep \( i \) in the year \( j \); \( YPC_{c} \) is YPC correction for sex; and \( h \) is heritability of YPC for the current flock (calculated as \( h^2 = 0.25 \pm 0.13 \)).

\[ YPC_{c} = \text{LSM}_{ij} - \left( \frac{\sum YPC_{ij}}{n} \right) \]

where \( \text{LSM}_{ij} \) is least square mean of YPC for the sex \( i \) in the year \( j \); \( n \) is total number of animals in the flock over years and sexes.

Averages of both groups, low YPC score’s BV (resistant) and high YPC score’s BV (susceptible) are presented in Table I.

The animals had midside wool samples taken at hogget age and then again at adult age, to double check their YPC scores. Selection of the animals for DD analyses was based on adult YPC scores. Skin samples were also taken from the midside patch during adult wool sampling.

**TECHNIQUES**

**RNA extraction**

Whole skin samples were homogenised with a Polytron homogeniser (Kinematica GmbH, Luzern, Switzerland) at speed 7 and RNA was extracted with TRIzol (Life Technologies, Auckland) according to the manufacturer’s instructions. RNA pellets were dissolved in 20 \( \mu l \) DEPC-treated water and the RNA concentration was measured at 260 nm (\( A_{260} \) nm) in a spectrophotometer. RNA samples were run in 0.8% agarose gel to check whether RNA was degraded or intact.

**Technique**

RNA samples were DNA decontaminated by digestion at 37\(^\circ\)C for 30 min with 10 \( \mu U \) DNase I RNase-free (Boehringer Mannheim), 1X DNase I buffer and 20 \( \mu U \) RNase inhibitor (Boehringer Mannheim), followed by phenol/chloroform extraction, and ethanol precipitation. RNA concentrations were measured at \( A_{260} \) nm and RNA integrity was checked again on 0.8% agarose gel.

**Technique**

DNA-free RNA (0.2 \( \mu g \)) was transcribed to complementary DNA (cDNA) in a total reaction volume of 20 \( \mu l \), with 20 \( \mu l \) 4dNTP (dATP, dGTP, dTTP, dCTP), 1X reverse transcriptase buffer, 10 mM DTT, and 1 \( \mu M \) 14-mer oligo (dT) primer (OP) (Table II). The reaction was incubated at 65\(^\circ\)C for 5 min to allow RNA denaturing, followed by a second incubation at 37\(^\circ\)C for 10 min to allow primer annealing. Ten units of reverse transcriptase (RT) from Moloney murine leukemia virus (MoMuLV; Life Technologies, Auckland) were added and the reaction was incubated at 37\(^\circ\)C for 50 min, for RNA reverse transcription into cDNA, followed by a final incubation at 95\(^\circ\)C for 5 min to inactivate the enzyme. The negative controls were the reaction without reverse transcriptase and the reaction without RNA.

**Technique**

PCR amplification of cDNA was conducted using 2 \( \mu l \) of the RT product in a 20-\( \mu l \) reaction mixture containing 2 \( \mu l \) 4dNTP (dATP, dGTP, dTTP, dCTP), 1X PCR buffer, 0.2 \( \mu M \) 10-mer arbitrary primer (AP) (Table II), 1 \( \mu M \) 14-mer oligo (dT) primer (OP) (Table II), 0.1 \( \mu Ci \) \( \alpha \)^32PdCTP, and 1 U Taq DNA polymerase (Boehringer Mannheim). Forty microliters of mineral oil was placed on the top of the reaction to avoid evaporation.

Four different oligo (dT) primers (Table II) were used in the current experiment, generating four different species of mRNA in the RT step. PCR amplification was performed using either an arbitrary primer and the oligo (dT) primer used for RT (OP-AP) or two different arbitrary primers (AP-AP) (Table II). The use of AP and OP primers in cDNA amplification has been described in Liang and Pardee (1992).

The PCR conditions were: 40 cycles of denaturation at 94\(^\circ\)C for 30 s, annealing of primers at 40\(^\circ\)C for 2 min, extension at 72\(^\circ\)C for 30 s followed by one final extension cycle at 72\(^\circ\)C for 5 min (Liang and Pardee, 1994).

**Technique**

PCR products (3.2 \( \mu l \) + 2 \( \mu l \) formamide loading dye) were run in a 6% polyacrylamide denaturing sequencing gel containing 7 M urea in 1X TBE buffer at 2000 V for 2.5 h. The gel was transferred to Whatmann No. 3 filter paper, vacuum dried at 80\(^\circ\)C for 1 h and exposed to X-ray film (Kodak) for 2 days. The filter paper was stapled to

**Table I** - Hogget yellow predictive colour (YPC) scores and breeding values (BV) of individuals used in the experiment.

| Group    | Mean YPC | YPC range | Mean BV | BV range |
|----------|----------|-----------|---------|----------|
| Resistant| 2.6      | 1-3       | 0.560   | -0.604 to 0.548 |
| Susceptible| 6.4    | 6-8       | 0.660   | +0.646 to +0.701 |

**Table II** - Oligo (dT) primer (OP) sequence used in the reverse transcriptase (RT) step and oligo (dT) and arbitrary primer sequences used in the PCR step.

| Steps | Oligo (dT) primers | Sequences | Arbitrary primers | Sequences |
|-------|-------------------|-----------|------------------|-----------|
| RT    | OP 2 5’T„AG 3’   | GT01 5’AGITTCGTTGCT3’ |
|       | OP 3 5’T„AC 3’   | GT03 5’CTCGGATACAC3’ |
|       | OP 4 5’T„AT 3’   | GT06 5’ATGTGGTTGTT3’ |
|       | OP 5 5’T„GA 3’   | GT09 5’TCTGCCGTTGA3’ |
| PCR   | OP 2 5’T„AG 3’   | 267/2 5’ACATAAGAGG3’ |
|       | OP 3 5’T„AC 3’   | 267/4 5’GGAATGGTGA3’ |
|       | OP 5 5’T„GA 3’   | 371/1 5’GATAAAGCAC3’ |
the X-ray film and hole punched to allow cDNA recovery from the polyacrylamide gel after candidate genes had been identified in the autoradiograph.

The molecular weight marker used for polyacrylamide gels was the promoter region of the rabbit uteroglobin gene inserted in the EcoRI site of pBluescript KS(+) (DeMayo et al., 1991) digested with BanI (Boehringer Mannheim) and end labelled with α32P-dCTP using the Klenow fragment of DNA polymerase I (Amersham, Buckinghamshire, UK). The digestion generated DNA fragments of 1736 bp, 1231 bp, 1097 bp, 834 bp, 460 bp, 426 bp, 218 bp, 142 bp and 76 bp.

The differentially expressed cDNA bands, hereafter termed cDNA, were cut from the polyacrylamide gel with a scalpel blade. The cDNA was eluted from the gel in 100 µl TE buffer, pH 7.6, at 37°C for 12 h, followed by incubation at 100°C for 15 min. To the TE buffer were added 3 M sodium acetate to a final concentration of 0.3 M, 10 mg/ml glycogen to a final concentration of 0.5 µg, and 400 µl 100% ethanol to precipitate the DNA. The samples were incubated at -70°C for 30 min and then centrifuged at 14,000 rpm for 10 min, after which the DNA pellets were washed with 85% ethanol and dissolved in 20 µl of sterile water.

PCR amplification of the recovered cDNA band was done in a 40-µl reaction mixture using the same PCR conditions and final reaction concentrations as previously described, except for the absence of the radioisotope and the concentration of the dNTPs being 20 M. These bands were subcloned in the pCR II vector (TA Cloning kit, Invitrogen, San Diego, CA). Transformation was done using E. coli DH5α competent cells using blue and white selection. A modified alkaline lysis/PEG precipitation protocol (PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit Protocol, Perkin Elmer Corporation) was used. DNA samples (200 ng/µl each) were sequenced in the automated sequencing facility of Auckland University using M13 and reverse M13 primers.

The forward and reverse directions of the cloned sequences were checked for homology using the DNAMAN for Windows programme Version 2.5 (Lynnon BioSoft, Quebec). The cloned cDNA sequences were then compared with DNA sequences from international databases in order to search for homology with known DNA sequences by using the BLASTN programme (Altschul et al., 1990).

Those DNA sequences identified by the BLASTN GenBank and TIGR searches as having short homologies with the cloned sequences were searched in the ENTREZ Nucleotide query browser provided by the National Center for Biotechnology Information [http://www3.ncbi.nlm.nih.gov/htbin-post/Entrez/] in order to identify their protein coding sequences (CDS).

The DNA sequences with CDS present within the region of homology between the cloned sequences and the DNA sequences were then searched in the PC/GENE program Release 6.8 under the PROSITE option (Bairoch, 1993) to find the likely function of the protein.

RESULTS

The Merino flock studied presented a wide range of YPC scores, from 1 to 10, and averaged 4.47 ± 1.38. Twenty-eight percent of the animals showed YPC scores between 6 and 10 (Table III). Sheep with YPC scores of 6 and above were considered to be highly susceptible to wool yellowing. The genetic and environmental variances of YPC scores observed for the Merino flock were 0.50 and 1.91 (not tabulated), respectively.

The two groups of sheep selected because of their extreme breeding values for YPC scores did not differ significantly (P ≥ 0.05) for GFW, CFW, Yield, or MFD. The only apparent significant difference (P < 0.001) was for YPC and visual wool colour assessment. The analysis of variance of YPC scores showed that the score averages significantly differed between years of birth (P < 0.001) and sexes (P < 0.01), with females presenting higher YPC scores than males.

The heritability of YPC, as estimated by the sire model, was found to be 0.25 ± 0.13, and was used to calculate YPC breeding values from which the resistant and the susceptible groups of sheep were selected to participate in the current experiment.

Four mRNA species generated by reverse transcriptase using 4 different OP primers (Table II) were used in 53 PCR rounds with different OP-AP primer and AP-AP primer combinations. An average of 52 cDNA fragments with sizes varying between 1700 bp and 30 bp were observed per gel. Fifty percent of the bands amplified were fragments shorter than 600 bp. Only 8.6% of the cases showed amplified bands with sizes greater than 1500 bp.

The majority of the differential display results (92.5%) showed a similar banding pattern between the lanes, i.e., between individuals, with only 7.5% of the differential displays showing a wide variability of band patterns between lanes. This pattern occurred both when OP primers and when AP primers were used as 3’-end primers at the PCR step, being 10.5% of the OP-AP combinations and 5.88% of the AP-AP combinations.

A differentially displayed band was amplified with the primer combinations: OP3-GT09, a 232-bp long cDNA band expressed in four of the five resistant individuals (hereafter termed cDNA#R; Figure 1).

| Score | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-------|---|---|---|---|---|---|---|---|---|---|
| YPC   | 1.8 | 9.1 | 19.2 | 21.0 | 20.8 | 18.6 | 7.2 | 1.3 | 0.9 | 0.1 |
| Visual score | 0.6 | 6.6 | 30.5 | 34.7 | 13.7 | 9.8 | 3.6 | 0.5 | 0 | 0 |
mology with some database sequences. Three database sequences from the GenBank database were found to have short homology with the cDNA#R sequence. Two database sequences belonged to the same family: metalloprotease-like, disintegrin-like proteins IVb and IVc. The PROSITE analysis of these database sequences homologous with cDNA#R did not detect any protein sites or signatures within the homologous region. No matches were found for cDNA#R in the TIGR database.

DISCUSSION

The DD technique was used in this experiment since it allows the identification of non-polymorphic genes through side-by-side comparisons between groups of animals having the same phenotypic characteristics, as well as for the identification of polymorphic genes.

Despite most of the reported differential display experiments having targeted two populations of cultured cells (Liang and Pardee, 1992; Liang et al., 1992, 1993; Sager et al., 1993; Zimmermann and Schultz, 1994; Donohue et al., 1995; Wang and Feuerstein, 1995), tissue comparisons involving multicellular structures have been published with positive results (Utans et al., 1994).

The strategy used to maximise the chances of observing group-related differences was to compare groups of animals with extremely low- and high-YPC score breeding values (resistant vs. susceptible). This approach has been described by Tanksley (1993) as the “distributional extremes method”, which assumes that individuals in the extreme tails of the Gaussian distribution (i.e., the lowest and the highest breeding values for a particular trait) are more likely to express differences in genotypes.

The use of sheep with extremes of YPC score breeding values also made it possible to reduce the number of animals to be screened in the current study. In this particular case the use of sheep with extreme YPC scores also avoided the inclusion of animals with intermediate YPC values that could have resulted in difficulties in the interpretation of the results. The low number of sheep used in the current experiment was a consequence of the high cost of screening large populations for genetic markers.

The cDNA#R cloned sequence found in this study showed to have short homology, but no identity, with 3 database sequences from the GenBank. The lack of identity between the database sequences and the cDNAs cloned here means that these cDNAs were unknown sequences. Nonetheless, the search in the GenBank database was important because it gave some indication of the possible biological function of the cloned cDNAs.

Interestingly, a group of proteins with similar physiological functions was observed amongst the sequences with short homologies found in GenBank searches. The three database DNA sequences found for cDNA#R: mast cell function associated antigen (MAFA) (Guthmann et al., 1995), metalloprotease-like disintegrin-like IVb (Perry et
Identification of resistant genetic markers for sheep wool

al., 1995) and metalloprotease-like disintegrin-like IVc (Perry et al., 1995), have been reported to act in transmembrane signalling processes.

A possible link between proteins related to transmembrane functions and wool yellowing would be through the high potassium content in the sudoriparous glands secretion (suint) which has a strong positive phenotypic and genetic correlations with wool yellowing (Aitken et al., 1994; Benavides, 1997). Suint covers the wool follicle and if it has a high pH it may lead to an increase of wool yellowing (Acharya and Singh, 1976).

A possible mechanism where K can be at higher levels in the sudoriparous gland cells is through the sodium-pump system, which actively transports Na⁺ and K⁺ ions across the cellular membrane through the action of the Na⁺-K⁺-ATPase enzyme (Sato and Dobson, 1970). Despite Na⁺-K⁺-ATP being the main K⁺ transport mechanism, it is not the only known system since H⁺-K⁺-ATPase also controls the exchange of K⁺ from the plasma to the cells (Sachs et al., 1992). However, if there was a difference in Na⁺-K⁺-ATPase between resistant and susceptible sheep it would most likely have been due to a difference in the activity of this essential enzyme. In this case, if Na⁺-K⁺-ATP was the protein responsible for the higher K⁺ content in the sweat glands in susceptible sheep a cDNA band would be seen simultaneously in both susceptibility groups, since it is a vital enzyme, but with different band intensity which could indicate a difference in gene expression between the two groups. At the time of this study it was not possible to draw any specific relationship between transmembranal functions or activity and wool yellowing susceptibility and the database sequences found to have homology with cDNA#R in this experiment.

One way of better understanding the function of the cloned sequences would be to clone their corresponding full length cDNA. Amplification of partial sequences is a characteristic of the differential display technique because it uses oligo d(T) primers and amplification will not occur unless the arbitrary primer is situated within 2 to 3 kb of the oligo d(T) primer (Liang and Pardee, 1992). The fragments amplified in this experiment were relatively short, 1700 bp being the biggest fragment size, but 50% of the bands were ≤ 600 bp. The cloning of full length sequences would be necessary to confirm their identity with any known sequence.

Further confirmation of the differentially displayed cDNA#R is essential to allow confidence in the use of this marker in order to identify sheep according to their degree of susceptibility. However, cDNA#R would not be expected to identify 100% of resistant or susceptible sheep since this candidate gene was present in only four out of the five resistant sheep studied. This means that in practice not all resistant sheep will have been identified by the use of this probe. Wool yellowing is a continuous trait, therefore it might be affected by many genes; so, the failure of a single candidate gene for susceptibility to wool yellowing to explain all the genetic variance for this trait was an expected result.

Figure 2 - Forward and reverse sequences of cDNA#R.

Table IV - Regions where possible open reading frames (ORF) in cDNA#R occur (forward: 1 to 232 bp). The table also shows the strand (plus or minus), number of amino acids present in each ORF and their respective position in the strand of the cDNA#R sequences.

| Strand | Frame | Number of amino acids | Position | Start codon | Stop codon |
|--------|-------|-----------------------|----------|-------------|------------|
| Minus  | 2     | 19                    | 116-175  | ✓           | ✓          |
|        | 3     | 15                    | 186-232  | ✓           |            |
|        | 1     | 11                    | 1-36     | ✓           |            |
|        | 2     | 8                     | 191-217  | ✓           | ✓          |
|        | 3     | 7                     | 3-26     | ✓           |            |
CONCLUSIONS

One differently expressed band was found in the group resistant to wool yellowing with no identity with DNA sequences available at GenBank and TIGR databases. The differentially displayed sequence showed short homologies that might point at proteins with transmembrane signalling functions in the wool yellowing mechanism.

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RESUMO

Ovinos resistentes e suscetíveis para predição da cor amarela na lã foram amostrados de um rebanho da raça Merino Australiano com o objetivo de comparar diferenças em cDNAs através da técnica de exposição diferencial de mRNA (differential display of mRNA). Uma banda de cDNA foi expressa somente no grupo dos animais resistentes. Não houve identidade desta banda com sequências do banco de dados do GenBank ou TIGR, no entanto a banda chamada de cDNA#R mostrou homologias curtas com três sequências que codificam proteínas envolvidas em funções de membrana. O uso deste gene candidato como marcador genético de animais resistentes ao amarelamento necessita ser avaliado em rebanhos mais numerosos para confirmar os resultados.

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