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# Detecting changes in the nutritional value and elemental composition of transgenic sorghum grain

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ABSTRACT

We have previously demonstrated that poor digestibility in sorghum can be addressed by using RNA interference (RNAi) to suppress kafirin synthesis. The approach resulted in a twofold improvement in overall protein digestibility levels. In the present study, the effect of this targeted kafirin suppression on other grain quality parameters was investigated. Several significant changes in the proximate composition, amino acid profile and the bulk mineral content were detected. Importantly, the most limiting amino acid, lysine, was significantly increased in the transgenic grains by up to 39%; whilst mineral elements in the bulk, such as sulphur (S) and zinc (Zn) were reduced by up to 15.8% and 21% respectively. Elemental mapping of the grain tissue, using micro-PIXE, demonstrated a significant decrease in Zn (>75%), which was localised to the outer endosperm region, whilst TEM revealed important changes to the protein body morphology of the transgenic grains.



## 1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is Africa's contribution to the elite cereal crops of the world and, as such, it is ranked as the fifth most important human staple after wheat, rice, maize and potatoes [2]. Although sorghum is important for food security, it is generally viewed as nutritionally inferior to other major cereals, because of its dominant proteins, the kafirins, which are difficult to digest, and are furthermore deficient in the essential amino acids lysine, methionine and tryptophan [3]. To improve the nutritive value of this crop, a recent study utilised RNA interference (RNAi) technology to suppress kafirin synthesis in the public Sorghum line P898012 [1]. Several of the resultant transgenic lines demonstrated a significant increase in overall *in vitro* protein digestibility (of up to 53%) [1]. Although this was a welcome improvement to the nutritional value of sorghum, it was not clearly established if the genetic alteration had any unintended effects on other important grain quality characteristics. To address this concern the present study was initiated to evaluate potential differences in the grain of two independent transgenic lines (featuring kafirin suppression) and their non-transgenic parental counterpart at the level of the proximates, the bulk mineral content and the total amino acid profile. Transmission electron microscopy (TEM) was also utilised to compare differences in the morphology of grain protein bodies, whilst micro-proton-induced X-ray emission (micro-PIXE) spectroscopy was used to resolve spatial differences in mineral concentrations within individual grains. The results of this study will serve to enhance our present understanding of the effects of kafirin suppression on sorghum grain quality, and will further highlight if there are any inadvertent changes in the transgenic grain material that may warrant closer investigation.

## 2. Materials and methods

### 2.1. Plant material

Plants of two independent transgenic $T_5$ sorghum lines, featuring the pABS044 construct, for the targeted suppression of select gamma- and alpha-kafirins (full details of the original transformation reported in [1]) and their non-transgenic parental counterpart, P898012, were grown under controlled conditions in a

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containment glasshouse located at the Biosciences Division of the Council for Scientific and Industrial Research (CSIR, South Africa). Plants were moved randomly at each watering to minimise positional effects, and before anthesis, individual panicles were bagged to prevent outcrossing. At full maturity, grains from the transgenic (designated herein as TG2 and TG3) and non-transgenic (designated herein as wild-type WT) plants were hand-harvested, cleaned and milled to fine flour; or kept as whole grains as required for the intended analysis. The two independent transgenic lines TG2 and TG3 were selected because of the results of Western Blot analyses which indicated that there was complete suppression of the targeted kafirins, namely, gamma-1 (25 kDa), gamma-2 (50 kDa) and alpha-kafirin A1 (25 kDa) [1].

### 2.2. Compositional analyses

#### 2.2.1. Proximate analysis

The proximate analyses of the samples for moisture, crude protein, crude fat and total ash were carried out in triplicate according to standard protocols. In brief, the weight difference method was used to determine the moisture content after drying the samples at 100 ± 5 °C for 24 h; and the ash content after sample ignition at 500 °C [4]. Crude protein (N × 6.25) was determined by the Dumas combustion method [5] and crude fat by means of ether Soxhlet extraction [4].

#### 2.2.2. Amino acid analysis

The protein-bound amino acid content of the samples was analysed in triplicate according to [6], using reverse phase-high pressure liquid chromatography (RP-HPLC). For cysteine and methionine determination, a separate hydrolysis step involving performic acid oxidation was performed. The amino acid analysis was carried out at the accredited facility of the South African Grain Laboratory (SAGL) in Pretoria, South Africa.

#### 2.2.3. Bulk mineral content

Approximately 5 g of the ground sorghum samples was freeze-dried to a constant dry weight over a period of four days. A half gram of each sample was then digested with 10 ml of $HNO_3$:HCl, 4:1, for destruction of organic matter using a microwave digester. The digested samples were then resuspended in 50 ml distilled water and thoroughly mixed before being analysed by inductively coupled plasma mass spectrometry (ICP MS) at the ICP Laboratory, Central Analytical Facility, Stellenbosch University. The instrument was calibrated using certified mixed standard reference materials from the National Institute of Standards and Technology (NIST). The results reported here were limited to the following main mineral elements: phosphorus (P), potassium (K), magnesium (Mg), sulphur (S), calcium (Ca), iron (Fe) and zinc (Zn).

### 2.3. Statistical analysis

For the compositional data, at least three independent determinations were made for each parameter investigated, and the results expressed as the mean ± standard deviation. To evaluate differences between the means at the 5% significance level, one way analysis of variance (ANOVA) and the Tukey mean separation test was performed using *Statistica* for Windows Version 12.6 (Statsoft Inc., USA).

### 2.4. Transmission electron microscopy (TEM)

To evaluate differences in the ultrastructure of the protein bodies in the transgenic and wild-type grain, TEM analysis was performed. In brief, small segments (1–2 $mm^3$) of the peripheral endosperm were fixed in 3% (v/v) glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2) at room temperature for 24 h, followed by post-fixation in 2% (v/v) osmium tetraoxide at 4 °C for 24 h. The samples were then dehydrated in a graded ethanol series, before being infiltrated and polymerised in Agar Low Viscosity resin at 70 °C for 16 h. Ultrathin sections were prepared using a ultramicrotome fitted with a diamond knife, stained with 2% (w/v) uranyl acetate and Reynold's lead citrate, and examined with a Tecnai G2 transmission electron microscope (situated at the Department of Physics, University of Cape Town). All of the TEM images reported depict protein bodies from the sub-aleurone layer of the grain endosperm.

### 2.5. Micro-PIXE analysis

Dry mature sorghum grains were selected for micro-PIXE analysis, and due to their low moisture content, no elaborate fixation treatment was deemed necessary [7]. Selected grains were embedded in EpoFix™ (Struers) commercial resin and longitudinally sectioned through the median using a rotating diamond-coated blade, operated at a low speed (~100 rpm), which cleanly cut the sample into half. Photomicrographs were then made of each half-grain sample using a Nikon SMZ1500 stereomicroscope fitted with a digital camera. A minimum of three different half-grain samples for each genotype was then coated with a thin layer of carbon before mounting for micro-PIXE analysis. Micro-PIXE on the half-grain samples was carried out using a proton beam of 3.0 MeV energy and a current of ~100 pA, at the Materials Research Department, iThemba LABS. The proton beam was focused to a 3 × 3 $\mu m^2$ spot and raster scanned over a sample area of ~2 $mm^2$, using square scan patterns and a data matrix of up to 128 × 128 pixels. Both micro-PIXE and proton backscattering (BS) spectra were collected simultaneously in event-by-event mode. Following data collection, quantitative elemental maps were generated, using the Dynamic Analysis method, as part of the GeoPIXE II software package [8]. Additionally, micro-PIXE spectra were extracted from a defined outer and inner region of the endosperm of each sample, to obtain average concentration values for particular mineral elements of interest. For data processing, each half-grain sample was treated as 'infinitely thick', and the main constituent of the biological matrix was assumed to be cellulose, following the similar approach of [9,10].

## 3. Results and discussion

The main nutritional components analysed in the transgenic and wild-type sorghum grain samples are shown in Table 1. Proximate analysis is an important tool for evaluating the quality of foodstuffs and is often used as the basis for establishing the overall nutritional value. The proximate components determined in this study included values for the moisture, crude protein, crude fat and the total ash content. The mean values of all measured parameters were found to be statistically equal (at the 5% level) for TG3 and WT grain. Grain from TG2 however, was found to be statistically different from the WT, in terms of its moisture, crude fat and total ash content. Higher levels of moisture (~25% increase) and crude fat (~16% increase) were recorded for TG2 grain; whilst total ash was significantly reduced (by ~21%), in comparison to the WT. According to the consensus document of the Organization for Economic Co-operation and Development (OECD), the following mean range values for grain sorghum are noted: moisture 9.2–12.5%; crude fat 0.8–4.3%; and total ash 1.5–3.3% [11]. The mean values for moisture (11.16%), crude fat (2.75%) and total ash (1.61%) in the TG2 sample therefore fall within the bounds of normal variation for grain sorghum, and as such, these differences are not regarded as biologically significant. The increased moisture