Full Length Research Paper

Establishment of sorghum cell suspension culture system for proteomics studies

Rudo Ngara, Jasper Rees and Bongani K. Ndimba*

Proteomics Research Group, Department of Biotechnology, University of the Western Cape, Private Bag X17, Bellville, 7535, Cape Town, South Africa.

This study describes the establishment of sorghum cell suspension culture system for use in proteomics studies. Friable sorghum callus was initiated from young shoots under completely dark conditions on MS medium supplemented with 3 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 2.5 mg/L 1-naphthaleneacetic acid (NAA). Additionally, sorghum cell suspension cultures have been initiated from the friable callus masses in liquid medium with the same composition as the callus initiation medium. Total soluble proteins (TSP) and culture filtrate (CF) proteins were extracted from the cell culture system and solubilised in urea buffer (9 M urea, 2 M thiourea and 4% CHAPS). Both one-dimensional (1D) and two-dimensional (2D) gel analysis of these two proteomes show that the TSP and CF proteomes have different protein expression profiles. The sorghum TSP proteome, which is highly complex, is best resolved when separated on large format, 18 cm, pH 4 - 7 isoelectric focusing (IEF) immobilised pH gradient (IPG) strips. On the other hand, the sorghum CF proteome (secretome) is less complex with most proteins being resolved on mini format, 7 cm, pH 3 - 10 IPG strips. Furthermore, narrowing down the pH range from 3 - 10 to 4 - 7 for the CF proteome resulted in improved protein spot resolution.

Key words: Sorghum, proteomics, callus, cell suspension cultures, total soluble protein, secretome.

INTRODUCTION

Sorghum, a cereal crop native to Africa, is drought-tolerant, surviving periods of water deficit (Rosenow et al., 1983). The crop is grown in the semi-arid regions of Africa and Asia primarily as a human food source and in the United States as stock-feed. In terms of both area planted and production yields, sorghum is ranked the fifth most important cereal crop in the world (Doggett, 1988) and second after maize in Africa (FAO, 1995). Sorghum survives in hot and dry environments least suitable for other crops, thus making it an important food and energy source for the future. Sorghum is also a potential model plant for studying the complex mechanisms of drought tolerance in cereal crops.

Over the years, mechanisms of plant stress tolerance have been studied largely through measurements of

mRNA expression profiles of genes of interest between stressed and unstressed plants. Although these transcriptomic studies provide important information regarding gene expression (Dubey and Grover, 2001) in particular, which genes are turned on and when (Abbott, 1999), there have been reports of the possibility of poor correlation between mRNA and protein levels (Gygi et al., 1999). This poor correlation may be attributed to the different rates of degradation of individual mRNAs and proteins as well as to the post-translational modification of many proteins (Abbott, 1999). In such cases therefore, the functions of genes may be better understood through the direct analysis or measurement of protein levels using proteomics technologies.

The field of proteomics is increasingly gaining momentum in plant sciences with several studies having been made and reported on agriculturally important crops such as maize (Riccardi et al., 1998), rice (Rakwal and Agrawal, 2003; Parker et al., 2006) and *Medicago truncatula* (Watson et al., 2003; Lei et al., 2005). Using

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*Corresponding author. E-mail: bndimba@uwc.ac.za. Tel: +27 21 959 2468. Fax: +27 21 959 1551.

proteomic approaches, both cell and tissue specific protein expression profiles under various stress conditions may be studied. Although there has been major proteomic advances using several other plant species, much of the knowledge of plant stress response mechanisms has been gained from work using *Arabidopsis* (Chivasa et al., 2002; Ndimba et al., 2005; Oh et al., 2005) mainly because of its small and fully sequenced genome (The Arabidopsis Genome Initiative, 2000). Even though *Arabidopsis* provides for an excellent model system for studying various plant processes under either natural or stress conditions, this plant is a dicot and is agriculturally unimportant (van Wijk, 2001). For this reason, there is need to understand stress tolerance mechanisms from the perspective of agriculturally significant monocots.

Despite the vast economic potential of sorghum in semi-arid areas as well the promising technique of proteomic approaches in understanding proteome expression profiles during abiotic stress conditions, no global sorghum proteomics studies have been reported to date. Several researchers have reported on the physiological distinction between pre- and post flowering drought tolerance (Rosenow et al., 1983), mRNA expression of dehydrin proteins (Cheng et al., 1993; Buchanan et al., 2005), dehydrin protein expression (Wood and Goldsborough, 1997), the increased accumulation of osmolytes such as glycine betaine (Wood et al., 1996) and soluble carbohydrates and organic acid (Newton et al., 1986) among various cultivars of *Sorghum bicolor*. It is our motivation therefore, to begin a comparative analysis of the global proteome expression of sorghum cell suspension cultures under a range of osmotic and salinity stress conditions. These efforts are encouraged by the ongoing sorghum sequencing project (Sorghum Genomics Planning Workshop Participants, 2005).

In this study, we report the establishment of sorghum callus, the initiation and maintenance of sorghum cell suspension cultures, as well as the evaluation of their potential for application in proteomics studies. Callus is an unorganised mass of undifferentiated cells which forms as a plant's protective response mechanism to seal off damaged plant tissue (Evans et al., 2003). In plant tissue culture, callus may be induced from various parts of the plant grown on solid media with appropriate combinations of plant hormonal supplements. Callus is particularly useful for the production of cells in suspension, which are a population of undifferentiated cells grown in liquid culture. In proteomics studies, cell suspension cultures provide an unlimited supply of uniform cells, which are grown in liquid culture. In comparison with whole plant systems, which have a relatively longer growth cycle as well as complex tissue specific proteomes, cells in suspension have a relatively shorter life cycle and remain undifferentiated. The shorter life cycle provides for a continuous supply of experimental units, which are grown under tightly controlled environmental conditions thus increasing reproducibility within and between experiments.

MATERIALS AND METHOD

Plant material

White sorghum seeds were purchased from Agricol, South Africa.

Plant tissue culture

White sorghum seeds were surface sterilised using 70% ethanol followed by 2.5% sodium hypochlorite solution for 20 min and then rinsed three times with sterile distilled water. The seeds were air-dried on filter paper before plating on half-strength Murashige and Skoog (Murashige and Skoog, 1962) medium supplemented with 1% (w/v) sucrose, 5 mM 2-(N-Morpholino)ethanesulfonic acid (MES) and 0.8% (w/v) agar, pH 5.8. Seeds were left to germinate in completely dark conditions at 25°C for about 3 - 4 days. Young shoots were cut into pieces of approximately 5 mm in length and used as explants for callus initiation on full strength MS medium supplemented with 3% (w/v) sucrose, 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 2.5 mg/L 1-naphthaleneacetic acid (NAA) and 0.8% (w/v) agar, pH 5.8 in the dark at 25°C on 10 cm diameter tissue culture dishes (Techno Plastic Products, Switzerland). Callus growth was visually assessed over a 4 week period. Friable callus was maintained in culture by sub-culturing 4-week old callus onto fresh callus initiation media.
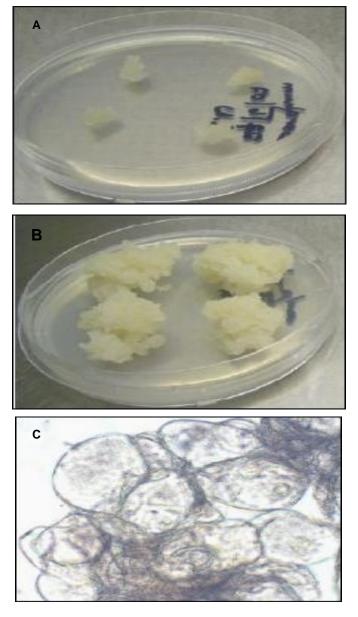
Friable callus cultures were used to initiate cell suspension cultures in liquid MS media with the same composition and plant hormone supplements as the callus induction media. About 3 large clumps of 4-week old actively growing friable callus were placed in a 250 ml conical flask containing 50 ml of media. Flasks were incubated on a horizontal incubator-shaker under completely dark conditions at 25°C with agitation at 130 rpm until cultures reached the desired density. Cells were sub-cultured into fresh media by transferring about 40 ml of the cell suspension into a 250 ml flask containing 60 ml of fresh media.

Protein extraction

Sorghum cell suspension culture total soluble proteins

Ten-day old cell suspension cultures were separated from culture media by filtering over 4 layers of Miracloth (Merck, Darmstadt, Germany) and washed once with sterile distilled water. The cells were transferred into sterile tubes, pelleted by centrifugation at 2500 x *g* for 5 min and were then frozen in liquid nitrogen and stored at -20°C until use in protein extraction procedures.

Liquid nitrogen frozen cells (approximately 2 g) were ground using a pestle and mortar, transferred into 15 ml sterile tubes and precipitated with 10 ml of 10% trichloroacetic acid (TCA) in acetone. The cell debris and precipitated protein were collected by centrifugation at 13400 x *g* for 10 min at room temperature. The pellet was washed 3 times with 10 ml of ice-cold 80% acetone by centrifuging at 13 400 x *g* for 10 min per wash. The pellet was then air dried at room temperature and resuspended in 2 ml of urea buffer [9 M urea, 2 M thiourea and 4% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)] for at least 1 h with vigorous vortexing at room temperature. Soluble protein was collected by vortexing at 15700 x *g* for 10 min. Protein content of total soluble protein was estimated by a modified Bradford assay using BSA as standards. One-dimensional 12% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed to evaluate the quality of protein extracts.

Figure 1. Sorghum callus cultured on tissue culture dishes (∅ 10 cm) and suspension culture cells grown under complete dark conditions on MS media supplemented with 2.4D and NAA. A. Callus masses used for subculturing and maintenance of callus. B. Friable sorghum callus masses after a 4 - 5 week incubation period. C. Microscopic pictures of sorghum suspension culture cells growing in liquid culture taken using a Nikon Inverted Light Microscope fitted with a Leica Firecam digital camera at a magnification of 10X.

Culture filtrate proteins

The culture medium was collected after filtering suspension cell cultures through 4 layers of Miracloth. Cell free culture filtrate was collected by centrifuging the culture medium at 2500 x *g* for 10 min. Culture filtrate proteins were precipitated in 80% acetone for at least 1 h at -20°C and collected in the pellet fraction after centrifu-gation at 15700 x *g* for 10 min. The pellet was washed 3 times using ice-cold 80% acetone, air dried at room temperature and re-suspended in 2 ml of urea buffer as described above.

2D Electrophoresis of protein

Total soluble proteins

Total soluble protein samples (400 and 800 μg) were mixed with 0.8% (v/v) dithiothreitol (DTT), 0.2% (v/v) ampholytes (BIO-RAD, Hercules, CA), a tiny pinch of bromophenol blue and made up to a final volume of 315 μl using urea buffer. The sample was then used to passively rehydrate linear 18 cm IPG strips, pH 4 - 7 (BIO-RAD) overnight at room temperature. The strips were subjected to isoelectric focusing (IEF) on an Ettan™ IPGphor II™ (GE Healthcare, Amersham, UK) in a step wise programme for a total of 66 000 Vhrs at 20°C. After IEF, the strips were equilibrated twice in an equilibration base buffer (6 M urea, 2% SDS, 50 mM Tris/HCl, pH 8.8 and 20% glycerol), firstly containing 2% (w/v) DTT followed by 2.5% (w/v) iodoacetamide for 15 minutes each with gentle agitation. Equilibrated strips were placed on 12% SDS PAGE gels (18 x 18 cm with 1 mm spacers) and electrophoresed on an Ettan™ DALTtwelve System (GE Healthcare), initially at 5 W/gel for 30 min and then at 17 W/gel (at a constant temperature of 25°C) until the bromophenol blue dye reached the bottom of the gel plates as described by Ndimba et al. (2005). The gels were stained overnight in modified Coomassie Blue stain, destained and imaged using a Molecular Imager PharosFX Plus System (BIO-RAD).

Culture filtrate proteins

CF protein (100 ∝g) was mixed with 0.8% (v/v) DTT, 0.2% (v/v) ampholytes (BIO-RAD), a tiny pinch of bromophenol blue and made up to a final volume of 125 μl using urea buffer. The sample was then used to passively rehydrate linear 7 cm IPG strips either pH 3 - 10 or pH 4 - 7, (BIO-RAD) overnight at room temperature. The strips were subjected to IEF on an Ettan™ IPGphor II™ (GE Healthcare) in a step wise programme for a total of 12 000 h at 20°C. After IEF, the strips were equilibrated in DTT and iodoacetamide as described above, placed on 12% SDS-PAGE and electrophoresed using the Mini-PROTEAN 3 Electrophoresis Cell (BIO-RAD). After the second dimension, gels were Coomassie stained, destained and imaged using a Molecular Imager PharosFX Plus System (BIO-RAD).
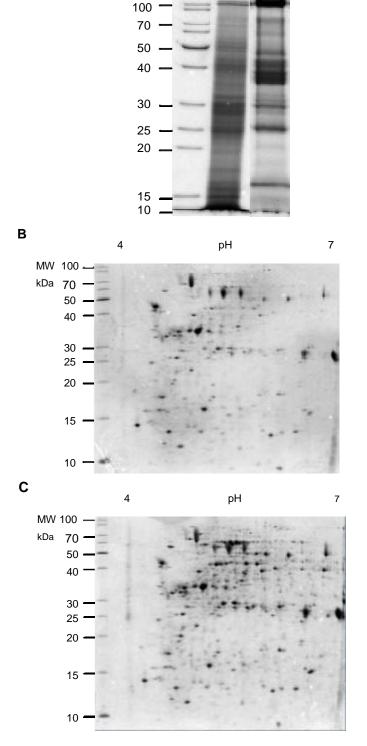
RESULTS AND DISCUSSION

Plant tissue culture

Sorghum callus was successfully initiated from young shoot explant material cultured on MS media supplemented with 2,4-D and NAA. Visual assessment of callus growth under alternate 16 h light and 8 h dark conditions versus completely 24 h dark conditions showed that sorghum callus grew better under dark conditions. White friable callus masses consisting of soft easily separable cells were maintained in culture by transferring approximately 3-4 mm callus masses (Figure 1A) onto fresh media every 4 - 5 weeks. Suspension culture cells were also established from friable callus masses (Figure 1B) and maintained in liquid culture over several generations.

Microscopic analysis of cultured cells showed cell clusters and aggregates of various sizes (Figure 1C). Individual cells were also observed having a dense cytoplasm with numerous cytoplasmic vesicles possibly indicating high cell metabolic activities. The cells grew

Figure 2. 1D and 2D SDS PAGE protein expression profiles of urea- buffer extracted total soluble proteins of sorghum suspension culture cells stained with Coomassie blue. A. 1D SDS-PAGE profile of sorghum total soluble protein (TSP) and culture filtrate proteins (CF). B. 2D expression profile of sorghum total soluble protein resolved on 18 cm IPG strips, pH 4-7 re-swelled with 400 µg. C. 800 µg of protein.

from a thin density immediately after subculturing, to a thick suspension of cells within 14 days. However, the measurement of cell growth by packed cell volume (PCV) has been rather difficult mainly because of two reasons. Firstly, sorghum cells occur in clusters of various sizes, making sampling even with a wide-bore pipette rather challenging. Secondly, although the cell clusters could be fairly distributed when agitated, they settled quickly resulting in inconsistencies in packed cell volume readings. So far therefore, we have not yet established a more reproducible growth curve of our sorghum cell suspension culture system.
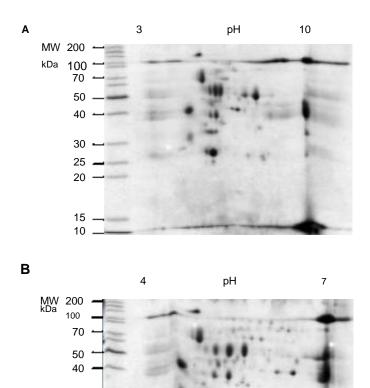
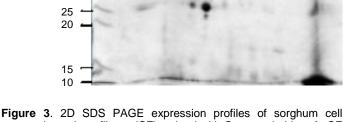**1D and 2D expression profiles of sorghum proteomes**

The sorghum cell suspension culture system established here is an invaluable system, which provides for an undifferentiated cell type that can be subjected to various experimental conditions. 1D gel analysis of total soluble protein (TSP) and culture filtrate (CF) showed that the two proteomes display different expression profiles (Figure 2A). The TSP exhibits numerous low abundant proteins while CF is composed of fewer but relatively higher abundant proteins. The isolated proteomes were separately subjected to 2D gel analysis on IPG strips of different lengths and pH ranges depending on the nature, composition and complexity of the protein mixture.

The TSP profile was better resolved on 18 cm strips, pH range of 4-7. Doubling TSP protein load on the IPG strips from 400 µg (Figure 2B) to 800 µg (Figure 2C) resulted in an increased intensity of Coomassie stained protein spots without causing any spot streaking due to protein overloading. Due to the less complex nature of sorghum CF fractions, mini gels (7 cm) were sufficient to resolve the whole CF proteome without any spot overlaps. Although the whole CF fraction could be resolved on the broad range IPG strips, pH 3 - 10 (Figure 3A), narrowing down the pH range to 4 - 7, resulted in an increase in spot resolution (Figure 3B). Protein spots, which were observed to be either closely migrating or co-migrating on IPG strips pH 3 - 10, became relatively well separated on IPG strips pH 4 - 7.

The observed differences in 2D proteome expression profiles of these fractions (TSP and CF) reinforces the fact that different proteomes, even if from the same plant system, are dynamic and heterogeneous, being composed of a complex mixture of proteins with different chemical and functional properties. The suspension culture system here described provides for two sets of proteomes, which although different in complexity play distinct but equally vital roles in maintaining biological processes in plant cells. Additionally, this experimental system demonstrates that we can separate hundreds of cellular soluble proteins and dozens of secreted proteins with high proficiency and efficiency.

Cell suspension culture systems have also been established for a wide range of plant species and applied

Figure 3. 2D SDS PAGE expression profiles of sorghum cell suspension culture filtrate (CF) stained with Coomassie blue. A. CF proteins resolved on 7 cm IPG strips pH 3 -10. B. pH 4 - 7. Each strip was passively re-swelled with urea- buffer containing 100 µg of protein.

in several proteomic-based studies (Slabas et al., 2004; Lei et al., 2005; Ndimba et al., 2005; Oh et al., 2005). There is no doubt therefore, that the culture system here described would provide the experimental material required for use in global proteomic studies based on both biotic and abiotic stress responses. The fractionation of culture systems into TSP and CF proteomes will help in the biochemical analysis of the separate biological processes that occur in the inter and intracellular matrix of plant cells.

The use of *Sorghum bicolor* suspension culture system, and high technology proteomics, provides a unique experimental model that will help in further understanding plant cell biology. Additionally, we intend to use this experimental system towards further understanding the molecular basis for sorghum's tolerance to drought. Future work will therefore involve further analysis of sorghum cell suspension culture proteomes by mapping and identifying as many expressed proteins as possible

via mass spectrometry. This exercise will provide invaluable reference maps of the sorghum soluble cellular proteome and secretome. We also intend to study stress responsive proteins, both in terms of their abundance and post-translational changes.

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