Chapter 8 – Proton induced X-ray emission and electron microscopy analysis of induced mutants of sorghum

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Abstract

Gamma irradiation induced alterations in the spatial distribution and localisation profiles of Fe, Zn, K, P, Cl, Ca, S, Mn, and Cu in mutant seeds of sorghum were determined using PIXE. The changes included enhanced or diminished accumulation of elements in preferential accumulation tissues and entire changes in cellular localisation. Transmission and scanning electron microscopy of the mutants resolved changes in size, shape, ultra-structure and packed cell volumes of protein- and starch bodies. The combined data suggest that induced mutations are an effective tool suitable for simultaneously targeting changes in multiple agronomic and nutritional traits that are crucial for human and animal health in important crops.

Keywords: sorghum, micronutrients, PIXE, protein body, starch body, irradiation

8.1 Introduction

Micronutrients are necessary for growth, development, immune functioning and health of all living organisms. Because they are functionally required in small quantities, they are often referred to as trace elements (Ager *et al.*, 2003). In higher concentrations some trace elements (for example iron and copper) are toxic to cells. Many organisms therefore have evolved mechanisms to regulate the uptake of trace elements, their excretion and intracellular translocation and localisation within cells. These regulatory mechanisms are intended to assist in balancing out the need for protection against toxic reactivity of some elements in cells, as well as the need to ensure adequate availability when cells require them (Ralle and Lutsenko, 2009). While plants obtain their elements from the environment and soil, humans and animals obtain them from diet.

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Important staple cereals like sorghum, maize, wheat and rice are deficient in micronutrients. In the case of Fe and Zn, these cereals contain significant amounts, but, in unavailable form because both are bound up by phytate and phenolic acids (Kayode *et al.*, 2007). Considerable resources have therefore been devoted to the biofortification and physical fortification of staple foods to improve their nutritionally critical elements in diet.

An additional field of research that is gaining importance is the area of phytoremediation/ phytoextraction of toxic elements in contaminated soils. Bioremediation and enhanced nutritional value of crops have been ranked in the top ten biotechnologies for improving human health in developing countries (Daar *et al.*, 2002). Both fields require an understanding of the physiology of specific cell types and their ability to accumulate elements in storage vacuoles (Baxter, 2010; Guerinot and Salt, 2001; Rugh, 2004). Our study primarily focused on investigating the distribution, accumulation patterns and localisation profiles of a selected group of macro- and microelements in a mutant population of sorghum generated through gamma irradiation. We previously reported that this mutant population displayed protein polymorphisms and significant changes in protein and amino acid profiles beneficial to human and animal health (Mehlo *et al.*, 2013).

Plants acquire elements from the environment, often in excess of the plants' own immediate requirements. The excess is usually stored in vacuoles until needed (Ager *et al.*, 2003). However, when such plants are consumed, the utility and importance of the accumulated elements depends largely on the nature of the element, its chemical form (bound vs free form) and its accumulation dynamics in certain body parts after consumption (Ager *et al.*, 2003). Even though very little is known of the molecular mechanisms and the biology of accumulation of elements in plants, several methods have been employed to alter the accumulation dynamics, concentrations, and forms of elements in plants and their quantification. Making use of proton induced X-ray emission (PIXE) and electron microscopy, alterations in the profiles and elemental distribution of 9 elements, and the protein- and starch- body ultra-structure of gamma-irradiation mutants of sorghum have been resolved (Mehlo *et al.*, 2013). These results prove that induced mutations can be a useful tool, in addition to other techniques like genetic engineering and breeding for traits associated with elemental accumulation. Our data suggests that induced mutations in particular, is suitable for effecting simultaneous alterations of multiple nutritional and agronomic traits in important cereal crops. We postulate further that in the near future, it may be possible to meet elemental requirements in nutrition if the remarkable influence of the plant genome in governing concentration of heavy metals essential for growth and development from the environment by some hyperaccumulators is fully understood.

8.2 Materials and methods

8.2.1 Plant material

The gamma irradiation induced sorghum mutants and initial genotypes, controls used in this study, were previously described in a study that focused on induced protein polymorphisms

and nutrition (Mehlo *et al.*, 2013). Briefly, the parental wild type sorghum accession P898012 is a purple, type II tannin (low tannin) public sorghum line originally obtained from John Axtell of Purdue University. The seeds of P898012 sorghum are chalky white in colour. The gamma irradiation mutants of P898012 included, non-tannin white sorghum (T120), a soft endosperm lemon yellow tannin sorghum (SY), a mutant with seeds whose pericarp is red (RED), a mutant sorghum whose seeds have a brown pericarp (BR) and a white tannin sorghum mutant producing many tillers (BIO). Three additional non-irradiated sorghum lines were used in the analysis (specified as control lines for this study). These control lines included, Tx430, a high anthocyanin black sorghum; Macia, a white food-type non-tannin sorghum line and SK5912, a yellow endosperm, malting sorghum line (YEL).

8.2.2 Nuclear microprobe analysis

Microanalysis to determine elemental distribution profiles of Fe, Zn, K, P, Cl, Ca, S, Mn and Cu was carried out using a nuclear microprobe at the Materials Research Department, iThemba LABS, South Africa. A detailed description of the nuclear microprobe setup at iThemba labs was previously outlined by Prozesky *et al.* (1995). Representative samples from each sorghum line were used to examine possible differences in elemental distribution. The grains selected for Micro-PIXE analysis were first embedded in a commercial resin (EpoFix, Struers) and then longitudinally sectioned with a rotating diamond-tipped blade before being carbon coated. The analysis was performed using a proton beam of 3.0 MeV energy and a current of ~100pA, focused to a 3×3 μm^2 spot around the germ. The seeds were then scanned over a sample area of ~50×124 μm^2 using square scan patterns with a variable number of pixels. The two complementary techniques: proton-induced X-ray emission (PIXE) and Rutherford proton backscattering spectrometry (RBS) were used simultaneously in event-by-event mode. Elemental concentration was obtained using GeoPIXE II software (Ryan *et al.*, 2002).

8.2.3 Transmission electron microscopy and scanning electron microscopy

For transmission electron microscopy (TEM), longitudinal sections of the seeds were made with sharp scalpels. The pericarp was then scraped from the top of the kernel directly opposite the germ, to leave sub-pericarp and aleurone layers intact. Small sections (1 to 2 mm thick) of cleaned peripheral endosperms were then taken off using a sharp scalpel and embedded in glutaraldehyde (2.5%) in pH 7.4 for 24 h, followed by 3× 5 min of 0.075 M phosphate buffer wash before staining with 0.5% aqueous osmium tetraoxide for 2 h. Fixing was as previously described (Da Silva *et al.*, 2011). The specimens were then dehydrated in a graded aqueous acetone series before infiltration with Spur's resin. Ultrathin sections were cut with an ultra-microtome fitted with a diamond knife. Sections were stained with aqueous uranyl acetate and then further stained in Reynold's lead citrate before being examined either with a Phillips EM301 or Phillips CM10 TEM (Eindhoven, the Netherlands). To visualize the samples under scanning electron microscopy (SEM), kernels were freeze fractured longitudinally in liquid nitrogen using a scalpel cooled to liquid nitrogen temperature. The samples were then mounted to an aluminium stub using double

sticky tape before being coated with gold palladium sputter coater (Technics Hummer 1). The samples were viewed on a JEOL SEM (JSM 5800 LVSEM, Tokyo Japan).

8.3 Results

8.3.1 Alterations in the spatial distribution and profiles of elements in seed tissues of mutant sorghum

Changes in the localisation and accumulation profiles of the elements Fe, Zn, K, P, Cl, Ca, S, Mn and Cu in seed tissues of 5 gamma irradiation mutants of sorghum were determined using PIXE analysis. The data indicates that Fe and Zn preferentially accumulated in the vacuoles of cells in the germ scutellum tissue of both the mutants and the parental P898012 sorghum line (Figure 8.1). Trace amounts of zinc were however also localized in the vacuoles of the pericarp and epicarp cells. The mutants designated RED, BR and BIO accumulated significantly higher concentrations of Fe than the parental P898012 sorghum line. With respect to zinc, the mutants SY, BR and BIO accumulated significantly higher amounts of Zinc in mutants, with the highest recorded in SY.

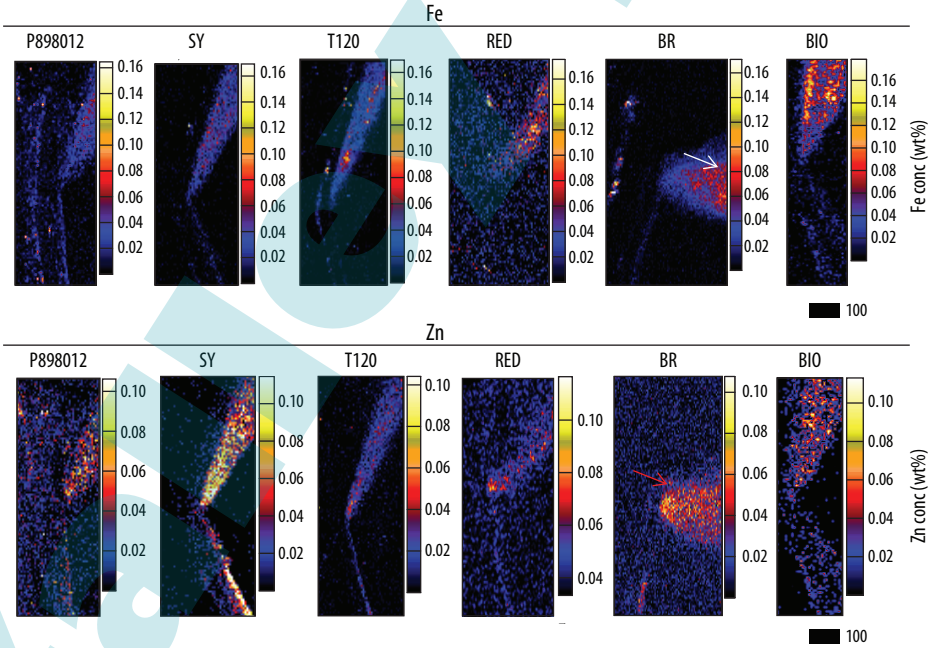

Figure 8.1. PIXE analysis of the spatial distribution and concentrations of iron and zinc in mutant sorghum seeds. The scutellum area and the epicarp are pointed out with a white and red arrow respectively. Concentrations are given in wt %, and the scale bar in µm.

Similarly, the macro-elements K and P were also predominantly localized in the vacuoles of the scutellum tissue of the sorghum germ. Traces of K were also mapped to the pericarp of RED, BR and BIO mutants (red arrows in Figure 8.2). The mutants RED and BIO similarly accumulated trace amounts of K in the endosperm (white arrow in Figure 8.2). In the mutant SY, two distinct layers of trace K localisation could be identified in the epicarp and pericarp of vacuoles in cells of the scutellum tissue (denoted 2DL, i.e. two distinct layers in Figure 8.2 as indicated by yellow arrow). Mutant T120 accumulated the lowest amounts of K and P with the lowest amounts recorded for P when compared across all the mutants and the control parental line P898012 (Figure 8.2). The detected levels of (K and P) were higher than those of (Fe and Zn) across all mutants and the control parental P898012 line (Figure 8.1 and Figure 8.2).

The elemental distribution and profile maps for Cl, Ca, S and Mn are shown in Figure8.3. Levels of Cl that were slightly higher than those observed in the control P898012 sorghum line were observed in the mutants RED and SY (Figure 8.3). Calcium concentrations were low across all mutants and the control P898012 lines analyzed, with exception of the mutant BR and SY where trace amounts were localized in the aleurone and sub-aleurone area (Figure 8.3). Sulphur

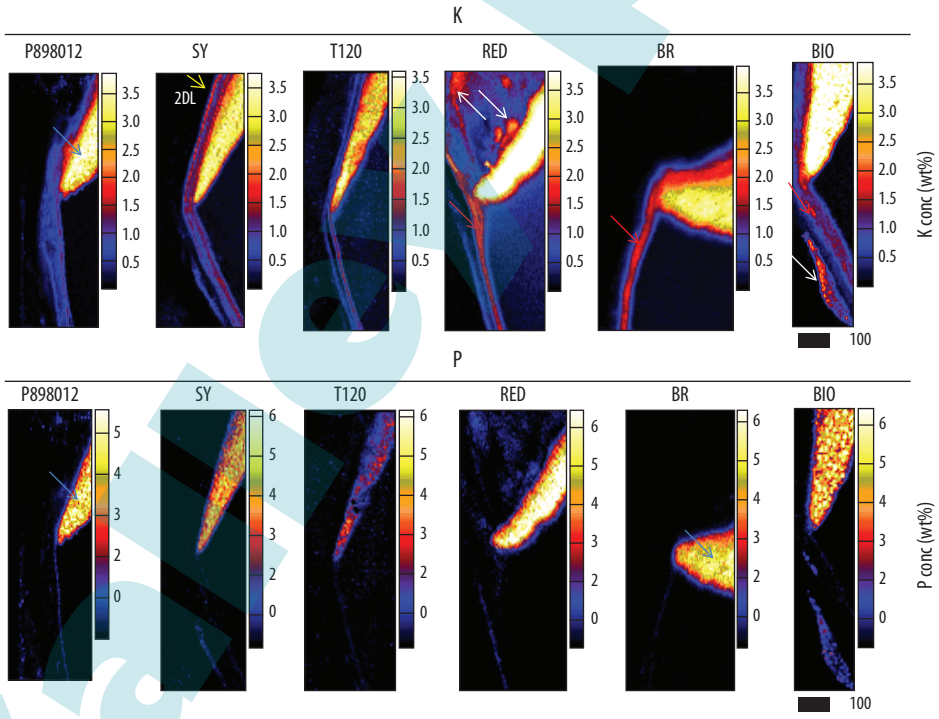

Figure 8.2. PIXE analysis of the spatial distribution and concentrations of potassium and phosphorus in mutant sorghum seeds. The scutellum and the pericarp are pointed out by the blue and red arrows, respectively. The white arrows indicate traces of K in parts of the endosperm. Concentrations are given in wt %, and the scale bar in µm.

accumulation was also low, with trace amounts localised specifically in the pericarp. As was the case with Cl and Ca, the mutant BR had the highest amounts of S, followed by the mutant SY, BIO, RED, T120 and the control parental line P898012 in that order. Manganese concentrations were highest in the pericarp of the parental P898012 sorghum line, with all the mutant lines accumulating trace amounts. Levels of Cu accumulation were very low across all the mutants and the parental control lines, and as such were not included in the maps displayed in Figures 8.1-8.3.

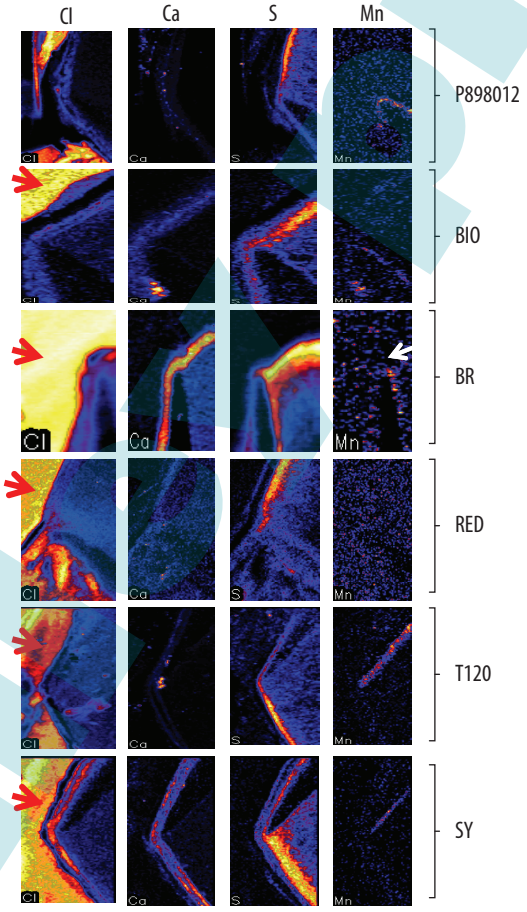

Figure 8.3. PIXE analysis of the spatial distribution of chlorine, calcium, sulphur and manganese in mutant seeds of sorghum. High concentrations of Cl in the embedding resin and the germ area of the seed are pointed out with red and white arrows, respectively.

8.3.2 Electron microscopic analysis of induced changes in protein- and starch-body ultra-structure

The ultra-structure of protein bodies of mutant sorghum grains were studied by TEM. Three very significant alterations to the protein body structure were observed in the form of invaginations, shape and size (Figure 8.4). The protein bodies of the mutants SY, RED, and BIO had invaginations on their surfaces (illustrated by an arrow in the SY mutant). Invaginations observed on the protein bodies of the mutant SY were extensive, deep almost permeating to the core of the protein bodies. The invaginations of mutant RED were also extensive but were confined to the surface layers of the protein bodies. Mutant BIO's protein bodies had mild invaginations confined to the surface layers of the protein body. Mutants BR, T120 and the parental P898012 lines had protein bodies with very few invaginations. As far as the shape of the protein bodies was concerned, the mutant BIO and the parental P898012 had rounded protein bodies, whereas SY, RED, BR and T120 all had irregular shaped protein bodies. Mutants RED and SY had the smallest size protein bodies, whereas the biggest protein bodies were observed in mutant BR. Dense packaging of the protein bodies was observed in all the mutants (Figure 8.4).

The starch body ultra-structure of the mutants was investigated via SEM. Three crucial observations relating to altered phenotype of starch body ultra-structure were made and these, as was the case with protein bodies were in the form of the size, shape and packed cell volume (Figure 8.5).

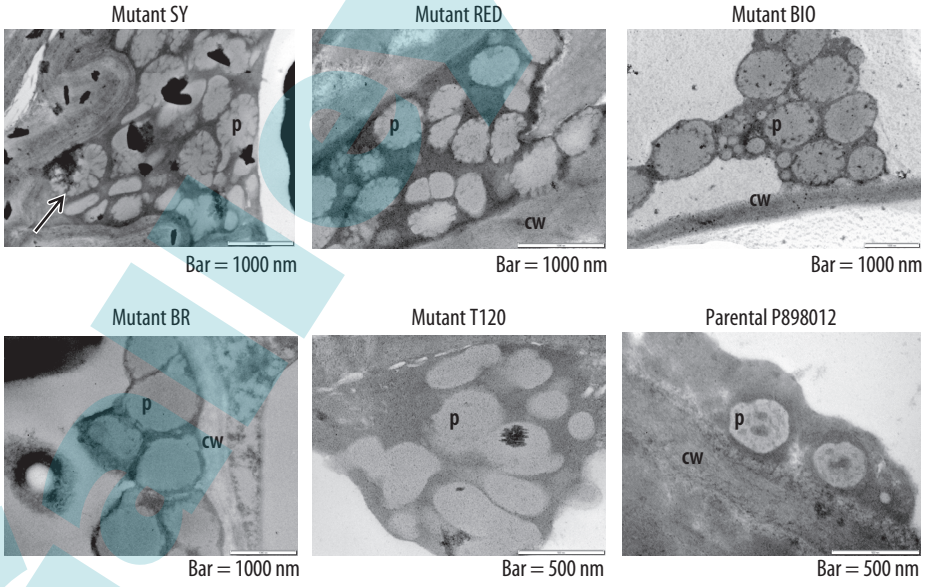

Figure 8.4. Transmission electron microscopy analysis of the protein bodies of mutant sorghum seeds. cw = cell wall; p = protein body. Extensive invaginations in protein bodies of SY are indicated by an arrow.

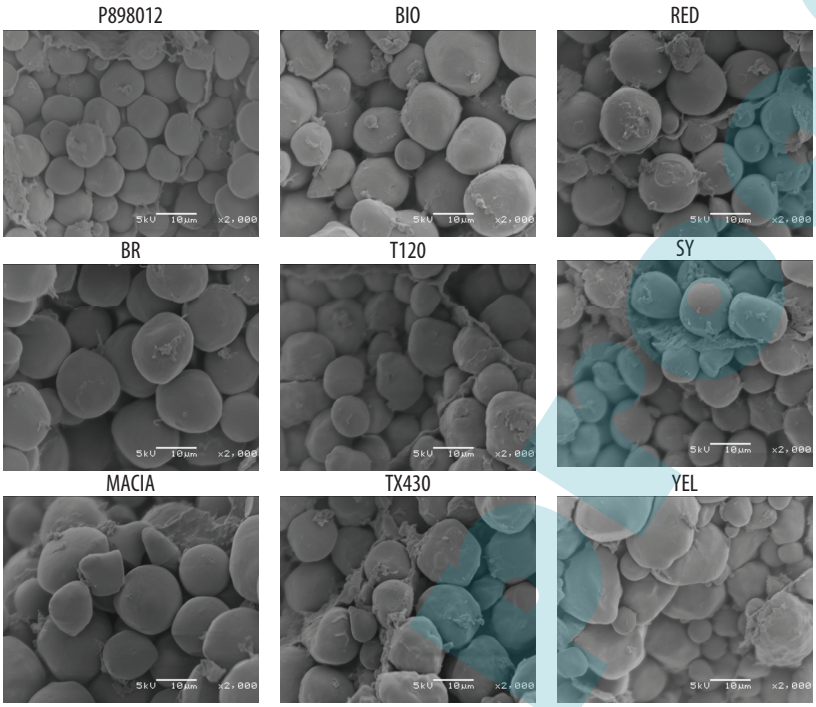

Figure 8.5. Scanning electron microscopy analysis of starch bodies of mutant sorghum seeds. PB = protein body; S = starch body.

The order of size of starch bodies, from the largest to the smallest was as follows: YEL>BIO >Macia>BR>RED>TX430>T120>SY>P898012, respectively (Figure 8.5). The control line YEL contained many small starch bodies interspersed with the large ones. The mutant RED had almost perfectly spherical starch bodies followed by BR, BIO and SY respectively. Those of YEL were highly irregular and ovoid, followed by those of TX430, T120 and P898012 sorghum lines. As a consequence of this irregularity in shape, the starch bodies of the lines (YEL, TX430, T120 and P898012) packed densely per cell volume leaving smaller spaces in between (Figure 8.5).

8.3.3 Analysis of endosperm macrostructure

Sectioned seeds of mutant sorghum revealed three types of endosperm macrostructure when visualized under a light microscope (Figure 8.6). Mutant T120, along with the sorghum control lines YEL, Macia and TX430 possessed a large proportion of hard corneous endosperm. The mutant line BIO, together with the parental line P898012 had approximately equal proportions of the hard and soft floury endosperms, and as such, were classified as 'intermediate hard endosperm' in this study. The mutants RED and BR had floury soft endosperms. An extremely floury endosperm with a hollow lumen was observed in the SY mutant line (Figure 8.6).

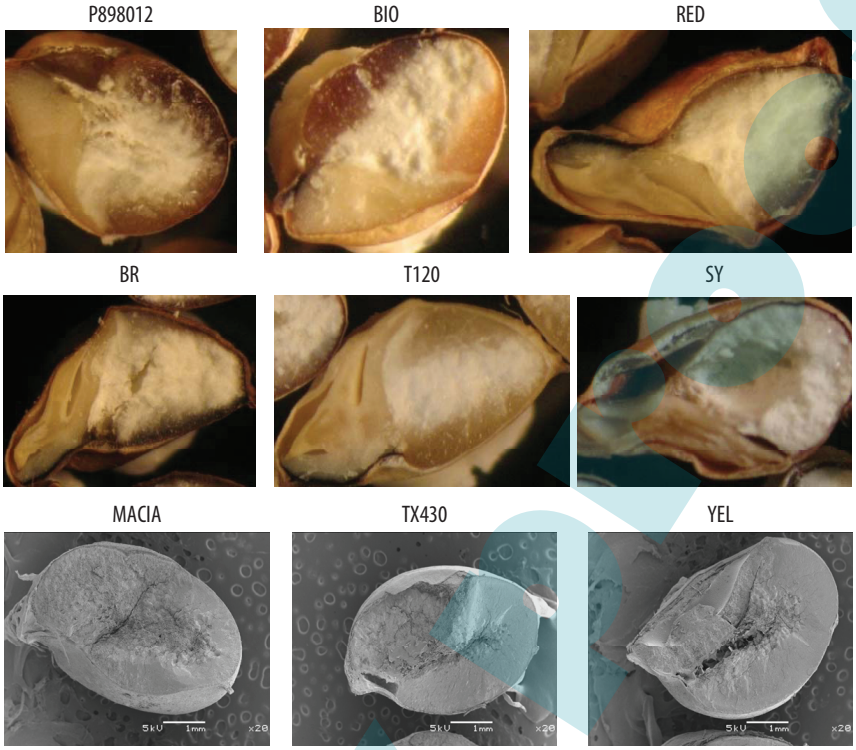

Figure 8.6. Light and scanning electron microscopy analysis of endosperm macrostructure of mutant sorghum endosperm.

8.4 Discussion

The discovery of natural metal hyper-accumulator plant phenotypes, especially those with the remarkable ability to accumulate exceptionally high amounts of metals at levels 100-fold higher than common non-accumulators have opened up two possible fields of applied research. One application relates to solving micronutrient deficiency in staple diets (alleviating the hidden hunger), and the other in the field of phytoremediation of metal-polluted soils (Ager *et al.*, 2003). Further efforts have therefore been directed towards genetic transformation of plants in order to enhance their ability to accumulate nutritional elements (in the case of health), and to tolerate metal polluted soils through hyper accumulation and sequestration of metals (Ager *et al.*, 2002).

Here, we report on the utility of induced mutations in effecting the simultaneous alteration of a number of important agronomic and nutritional traits of sorghum, including changes in the profile and distribution of macro- and microelements, protein- and starch-body ultra-structure. Previous study demonstrated that gamma irradiation mutants of sorghum displayed induced protein polymorphisms that were associated with improved amino acid content and nutrition (Mehlo *et al.*, 2013). In the current report, we present further evidence that the same mutants

also possessed alterations in elemental distribution and spatial profiles using PIXE (Figures 8.1-8.3). TEM and SEM also allowed us to explore further changes in size, shape and ultra-structure of protein bodies and starch bodies (Figures 8.4 and 8.5). Changes in protein-body and starch-body ultra-structure directly impacts a number of crucial factors governing sorghums' limited nutritive value; for example protein and starch digestibility, quality of protein and grain hardness (Kumar *et al.*, 2012). A unique microstructure of protein bodies induced via chemical mutagenesis in sorghum (Opaque-721 mutant) was previously characterized as being irregular and invaginated; a trait consistently correlated with high protein digestibility (Oria *et al.*, 2000). The invaginations are assumed to increase protein-body surface area, thus favouring easy accessibility to proteolytic enzymes.

Kafirin suppression, particularly involving a combination of gamma and alpha species is also reportedly associated with distorted protein bodies (Kumar *et al.*, 2012). This is a more likely explanation for the observed changes in protein bodies reported here (Figure 8.4). Much of the protein polymorphisms have been documented in an earlier study of these mutants involved kafirin suppression (Mehlo *et al.*, 2013). The floury endosperm on the other, as observed in mutants RED, BR and SY (Figure 8.6), is a trait attributed to a discontinuous protein matrix, smaller and fewer protein bodies and loosely packed starch granules with air-filled spaces that diffract light (Rooney and Miller, 1982). Our results are in line with this explanation. The starch bodies of the mutants RED, BR and SY for example, as shown in Figure 8.5, are round in shape, and thus leave large spaces in between when packing. Such large spaces contribute towards refracting light as previously reported (Oria *et al.*, 2000).

In this study, specific gene mutations as culprits for the altered elemental profiles and changes in protein- and starch-body microstructure were not determined. Determining the functional links between the genome, proteins, metabolites and mineral ions is very complicated because of the number of genes and epigenetic factors involved. In *Arabidopsis thaliana* for example, it is estimated that as much as 2-4% of the genome is involved in regulating plant nutrient and trace element content (Lahner *et al.*, 2003). There is therefore somewhat limited knowledge of which techniques would adequately unravel these functional linkages. However, many techniques have been used for the estimation of elements and the ionic concentrations of plant tissues at the whole plant level, or the sub-cellular level. Examples include flame atomic absorption spectroscopy and techniques based on the fluorescence or luminescence of indicator macromolecules like proteins, X-ray fluorescence detected directly from the element, radioactive emission from tracers, etc. (Ortega, 2005). The application of inductively coupled plasma-mass spectroscopy (ICP-MS) to a mutagenized population of 6,000 Arabidopsis plant lines, for example, revealed 51 mutants with altered elemental profiles (Lahner *et al.*, 2003).

PIXE and electron microscopy are ideal tools for imaging the profiles and spatial distribution of elements and for the visualisation of changes in protein- and starch-body ultra-structure of mutant seeds. Using a combination of PIXE, TEM and SEM we obtained results consistent with observations that different plants, parts of the same plant and even different cells of the same plant tissue may have different elemental profiles (Vega-Carrilloa *et al.*, 1997). Compartmentalisation

of certain elements, particularly those in soluble ionic form makes physiological and chemical sense for plants in order to maintain optimal function: some elements are toxic and some react with other elements within the cells, thus the need for cell-specific distribution of some elements (Leigh, 1997). Compartmentalisation of elements further provides a means to regulate cytosolic nutrient availability and accumulation (Miller and Smith, 2008). The results of this study demonstrate no adverse effects resulting from the changes in elemental distributions and spatial concentrations of the nine elements even in mutants where unexpected accumulation was observed (Figure 8.3).

The distribution of elements and the nature of protein- and starch-bodies of a stable plant type may act as signatures that identify the plant. Research has also shown that element accumulation patterns could also be influenced by expression of key ion or solute transporters in certain cells. Alterations therefore of solute transporters via genetic engineering, growth under stress, and indeed induced mutations can culminate in changes to these accumulation patterns (Conn and Gilliham, 2010). Elemental signatures, as suggested by Baxter *et al.* (2008), can be used as a diagnostic tool when analysis of a target element alone may be an insufficient indicator of nutritional status, thus reducing the proportion of plants that may be incorrectly labelled as having altered elemental profiles. In this report we discount the impact of redundancy and pleiotropic expression effects amongst the mutant lines studied by employing direct comparisons with the wild type P898012 sorghum line. In this regard, our assumption was that the major differences between the parental wild type plant (P898012) and the mutant lines would essentially reflect the impact of mutagenesis. But, as suggested by (Conn and Gilliham, 2010), the possibility that accumulation of one element could influence the accumulation of the others cannot be ruled out. Such phenomenon is driven by the plants' own mechanisms to maintain vacuolar and cytoplasmic osmolarity and charge balance plus related detoxification mechanisms following perturbations in the profile of one element or the other (Conn and Gilliham, 2010). This hypothesis is in line with our own previous observations within these mutants; that suppression of certain kafirin proteins for example, could be accompanied by the over-expression of albumins, globulins and other proteins as part of a compensatory mechanism (Mehlo *et al.*, 2013).

Plants are known to contain approximately 40 different cell types, each varying in its capacity to accumulate elements, thus, whole tissue analysis may have masked individual cell contributions to elemental profiles (Martin *et al.*, 2001). Observations in Figures 8.4 and 8.5 strongly support the conclusion that the alterations in the distribution of profiles of elements detected are consistent with the changes in the ultra-structure of protein- and starch-bodies as well as the alterations in the size, shape in mutant sorghum due to mutations conferred by gamma irradiation.

8.5 Conclusions and future perspective

In the present study we used PIXE and electron microscopy to demonstrate variations in elemental distribution and profiles, and changes in protein and starch body ultra-structure. These changes may have significant implications on the nutritional value of sorghum as a staple cereal. This study

thus contributes to the body of knowledge on how nutritionally important elements and traits in plants can be simultaneously altered to benefit human and animal health, and the environment through phytoremediation. However, the significance and potential of our results could only be fully exploited once the mechanisms of alteration of the micronutrient profiles and changes in protein and starch body microstructure in mutant plants, and its impact on nutrition and the environment are fully understood. Our further challenge in subsequent research therefore is to be able to effectively direct accumulation of these elements in important plants and into plant cells where they are easily available and not bound up by anti-nutrients as is the complex case of iron and zinc being bound by phytate and phenolic acids.

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