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# Use of the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique to analyse the *Anthocyanidin Synthase* (*ANS*) gene *Locus* in Zimbabwean sorghum landraces with different seed proanthocyanidin profiles

**Ngonidzashe Mangoma* and Zephaniah Dhlamini**

Department of Applied Biology and Biochemistry, National University of Science and Technology, Corner Gwanda Road/ Cecil Avenue, PO Box AC 939, Ascot, Bulawayo, Zimbabwe.

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**Studies on the effects of mutations within flavonoid pathway genes on the resultant flavonoid profiles in sorghum are important in the identification and characterisation of varieties with nutritionally superior flavonoid profiles. In this study, we aimed at determining the effect of mutations at one important flavonoid pathway *locus*, the *anthocyanidin synthase* (*ANS*) gene, on grain flavonoid profile in sorghum. Sequence polymorphisms at this *locus* were determined in sorghum varieties with different seed proanthocyanidin profiles. The proanthocyanidin profiles of 61 local landraces were determined by the DMACA stain and butanol-HCl assay. The *Anthocyanidin synthase* (*ANS*) gene was then amplified using PCR from a subset of 11 landraces, and the amplicons subjected to sequence polymorphism analysis using the restriction fragment length polymorphism (RFLP) technique. Results show that 89% of the brown landraces, 4% of the red and none of the white landraces had detectable proanthocyanidins in their grain. Grain proanthocyanidins ranged from 0.1 to 1.8 AU at 550 nm per gram of sample. Using the PCR-RFLP technique, no sequence variations were detected at the *ANS locus.* Consequently, the different proanthocyanidin profiles observed could not be attributed, according to the methods used, to events at the *ANS* gene *locus*. These could be due to mutations at other *loci* or a combination of genetic and environmental factors.**

**Key words:** Sorghum, flavonoid, flavonoid profile, mutation, restriction fragment length polymorphism (RFLP), condensed tannins, Zimbabwe.

## INTRODUCTION

*Sorghum bicolor* (cultivated sorghum) is a cereal crop of the hot, semi-arid tropics, believed to have originated from Africa (Taylor, 2006; Waniska, 2000). The crop can do well in a wide range of climatic conditions, including the arid and semi-arid tropics that characterise most of Africa. Despite this, sorghum has remained largely a subsistence crop in Africa, grown mainly by resource poor communal farmers for domestic consumption,

thus contributing to the generally low growth in sorghum production figures on the continent. Average yields have remained static, with an average less than 1.1 tonnes per hectare (Kumar et al., 2011; Taylor, 2006).

This apparent lack of growth in sorghum production in Africa is due to a number of reasons, including the use of traditional, and usually less efficient, methods of farming and lack of access to improved varieties with high yield potential. Studies have indicated that communal farmers would prefer varieties that show high pest and disease tolerance, drought tolerance, early maturity, palatability and storability (Nagaraj et al., 2013). Sorghum grain flavonoids play important roles in the uptake of the crop by communal farmers, due to their roles in conferring resistance to bird depredation and grain mold in developing sorghum grain, and in human and animal nutrition.

There are over 4000 known classes of structurally related flavonoids, and these include condensed tannins, anthocyanins, 3-deoxyanthocyanidins and flavanols (Patel, 2008). All sorghum varieties contain phenolic compounds in their grain, while only some contain condensed tannins (Dykes and Rooney, 2006). The types and quantities of flavonoids (flavonoid profile) that each variety accumulates in its grain determine how the variety would be utilised. Condensed tannins, and several other flavonoids, have important roles in human and animal nutrition and in the sorghum plant's physiology (Winkel-Shirley, 2001). However, some flavonoids, including condensed tannins, have both desirable nutritional as well as undesirable, anti-nutritional factors. Thus, a balance of flavonoids is required if sorghum is to fully reach its potential.

There are efforts to try and manipulate the flavonoid pathway in sorghum in order to identify and develop sorghum flavonoid pathway mutants that have desirable and nutritionally superior flavonoid profiles. Sorghum varieties for different applications would need to have flavonoid profiles specific to the application, e.g. flour milling and baking. Different applications would require the presence of different classes and quantities of flavonoids in sorghum grain. Such varieties can be obtained by screening a large number of mutants for those with desired flavonoid profiles.

It is important to understand how mutations within certain flavonoid pathway genes would affect the flavonoid profile in a given sorghum variety. The flavonoid pathway is a complicated pathway with over 10 known and unknown structural and regulatory genes (Winkel-Shirley, 2001). Studies on mutations at these different *loci* are yet to be carried out in sorghum, even though a lot of such work has been carried out in other plants (von Wettstein, 2007; Abrahams et al., 2002; Debeaujon et al., 2001). Due to the complex nature of the flavonoid pathway, it is difficult to accurately predict how a mutation in a given flavonoid pathway gene would impact on the flavonoid pathway in sorghum. However, it is worthwhile to carry out studies that relate any genetic polymerphisms at different flavonoid pathway *loci* to different flavonoid profiles, with a view to understanding how mutations in a given flavonoid pathway gene would affect flavonoid accumulation.

In this study, we aimed at studying how sequence variations at the anthocyanidin synthase (*ANS*) gene *locus* relate to observed differences in condensed tannin profiles in different sorghum varieties. This gene, also called leucoanthocyanidin dioxygenase (*LDOX*) catalyses the conversion of leucoanthocyanidins into anthocyanidins and sits at the branch-point between the PA-specific branch and the anthocyanin-specific branch of the flavonoid pathway (Abrahams et al., 2002). Our interest is to find out if any sequence polymorphisms at this *locus* may explain observed differences in grain proanthocyanidin profiles. A number of flavonoid *ANS* gene mutants in Arabidopsis, namely *tt 11*, *tt17* and *tt 18* (*tds-4*) have been identified and characterised in Arabidopsis and alfalfa, both at the biochemical and molecular level (Misyura et al., 2012; Bowerman et al., 2012; Abrahams et al., 2002; Buer and Djordjevic, 2009). All these mutants were characterised by altered flavonoid profiles and deposition patterns in the grain.

## MATERIALS AND METHODS

### Sample selection

A total of 61 sorghum landraces were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Research Station stationed at Bulawayo, Zimbabwe. Selection was based on availability and grain colour. Dwarf Wonder, a brown, inbred medium tannin line was the reference landrace in the study. Condensed tannins were assayed for in all 61 grain samples, while only 11 landraces, including Dwarf Wonder, were selected for *ANS* gene sequence studies.

### DMACA screening assay for sorghum grain proanthocyanidins

The aldehyde dye, dimethylaminocinnamaldehyde (DMACA) reacts with proanthocyanidins (PAs) and their immediate precursors, flavan-3-ols and flavan-3,4-diols, producing a blue coloured complex. It does not react with anthocyanidins and anthocyanins, making it suitable for use in tracing condensed tannin biosynthetic activity in fruits, seeds and tissues (Bogs *e*t al., 2005; Abrahams et

*Corresponding author. E-mail: ngonidzashe.mangoma@nust.ac.zw.

**Table 1.** Expected *ANS* gene PCR amplicon restriction profiles.

| Restriction enzyme | Frequency of cuts | Cut positions | Expected sizes of restriction products (bp) |
|---|---|---|---|
| *HinfI* | 1 | 1218 | 52, 1218 |
| *MboI* | 4 | 167, 740, 929, 1231 | 39, 167, 189, 302, 573 |
| *TaqI* | 6 | 312, 576, 774, 1059, 1125 | 48, 54, 69, 77, 138, 344, 540 |
| *AluI* | 5 | 77, 125, 179, 719, 788, 926 | 66, 145, 198, 264, 285, 312 |

al., 2002). In this study, DMACA was used to screen 61 sorghum landraces on the basis of PA accumulation in the grain samples available. The DMACA assay was performed as described by Abrahams et al. (2002), with minor modifications. About 20 healthy, dry seeds from each landrace were soaked in DMACA solution (2% [w/v] DMACA in 3M HCl/ 50% methanol (v/v)) in a microcentrifuge tube for seven days, followed by washing thrice with 70% ethanol. Washed seeds were dried and then analysed for the development of a blue/blue-black colour, indicating the presence of PAs and/or their precursors.

#### Quantification of proanthocyanidins in sorghum grain samples

Sorghum grain PAs were quantified in a grain total flavonoid extract. To obtain the extract, exactly 1.0 g of each sorghum grain sample was ground to a fine meal using a mortar and pestle, and then total flavonoids in the meal were extracted in 10 ml of 1% (v/v) HCl in methanol, with shaking. After extraction, the mixtures were centrifuged at 5000 rpm for 30 min and the clear supernatants refrigerated at 4°C until they were analysed. Total condensed tannins in each extract were then quantified by the butanol-HCl assay as described by Hagerman (2000). All samples were analysed in triplicate, and the results expressed as absorbance units at 550 nm per grain of sample. This assay is based on the acid catalysed hydrolysis of proanthocyanidins in a mineral acid to produce pink/red coloured anthocyanidins that absorb maximally at 550 nm. An iron reagent is normally included in the reagent to enhance colour formation and stability.

#### Sorghum genomic DNA extraction

Sorghum genomic DNA was extracted from the frozen leaves of 3-week old etiolated sorghum seedlings. The frozen tissue was ground to a fine powder using a chilled mortar and pestle. Genomic DNA was then extracted from the crushed material using the ZR Plant/Seed DNA Miniprep kit (Zymo-Research, Pretoria, South Africa), following the manufacturer's instructions. A 10 µl aliquot of the extracted DNA was run on a 0.8% agarose gel to determine DNA quality and quantity.

#### PCR amplification of the sorghum *ANS* gene in selected landraces

The *ANS* gene primers used in this study were designed for use in Arabidopsis complementation studies (Liu et al., 2010). The primer sequences are ANS 1 (5' - CGCGGCGATAGTGAATTAGT) and ANS 2 (5' - ATTGATGGATGGACGAAAGC). PCR amplification was performed in 50 µl reactions with the Phire Hot Start II DNA Polymerase (Thermo Scientific Inc., Pretoria, South Africa) using the following program: initial denaturation at 98°C (30 s); 30 cycles consisting of denaturation at 98°C (5 s), annealing at 55°C (5 s), and extension at 72°C (30 s); and then a final extension at 72°C (60 s), in a GENEAMP® PCR System 9700 thermocycler machine (Life Technologies). After cycling, a 10 µl aliquot of each reaction was electrophoresed on a 1% agarose gel for 1 h and PCR products were confirmed by viewing the gel under ultraviolet irradiation.

#### RFLP analysis of *ANS* gene amplicons

Restriction digestion analysis was performed directly on PCR amplicons as a preliminary screen for polymorphisms at the *ANS* gene *locus* in the selected landraces. The PCR amplicons were digested using the restriction enzymes *HinfI*, *MboI*, *TaqI* and *AluI* (Thermo Scientific Inc, Pretoria, South Africa), following the manufacturer's instructions. Enzyme selection was based on a virtual digest of the *ANS* gene sequence using several enzymes. Only those enzymes producing a sufficient number of well-spaced cuts were selected for use (Table 1).

## RESULTS

### Variation in seed PA content

The sorghum landraces used in this study showed significant variability in their seed proanthocyanidin profiles. Both qualitative and quantitative assays for proanthocyanidins were carried out, using the DMACA stain assay and the butanol-HCl assay, respectively.

### Qualitative assay for PAs in sorghum grain samples

The DMACA assay was used to characterise available sorghum landraces on the basis of the presence or absence of PAs in their grain. It is a qualitative assay that detects PAs or their immediate precursors. With the DMACA assay, 89% of the brown lines, 4% of the red lines and none of the white lines tested positive for PAs and/or their precursors (Figure 1). A few samples gave inconclusive results (Figure 2). Being a qualitative assay, the DMACA assay was used in combination with the butanol-HCl, a quantitative assay for PAs in order to get more insight about each sorghum landrace.

### Quantitative assay for PAs in sorghum grain samples

The butanol-HCl assay is based on the acid-catalysed depolymerisation of PAs into anthocyanidins. Due to the absence of a tannin standard, butanol-HCl assay results were expressed as absorbance units at 550 nm per gram of sample (AU@550 nm/g of sample). All 27 brown landraces had detectable grain PA levels, except for only

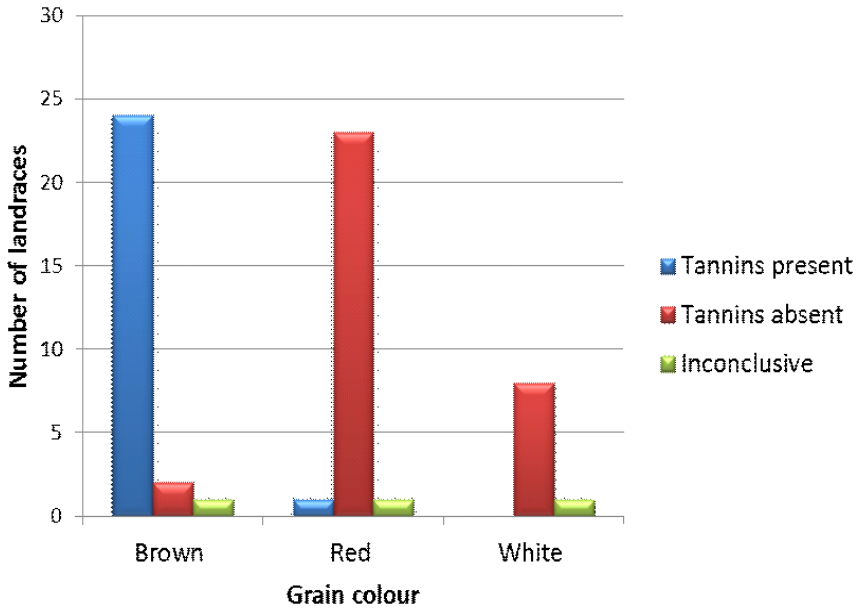


**Figure 1.** Number of tannin positive, negative and inconclusive sorghum landraces as determined by the DMACA assay.

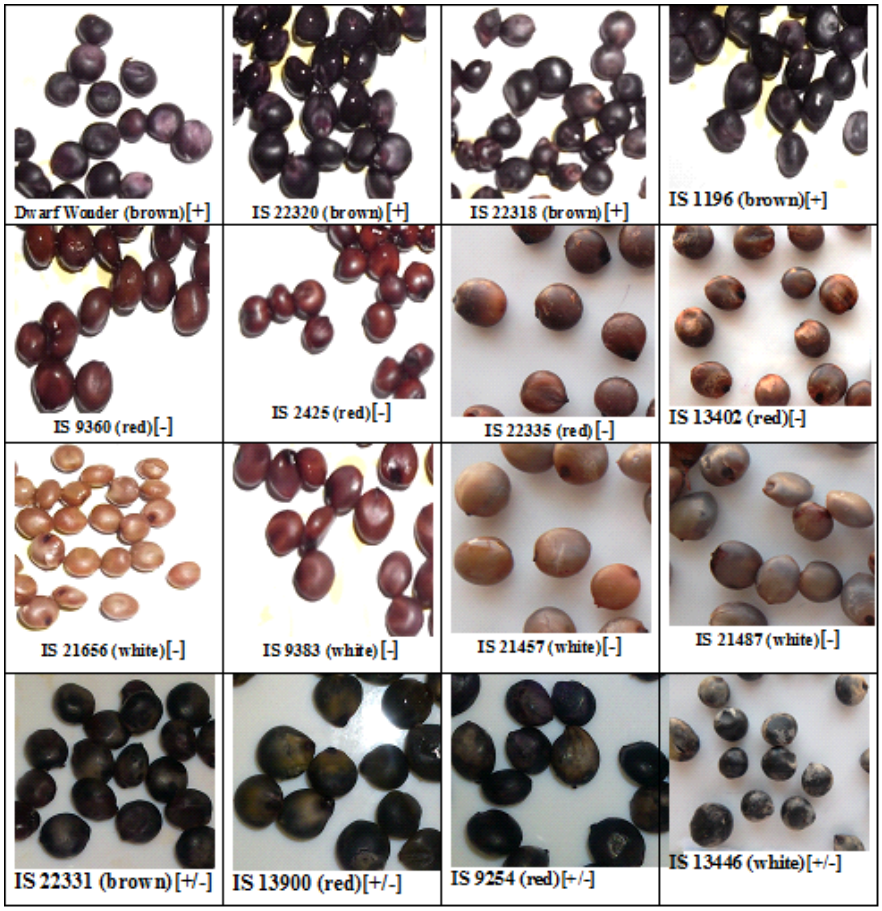


**Figure 2.** Sorghum seed stained with DMACA dye. [+] - DMACA positive; [-] - DMACA negative; [+/-] - inconclusive result.

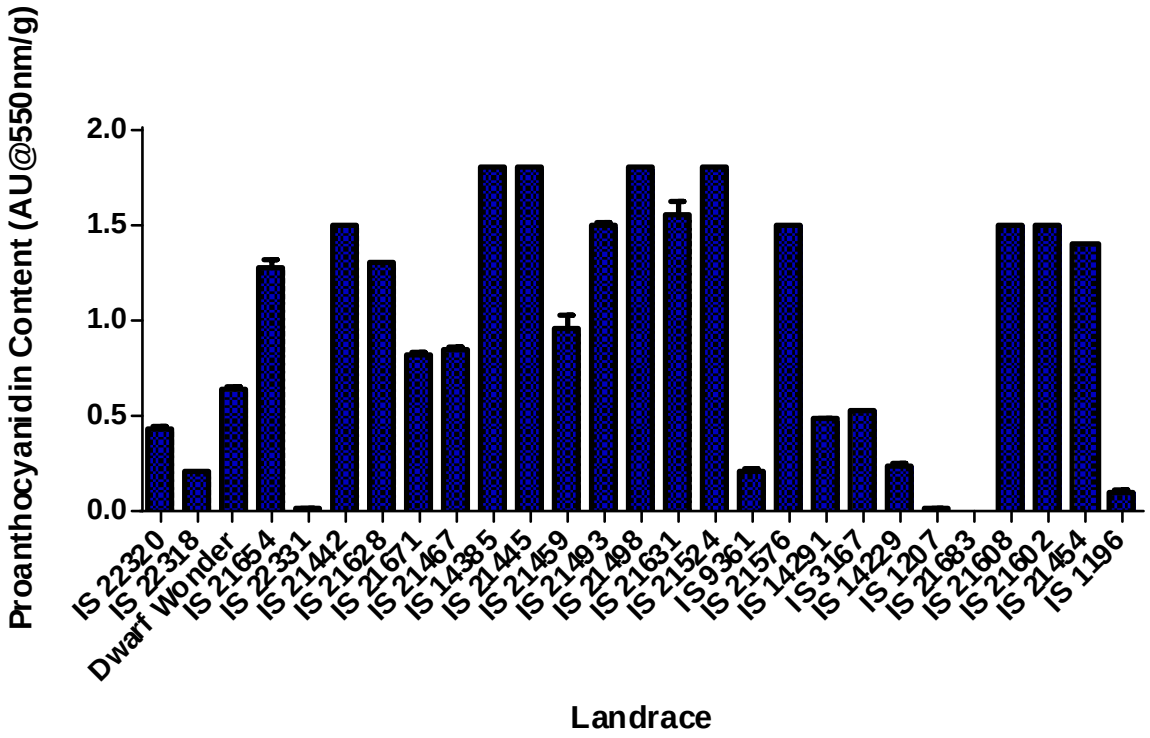


**Figure 3.** Levels of proanthocyanidins in brown sorghum grain samples.

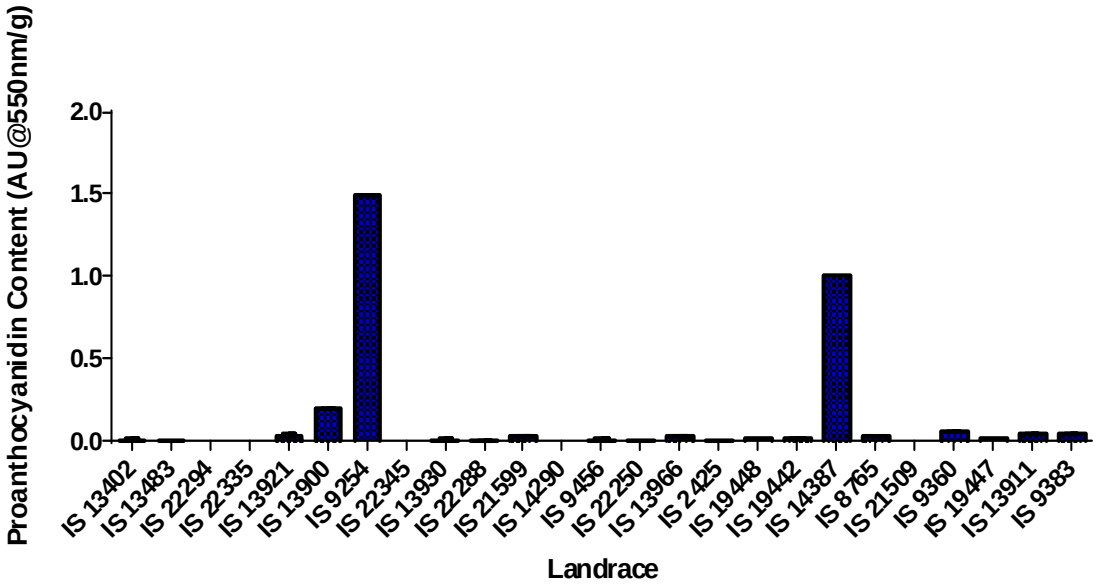


**Figure 4.** Levels of proanthocyanidins in red sorghum grain samples.

the three landraces IS 22331, IS 1207 and IS 21683 (Figure 3). The reference landrace, Dwarf Wonder, is a medium tannin line, thus in this study all landraces showing lower tannin levels were designated low tannin, while those accumulating higher quantities were designated high tannin lines. All but three red lines assayed for did not accumulate grain PAs. Of the three that did, IS 13900 accumulated very low levels while IS 14387 and IS 9254 accumulated high quantities (Figure 4). Meanwhile, all white lines tested PA negative by the butanol-

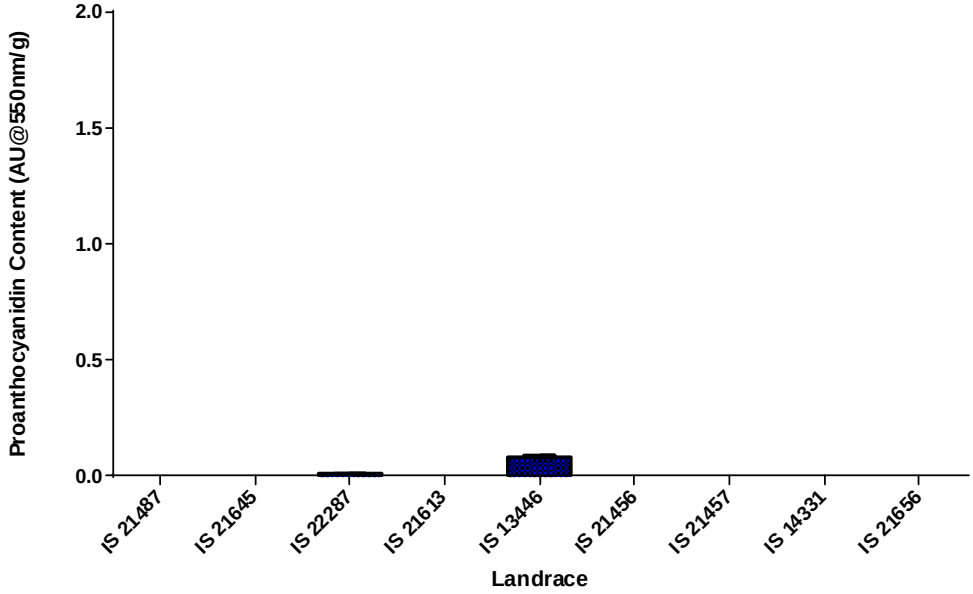


**Figure 5.** Levels of proanthocyanidins in white sorghum grain samples.

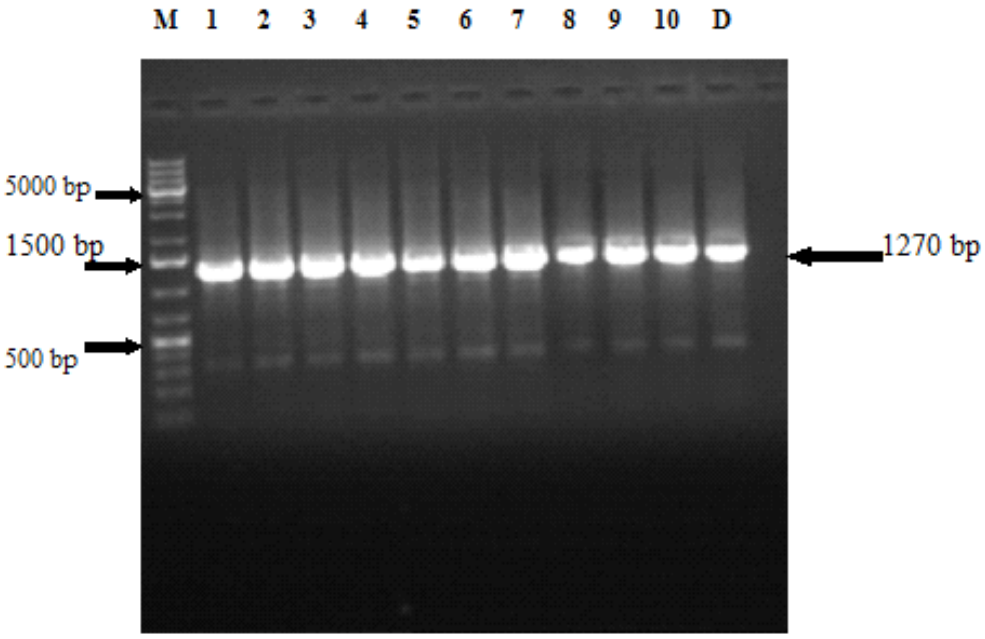


**Figure 6.** PCR amplification of the sorghum *ANS* gene. Lane marked M contains a 1 kb DNA ladder, lanes 1 - 10 contain the samples as follows: 1 - IS 22320; 2 - IS 13402; 3 - IS 21487; 4 - IS 22318; 5 - IS 13483; 6 - IS 22294; 7 - IS 1196; 8 - IS 22335; 9 - IS 21456; 10 - IS 21457; D - dwarf wonder.

HCl assay (Figure 5).

## PCR amplification of the sorghum *ANS* gene

Good quality *ANS* gene PCR amplification products were obtained in all samples (Figure 6). The *ANS* amplicon obtained was 1270 bp in size, as expected. This includes the whole of the gene's coding sequence (CDS) as well as 33 and 36 bp of the 5' and 3' UTR, respectively. The *ANS* gene has been cloned in sorghum and the sequence filed under accession number XM_002451291

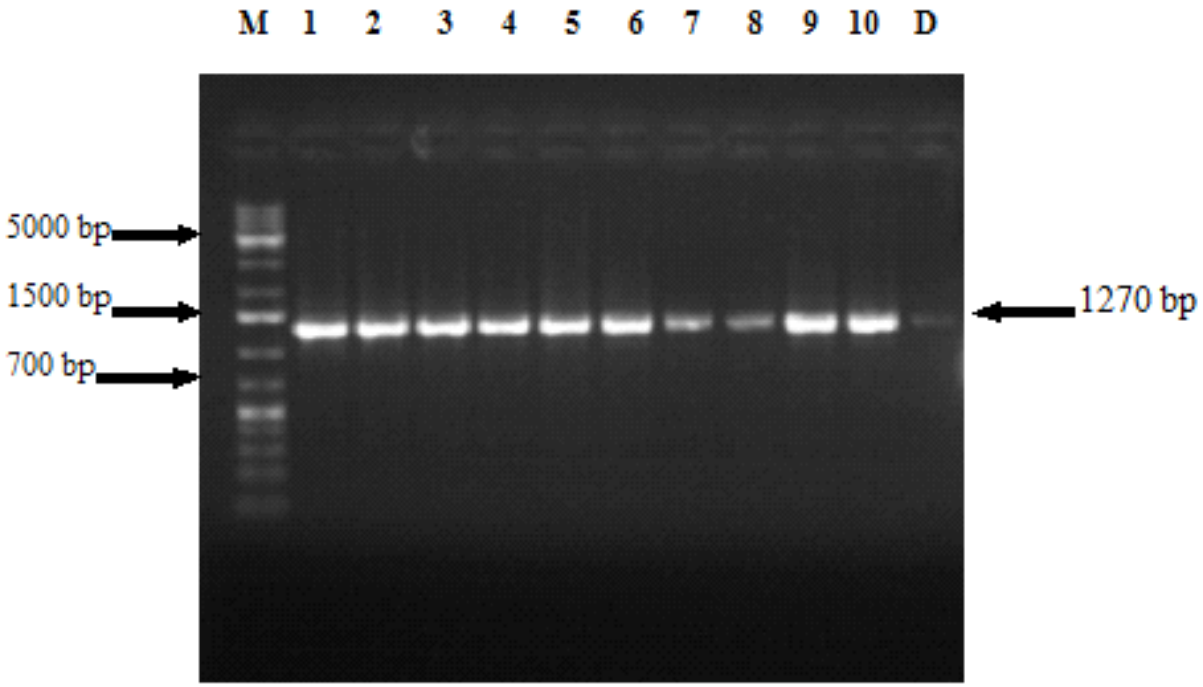


**Figure 7.** Sorghum *ANS* gene PCR product recovered from agarose gel. Lane marked M contains a 1 kb DNA ladder, and other lanes: 1 - IS 22320; 2 - IS 13402; 3 - IS 21487; 4 - IS 22318; 5- IS 13483; 6- IS 22294; 7- IS 1196; 8- IS 22335; 9- IS 21456; 10- IS 21457; D- dwarf wonder.

(www.ncbi.nih.nlm.gov). It is important to produce amplicons that include the whole of the CDS if any determinations involving how changes in a gene may affect its functionality are to be made. Also, high quality PCR amplicons were recovered and purified using the The Zymoclean™ Gel DNA Recovery kit (Zymo-Research, Pretoria, South Africa). Only 2.5 µl of the recovered and purified PCR amplicons were electrophoresed on 1% agarose gel, producing good images (Figure 7).

### *ANS* gene *locus* sequence polymorphism analysis

The aim of this project was to investigate the relationship between differences in seed PA profiles in local sorghum landraces and sequence variations at a key flavonoid pathway *locus*, the *anthocyanidin synthase* (*ANS*) gene. This gene is vital for the biosynthesis of condensed tannins in sorghum. The method used in this study is the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. This combines the DNA amplifying prowess of the PCR to a specific gene and the specific cleavage of the DNA amplicons by the action of a group of nucleases called restriction endo-nucleases.

Restriction digestion analysis of DNA is used to detect polymorphisms that cause the disappearance of existing, or emergence of new, restriction enzyme sites. In this study, the following restriction enzymes were used: *HinfI*, *MboI*, *TaqI* and *AluI*. The enzymes *MboI*, *TaqI* and *AluI* are all 4-base cutters, while *Hinf1* is a 5-base cutter. Each *ANS* gene fragment was cut sixteen times by all the enzymes combined. High resolution restriction data was generated for the enzymes used. However, with all these restriction enzymes, no differences in restriction patterns were detected, implying that the restriction maps for the amplicons under study using the available restriction enzymes were the same (Figures 8, 9 and 10).

## DISCUSSION

Selecting or breeding for flavonoids in sorghum is a fairly unexplored area. Unlike in other plants like *Medicago truncatula* (alfalfa), *Camellia sinensis* (the tea tree) and *Arabidopsis thaliana* where the flavonoid pathway is fully understood, a lot is still unknown in sorghum. This study is part of efforts aimed at the identification and characterisation of flavonoid pathway mutants in sorghum. In this study, 61 sorghum landraces which are part of the sorghum genebank, made up of Zimbabwean and other Southern African sorghum lines were used. The study showed that 44% of these landraces contained quantifiable condensed tannins in their grain. Another study which focused on the use of RAPD markers to determine the genetic relatedness of Zimbabwean sorghum varieties with different seed proanthocyanidin

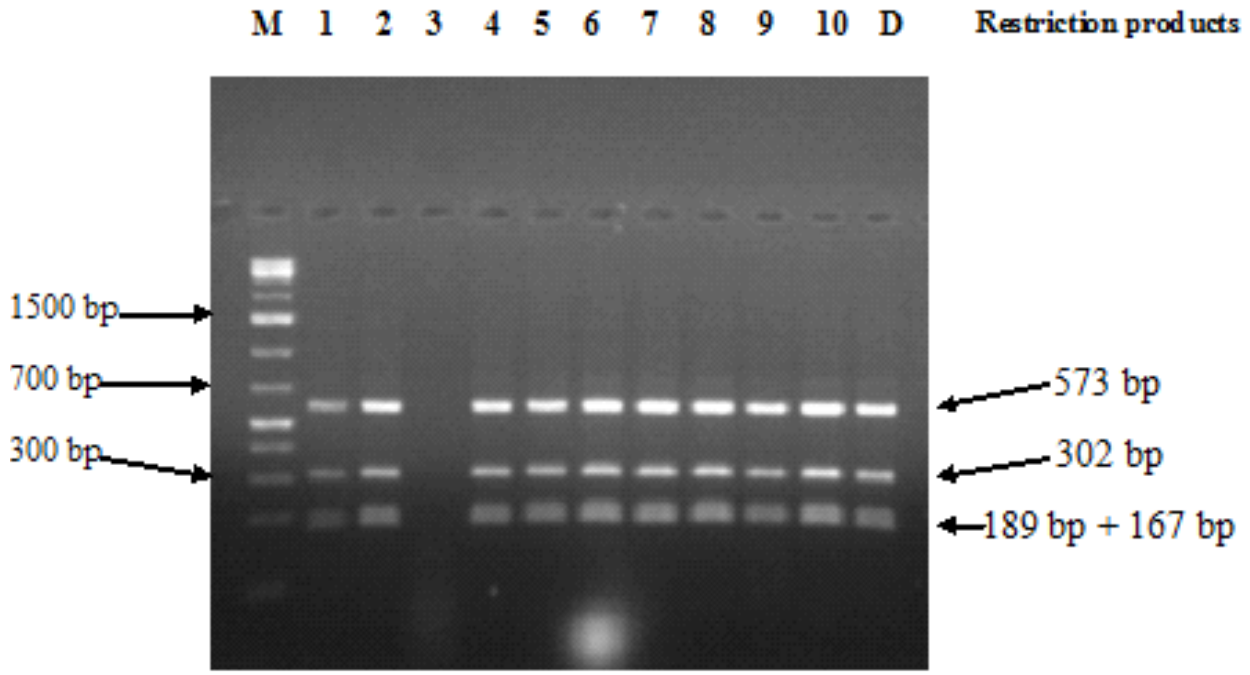


**Figure 8.** Sorghum *ANS* gene PCR product restricted by the enzyme *Mbo*I. Lane marked M contain a 1 kb DNA ladder, lanes 1 - 10 contain the samples as follows: 1 - IS 22320; 2 - IS 13402; 3 - IS 21487; 4 - IS 22318; 5 - IS 13483; 6 - IS 22294; 7 - IS 1196; 8 - IS 22335; 9 - IS 21456; 10 - IS 21457; D - dwarf wonder.

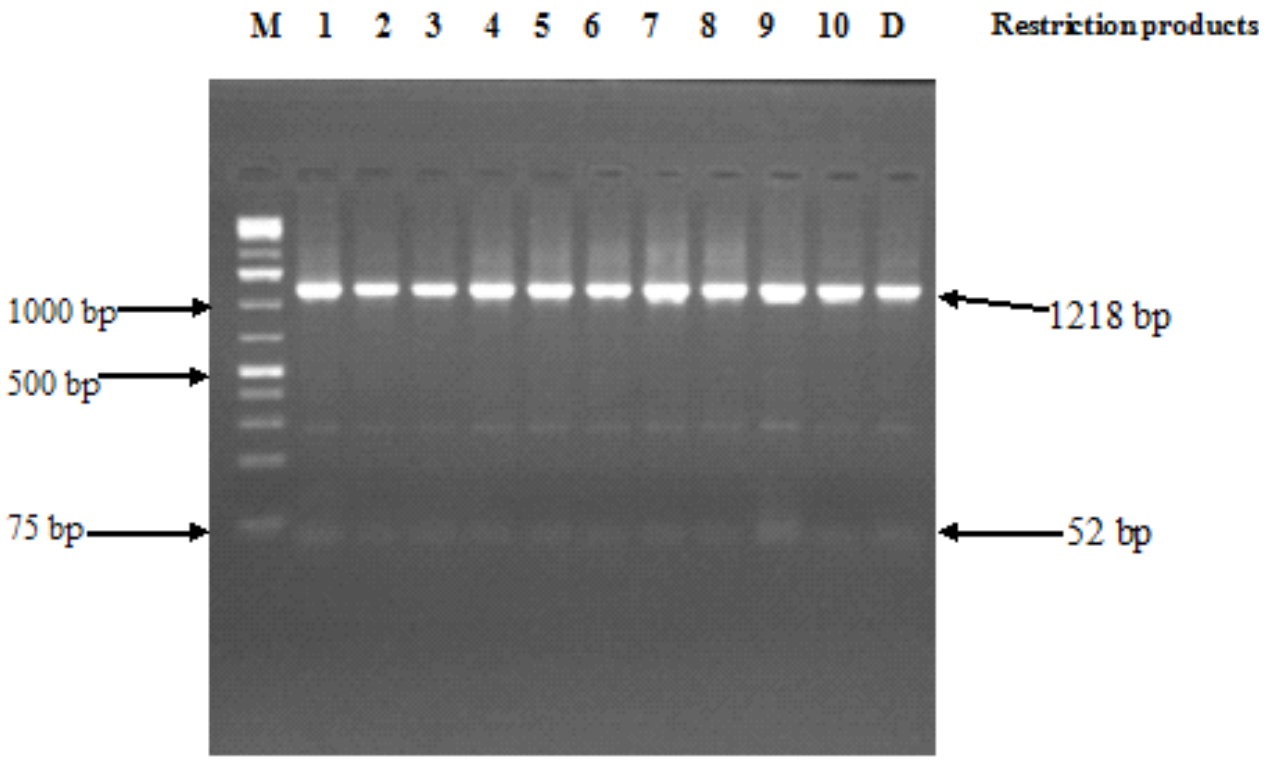


**Figure 9.** Sorghum *ANS* gene PCR product restricted by the enzyme *Hinf*I. Lane M contains a 1 kb DNA ladder, while: 1 - IS 22320; 2 - IS 13402; 3 - IS 21487; 4 - IS 22318; 5 - IS 13483; 6 - IS 22294; 7 - IS 1196; 8 - IS 22335; 9 - IS 21456; 10 - IS 21457; D - dwarf wonder.

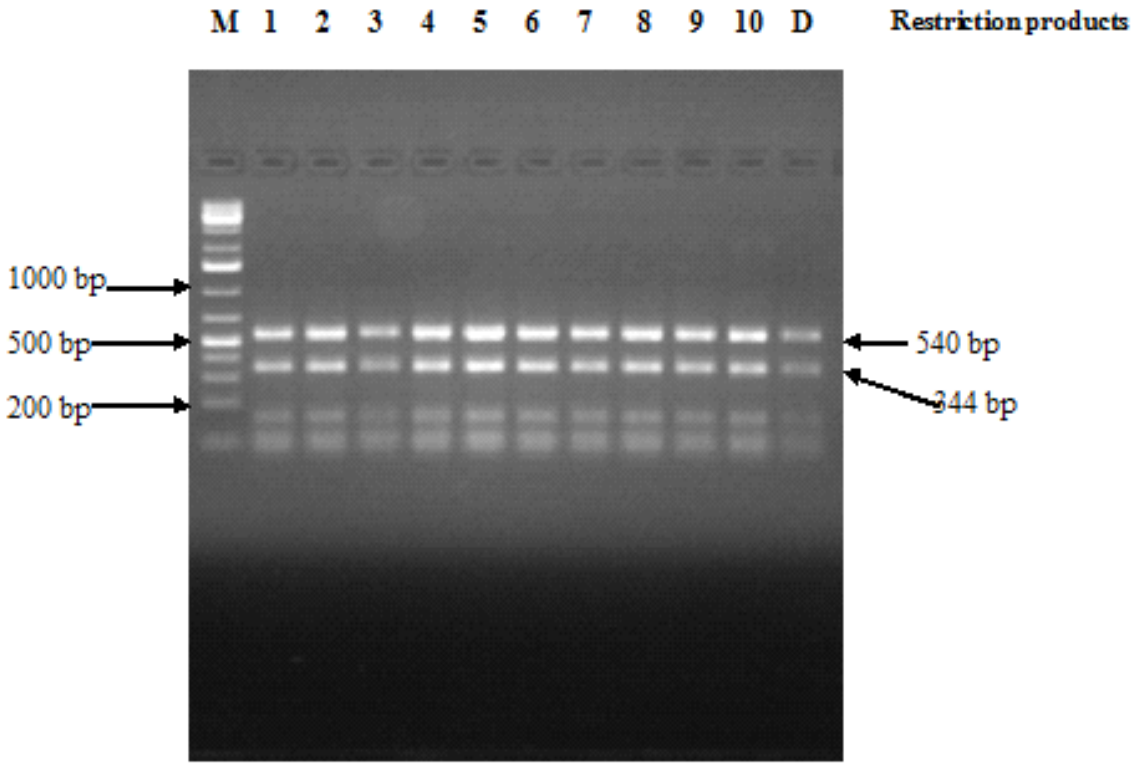


**Figure 10.** Sorghum *ANS* gene PCR product restricted by the enzyme *TaqI*. Lane marked M contain a 1 kb DNA ladder, lanes 1 - 10 contain the samples as follows: 1 - IS 22320; 2 - IS 13402; 3 - IS 21487; 4 - IS 22318; 5 - IS 13483; 6 - IS 22294; 7 - IS 1196; 8 - IS 22335; 9 - IS 21456; 10 - IS 21457; D - dwarf wonder.

levels concluded that 35% of the landraces used in the study had detectable proanthocyanidins in their grain (Dhlamini and Niang-Sithole, 2014). These results agree with the results obtained in this study, even though different batches of local landraces were used.

The DMACA assay was used not only as a preliminary screen for PAs in the selected sorghum landraces, but also to reveal those sorghum lines that have an altered PA distribution pattern. In other plants, altered seed DMACA staining patterns have been successfully used to identify flavonoid pathway mutants. In studies in Arabidopsis, several transparent testa (*tt*) and tannin deficient seed (*tds*) mutant lines were identified through the use of DMACA staining (*Abrahams et al., 2002). Fur*ther studies on these lines indicated that they were indeed flavonoid pathway mutants at several *loci* vital for PA biosynthesis and accumulation (Abrahams et al., 2002). Mutations in some flavonoid pathway genes, e.g. *ANS* and *TT12*, have been shown to not only affect presence and quantities of certain flavonoids, but also final PA deposition patterns (Abrahams et al., 2002; Debeaujon et al., 2001).

Using the DMACA assay, five landraces showing unusual staining patterns were identified. These are IS 22331 (brown non-tannin), IS 13446 (chalky-white), IS 9254 (red high-tannin), IS 13900 (red low-tannin) and IS 9361 (brown high-tannin). All showed a patchy appearance after DMACA staining, typical of some flavonoid pathway mutants. The butanol-HCl assay was used to further characterise these lines, showing in the process that of those lines showing unusual DMACA results; IS 22331 (pale brown) and IS 13446 (chalky white) did not contain any seed PAs, while the 2 red lines IS 9254 and IS 13900 contained PAs. The black and white DMACA colouration given in IS 13446 is typical with many other chalky white grain samples. The chalky-white appearance of the grain of some sorghum varieties is due to either the presence of starch grains in the mesocarp or the presence of a pigmented testa layer containing insoluble tannins underneath a colourless pericarp (Rooney and Murty, 1982). A sectioning examination has shown IS 13446 to indeed possess a pigmented testa underneath a clear pericarp.

From this study, it can be concluded that most red (92%) and all white (100%) sorghum grain samples tested negative for PAs, while 89% of the brown lines tested positive for grain PAs (Figures 1, 3, 4 and 5). Many white sorghum varieties are mutated at *loci* higher than the flavonoid pathway at or before points when coloured flavonoids begin to appear in the grain, thus

explaining why they stain negative for tannins and do not accumulate any assayable tannins. In tannin sorghum, tannins accumulate either in the pigmented testa only (type II) or in both the pigmented testa and pericarp (type III), meaning the presence of a pigmented testa is vital for tannin accumulation (Earp et al., 2004).

The DMACA assay was used in combination with the butanol-HCl assay. This is because use of the DMACA assay alone in the biochemical characterisation of tannin-producing mutants in sorghum is insufficient. Firstly, DMACA not only reacts with condensed tannins, but also with their immediate precursors, that is, flavan-3-ols and flavan-3,4-diols (Abrahams et al., 2002). This means any mutant expressing any one of the precursors will stain positive, giving a false positive result for the presence of tannins when in actual fact the mutant will be accumulating only the precursors. Secondly, studies have shown that those mutants that accumulate minute quantities of the soluble PA precursors tend to leach those precursors into the DMACA staining solution before the reaction is complete, leading to a false negative result (Koupai-Abyazani et al., 1993). As a consequence, the butanol-HCl assay was used to confirm the results of the DMACA assay.

Even though the flavonoid pathway has several structural and regulatory genes, any mutation on any one of these many *loci* has the potential effect of altering the flavonoid profile of the mutant line carrying the mutation. It is important to identify such mutations and establish their exact effects on the flavonoid pathway and on flavonoid profiles. Such studies have been carried out in other plants like arabidopsis, alfalfa and barley (Misyura et al., 2012; Abrahams et al., 2002; Bowerman et al., 2012; Buer and Djordjevic, 2009; von Wettstein 2007). However, similar studies are yet to be carried out in sorghum, hence this study is aimed at attempting to establish a link between sequence polymorphisms at the *ANS* gene *locus* and differences in proanthocyanidin profiles in sorghum.

Sequence polymorphisms at the *ANS locus* were determined using a combination of PCR and restriction digestion analysis (PCR-RFLP). Each *ANS* gene fragment was cut sixteen times by all the enzymes combined, translating to a DNA fragment of 65 bp in length. This covers the actual position of the cut and the other bases that make up the restriction site. Any mutation within this 65 bp region of the amplicon under study would have been detected by this method. However, with all these restriction enzymes no differences in restriction patterns were detected (Figures 8, 9 and 10).

This potentially means either of two things, firstly that there are no polymorphisms at this *locus* in the landraces under study, and as a result any observed differences in PA profiles may be due to mutations at other *loci* or due to environmental factors, or secondly that the RFLP technique probably missed some potential SNPs due to its inherent shortcomings.

The RFLP technique has one major handicap when used in the detection of sequence variations among similar sequences. The method targets only those mutations that occur within restriction sites ad ignores the rest of the gene. In this case, this method only focused on a 65-bp region in a PCR amplicon, 1270 bp in size. This translates to only 5% of the gene. Inasmuch as the method may be a very useful and cheap rapid screening tool for DNA mutations, it suffers from low coverage. The method can however be made more effective if more restriction endonucleases are used. This way the portion of the gene directly screened for any mutations significantly increased.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

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