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Full Length Research Paper

Mapping and characterisation of the sorghum cell suspension culture secretome

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Here we reported the first secretomic study of sorghum (*Sorghum bicolor*), a naturally drought tolerant cereal crop. In this study, we used a gel-based proteomic approach in combination with mass spectrometry to separate and identify proteins secreted into the culture medium of sorghum cell suspensions, a first step towards understanding their functions during plant growth and development. Proteins secreted into the culture medium of 10-day old sorghum cell suspension cultures termed culture filtrate (CF) proteins were separated by two-dimensional gel electrophoresis (2DE) and visualised using Coomassie brilliant blue (CBB) R-250 staining. Of the 25 visualised CBB stainable spots, 15 abundant, well-resolved and reproducible spots amongst the three biological replicates used were selected for identification using matrix assisted laser desorption/ionisation-time of flight/time of flight tandem mass spectrometry (MALDI-TOF-TOF MS). Of these spots, 14 were positively identified, representing four different protein classes: Peroxidases, germin proteins, oxalate oxidases and α-galactosidases. All the identified proteins are known secretory proteins, with predicted signal peptides, which target proteins for the secretory pathway. The identified proteins have known functions in signalling processes, defence mechanisms and cell wall metabolism which is consistent with their location outside the cell. Western blotting analysis of the CF protein extracts using an antibody against β-tubulin, a cytoplasmic protein, indicated that our CF protein preparations are free from any detectable amounts of this marker protein. Therefore, our sorghum cell culture system is ideal for use in the proteomic analysis of secreted proteins. The findings of this study are a step in the process of bridging the gap that currently exists in sorghum proteomics and also provides a foundation for future studies on understanding the roles played by secreted proteins during plant growth and development of the same crop.

Key words: Sorghum, cell suspension cultures, culture filtrate, secreted proteins, proteomics analysis, two-dimensional gel electrophoresis, β-tubulin.

INTRODUCTION

Sorghum (*Sorghum bicolor*), a naturally drought-tolerant cereal crop (Rosenow et al., 1983) is an important food source for people living in semi arid regions as well as a source of animal feed and biofuel. Genome sequencing of this crop has recently been completed (Paterson et al., 2009), and is available on Phytozome v5.0 (http://www.phytozome.net/), making sorghum the second cereal to be fully sequenced after rice (*Oryza sativa*) (International rice genome sequencing project, 2005). Despite this sequencing milestone in sorghum, to date, only limited sorghum protein sequences are available on sequence databases that can be searched via the Matrix Science server (MASCOT). As such, the identification of sorghum proteins in proteomics studies still largely relies on homology based protein identification.

Complete plant genome sequences are indeed invaluable resource tools for both genomics and proteomics studies as they help to landmark expressed genes and/or proteins in biological systems (Lin et al., 2003). In addition, genome sequence information is useful for the identification of variations in gene sequences that can be

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used in various plant breeding strategies (Sasaki and Antonio, 2009). In proteomics studies, rice is currently considered to be a model plant amongst the grasses with an appreciable amount of research work being reported on this crop (Jorrin et al., 2007; Jorrin-Novo et al., 2009; Salekdeh and Komatsu, 2007). Although sorghum proteomics is still virtually unknown outside our research group (Ngara et al., 2008), this crop has immense potential as a model for studying the molecular mechanisms of drought tolerance in plants.

Proteomics approaches using two-dimensional gel electrophoresis (2DE) in combination with mass spectrometry (MS) are powerful tools for the separation, visualisation and identification of proteins expressed in biological systems under defined conditions. Over the years, plant proteomics (either descriptive and/or differential expressional proteomics) of specific tissues, organelles, cellular compartments as well as secreted proteins has been reported. Secreted proteins also referred to as the secretome (Greenbaum et al., 2001; Hathout, 2007), form an important component of the extracellular matrix (ECM) of living cells (Showalter, 1993). The secretome and other components of the ECM play vital roles in cell-cell interactions, growth and development, as well as defence mechanisms upon wounding or pathogenic attack (Brownlee, 2002; Knox, 1995; Pennell, 1998; Showalter, 1993). However, the actual composition and function of the ECM varies with plant species, cell types, developmental stages as well as responses to both abiotic and biotic stresses (Ye and Varner, 1991).

The study of the secretome also termed secretomics (Hathout, 2007) is generally thought to be challenging because of difficulties encountered during protein extraction and solubilisation, the presence of an insoluble polysaccharide matrix in which proteins are embedded and heavy glycosylation of the secreted proteins (Jamet et al., 2006). Despite these technical difficulties, secreted proteins of various plant species such as tobacco (*Nicotina tabacum*) (Dani et al., 2005; Okushima et al., 2000), rice (*O. sativa*) (Zhang et al., 2009), chickpea (*Cicer arietinum* L.) (Bhushan et al., 2007) or *Arabidopsis thaliana* (Charmont et al., 2005; Ndimba et al., 2005; Oh et al., 2005) among others have each been studied. In these studies, the apoplast of whole plants or culture media of cell suspension cultures were used as source of secreted proteins.

The extraction of secreted proteins from the apoplast of whole plants mainly relies on the vacuum infiltration method (Dani et al., 2005; Soares et al., 2007; Zhang et al., 2009). However, this method has a potential of causing cell damage, which results in the contamination of apoplastic fractions with cytoplasmic proteins (Haslam et al., 2003). In subcellular proteomics studies, high sample purity is of prime importance as it reduces the chances of identifying cross organelle protein contaminants in the proteome under study. Without high sample purity, protein contaminants would otherwise be erroneously identified in their non-native cellular compartments. Since protein function is related to cellular localisation (Kumar et al., 2002; van Wijk, 2004), the identification of protein contaminants in specific compartments/proteomes ultimately affects the precision of assigning putative functions to these contaminants. In secretomics, the level of cytosolic protein contaminants in the secreted protein fraction can be determined by enzyme assays or western blotting analysis of known cytoplasmic marker proteins (Oh et al., 2005; Zhang et al., 2009).

The culture media of cells in suspension offer an easily extractable source of secreted proteins. Cell suspension cultures are a homogenous group of undifferentiated cells that are grown in liquid culture (Evans et al., 2003) and provide a large amount of biomass for proteomics studies (Slabas et al., 2004). In this study, we reported on the proteomic analysis of proteins secreted into the culture medium of sorghum cell suspension cultures. Proteins secreted into the culture medium of 10-day old sorghum cell suspension cultures were collected by filtration and acetone precipitation techniques. Using a combination of 2DE and matrix assisted laser desorption/ionisation-time of flight/time of flight tandem mass spectrometry (MALDI-TOF-TOF MS), we identified all the abundant and well-resolved secreted proteins, which represented four unique proteins classes, namely: Peroxidases, germin proteins, oxalate oxidases and α-galactosidases.

MATERIALS AND METHODS

Plant material

White sorghum (*S. bicolor*) seeds purchased from Agricol, Brackenfell, South Africa were used to generate sorghum cell suspension cultures as previously described (Ngara et al., 2008). The cell cultures were maintained by subculturing 40 ml of 14-day old cells into 60 ml of fresh Murashige and Skoog (MS) growth medium (Murashige and Skoog, 1962) [4.4 g/l Murashige and Skoog basal salt with minimal organics (MSMO) medium supplemented with 3% (w/v) sucrose, 3 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.5 mg/l 1-naphthaleneacetic acid (NAA), pH 5.8]. The cells were incubated in the dark at 25°C with agitation at 130 rpm.

Measurement of growth parameters of the sorghum cell suspension culture system

Growth curve measurements

The growth of sorghum cell suspension cultures was estimated using the settled cell volume (SCV) method. Established cell cultures were subcultured into fresh cell suspension culture medium as described earlier and SCV estimates were taken for three independently established cell cultures. At each sampling time point, the flask was gently shaken and 1 ml aliquot of the cell culture was pipetted into a graduated 1.5 ml microcentrifuge tube. The tubes were left to stand for 5 min after which the volume of the settled cells was estimated. Settled cell volume was presented as the percentage of the volume of settled cells over the sample aliquot. The SCV readings were taken at two-day intervals starting from day of subculture until day 18 when the suspension cultures became too dense for consistent and reproducible sampling using a

1 ml pipette.

Estimation of cell viability using the Evans blue test

The viability of the sorghum cell suspension cultures was determined using Evans blue as previously described (Baker and Mock, 1994) with minor modifications. Cell viability was estimated for three independently established cell cultures over a 20-day culture period. Twenty milligrams of suspension cells (wet wt. after draining excess medium) was mixed with 180 µl of distilled water and 20 µl of 0.5% (w/v) Evans blue solution, and incubated for 15 min with gentle shaking at room temperature. After incubation, cells were centri-fuged at 2300 *g* for 5 min. The supernatant was discarded and the cell pellet was washed x3 in 1 ml of distilled water by centrifugation at 2300 *g* for 5 min per wash. The cells were ground in 1.2 ml of 1% (w/v) sodium dodecylsulfate (SDS) in 50% (v/v) methanol using a plastic pestle and incubated at 50°C for 6 h. After incubation, the homogenate was centrifuged at 2300 *g* for 5 min and the released Evans blue stain was collected in the supernatant fraction. The optical density (OD) of the released stain was measured spectro-photometrically at 600 nm on a Milton Roy Spectronic GENESYS 5 Spectrophotometer (Spectronic Analytical Instruments, Leeds, UK) using 1% (w/v) SDS in 50% (v/v) methanol as a blank solution. To determine total cell death (0% viability), sorghum suspension cultured cells were deliberately killed by heating at 95°C for 10 min on a dry block heater (FMH Instruments). The deliberately killed cells were stained with Evans blue and absorbed stain was quantified in parallel with unboiled cells as described earlier. Cell viability was estimated as shown below:

$$\text{Cell viability (\%)} = \frac{A_B - A_{UB}}{A_B} \times 100$$

Where, A_B is the absorbance of stain released from boiled cells; A_{UB} is the absorbance of stain released from unboiled cells.

pH and conductivity measurements of the culture medium

The pH and conductivity of culture medium of sorghum cell suspension cultures were simultaneously measured using a Portable Multimeter MM40 (Crison Instruments, SA, Allella, Barcelona, Spain). The measurements were taken for three independently established sorghum cell suspension culture lines at each sampling time point over a 20-day culture period.

Protein extraction from the sorghum culture filtrate

The culture medium of 10-day old sorghum cell suspension culture was used as a source of secreted culture filtrate (CF) protein in this study. Protein extractions were carried out from at least three independently established cell cultures. The CF proteins were prepared from approximately 30 ml of sorghum cell suspension cultures by filtration over four layers of Miracloth (Merck, Darmstadt, Germany) and acetone precipitation techniques as previously described (Ngara et al., 2008). The protein precipitate pellet was washed x3 using ice-cold 80% (v/v) acetone by centrifuging at 15,700 *g* for 10 min per wash and air dried for 5 min at room temperature. The CF protein precipitate was then re-suspended in approximately 2 ml urea buffer {9 M urea, 2 M thiourea and 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)} for at least 15 h with vigorous vortexing at room temperature. The solubilised protein mixture was centrifuged at 15,700 *g* for 10 min and the supernatant containing CF soluble protein was collected and stored at -20°C until use in western blotting or 2DE procedures. Protein extracts were quantified using a modified Bradford assay (Bradford, 1976) as previously described (Ndimba et al., 2003).

Assessing the level of cytoplasmic contaminants in the culture filtrate protein extracts

The level of cytoplasmic contaminants in the CF protein extracts was assessed by western blotting analysis using antibodies against two cytosolic proteins, β-tubulin (Zhang et al., 2009) and heat shock protein 70 (Hsp70) (Cho et al., 2009). The CF protein extracts were prepared as described earlier, while the total soluble proteins (TSP) were prepared as previously described (Ngara et al., 2008). Protein extracts were separated on 1D SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and transferred onto polyvinylidene difluoride (PVDF) membrane (Hybond-P PVDF membrane; GE Healthcare, Amersham, UK) as previously described (Towbin et al., 1979). Electrophoretic transfer of proteins was performed using a Mini Trans-Blot® Electrophoresis Transfer Cell (Bio-Rad, Hercules, CA, USA) at 36 V, overnight at 4°C with cooling using the Bio-Ice Cooling unit (Bio-Rad). After protein transfer, the membrane was washed once in tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl, pH 7.5) for 10 min before blocking in blocking solution [1% (w/v) Elite fat free instant milk powder in TBS] for 1 h. The membrane was then incubated with the specific primary antibodies, anti-β-tubulin, (monoclonal anti-β-tubulin produced in mouse; Sigma-Aldrich, St. Louis, USA) diluted 1:2000 or anti-Hsp70 (human HeLa cells anti-Hsp70/Hsc70 monoclonal antibody raised in mouse; Stressgen Bioreagents Corp., Victoria, Canada) diluted 1:2500 in 0.5% (w/v) blocking solution for 1 h. The membrane was then washed x3 with TBST [TBS containing 0.1% (v/v) Tween 20] for 10 min per wash and once with 0.5% (v/v) blocking solution for another 10 min. Thereafter, the membrane was incubated with the secondary antibody [goat anti-mouse IgG (H and L) horseradish peroxidase conjugate; Invitrogen Corp., Carlsbad, CA, USA] diluted 1:1000 in 0.5% (w/v) blocking solution for 1 h. After the secondary antibody incubation, the membrane was washed x3 in TBST for 15 min per wash. The β-tubulin and heat shock proteins were detected using a SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL, USA) according to the manufacturer's instructions. The Amersham Hyperfilm ECL (GE Healthcare) was exposed and automatically developed using the Curix 60 (Agfa- Gevaert, N.V., Mortsel, Belgium).

Two-dimensional gel electrophoresis of culture filtrate proteins

Two-dimensional gel electrophoresis of the sorghum CF protein extracts was performed using 7 cm, ReadyStrip™ immobilised pH gradient (IPG) strips of linear pH of range 3 - 10 and 4 - 7 (Bio-Rad). Three biological replicate gels were run for each of the two pH range IPG strips used. Culture filtrate proteins were separated by 2DE following the procedure previously described (Ngara et al., 2008). Protein extracts (100 µg) were mixed with 0.8% (v/v) dithiothreitol (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 samples were used to passively rehydrate 7 cm strips of pH range 3 - 10 or range 4 - 7 (Bio-Rad) in an Immobiline™ Dry Strip Reswelling Tray (GE Healthcare) overnight at room temperature. Isoelectric focussing (IEF) of the strips was performed on an Ettan™ IPGphor II™ (GE Healthcare) using a stepwise programme starting at 250 V for 15 min, followed by 4000 V for 1 h

and lastly, 4000 V until it reached 12,000 Vh. After IEF, the IPG strips were each equilibrated with 2.5 ml of equilibration buffer [6 M urea, 2% (w/v) SDS, 50 mM Tris/HCl, pH 8.8 and 20% (v/v) glycerol], firstly containing 2% (w/v) DTT for 15 min followed by 2.5% (w/v) iodoacetamide for another 15 min with gentle agitation at room temperature. Equilibrated 7 cm IPG strips were placed on top of 10.1 x 8.3 x 0.1 cm, 12% SDS polyacrylamide gels and electrophoresed using the Mini-PROTEAN® 3 Dodeca™ cell (Bio-Rad) at 100 V for the first 30 min and then at 150 V until the bromophenol blue dye reached the bottom of the gel plates. The 2DE gels were stained with Coomassie brilliant blue (CBB) R-250 and imaged using a Molecular Imager PharosFX Plus System (Bio-Rad). Only the highly abundant, well-resolved and reproducible protein spots across all three biological replicates were selected for identification by MALDI-TOF-TOF MS.

Protein identification using MALDI-TOF-TOF MS

Tryptic digestion of protein spots was performed using a ProGest robot (Genonic Solutions, Huntingdon, Cambridgeshire, UK) programmed with the long trypsin digestion method. Protein spots were picked from the CBB R-250 stained gels using an ExQuest™ spot cutter (Bio-Rad) and transferred to the wells of a 96 well microtitre plate. This plate is designed with microscopic holes at the bottom of the wells, which allow for positive displacement of liquids during reagent changes on the robot. Briefly, the gel pieces were first equilibrated in 50 µl of 50 mM ammonium bicarbonate and proteins were reductively alkylated with 10 mM DTT and 100 mM iodoacetamide. The protein spots were then destained and gel plugs desiccated with acetonitrile. Gel plugs were rehydrated with 50 mM ammonium bicarbonate containing 5% (w/v) trypsin and the proteins were digested for 12 h at 37°C. Resulting peptides were extracted from the gel plugs twice with 25 µl washes of 50% acetonitrile and 0.1% (v/v) trifluoroacetic acid (TFA). Peptide extracts were freeze-dried and then re-suspended in 10 µl of 0.1% (v/v) formic acid. A saturated solution of α-cyano-4-hydroxy-cinnamic acid (matrix) was prepared in 50% acetonitrile, 0.1% (v/v) TFA, 10 mM ammonium acetate. For each sample, 1 µl of the matrix solution was spotted on the MALDI target, immediately followed by 1 µl of protein sample into the matrix spot. The sample/matrix droplet was allowed to slowly air dry. Matrix-assisted laser desorption/ionization time of flight-time of flight analyses were carried out on a 4800 proteomic analyser (Applied Biosystems, Boston, MA, USA). Firstly, MALDI-TOF MS analysis was performed on all the target spots using automated data acquisition and processing under the control of Applied Biosystems 4000 series Explorer software (version 3.5) using reflector mode, a mass range of 700 - 4000 m/z, 1000 total laser shots per spectrum and a laser intensity of 3300 V. Following acquisition, the TOF-MS spectra were noise corrected, peak de-isotoped and internally calibrated using the trypsin autolysis peaks 842.500 and 2211.100 m/z. The ten most abundant precursor ions from each spectrum were then selected by the software for fragmentation and MS-MS analysis using a 1 kV collision-induced dissociation fragmentation method collecting 4000 laser shots per spectra with a laser intensity of 3800 V over the mass range. Peak lists of ion masses were generated by GPS Explorer software version 3.6 (Applied Biosystems) from the calibrated and de-isotoped MS and MS-MS spectra for each sample. Combined lists of the MS and MS-MS data were used for database searching with MASCOT version 2.2 against all entries in the National Center for Biotechnology Information (NCBI) database. Database search parameters used were as follows: Digestion enzyme trypsin, single missed cleavage allowed, variable modification of carboxymethyl cysteine and oxidised methionine, precursor mass tolerance of 50 ppm and fragmentation ion tolerance of 0.2 Da. The MASCOT outputs were generated for each sample.

Bioinformatic analysis

Sorghum homologs of all the NCBI protein accessions (Table 1) were searched using the BLASTp programme against the *S. bicolor* proteome found on the Phytozome v5.0 database (http://www.phytozome.net/). The presence and location of cleavable N-terminal signal peptide domain was predicted using the SignalP 3.0 prediction server (http://www.cbs.dtu.dk/services/SignalP/) (Emanuelsson et al., 2007) as well as literature sources.

RESULTS AND DISCUSSION

Growth characteristics of sorghum cell suspension cultures

The growth of sorghum cell suspensions over time in culture was estimated using the SCV method as described earlier. A typical sigmoidal shaped growth curve with three distinct phases: lag, exponential and stationary phases were observed (Figure 1A). The lag phase, which begins on the day of subculture until about day 8 is characterised by a very slow increase in SCV. After day 8, the SCV increases exponentially until about day 14 thus representing the exponential phase. Thereafter, the cells go through a stationary phase where the rate of cell growth and/or multiplication is slower.

The viability of sorghum cells was also estimated throughout the growth cycle using the Evans blue staining method as described earlier. Evans blue is selectively excluded from the interior of live cells, but penetrates through damaged membranes of dead cells (Baker and Mock, 1994). As such, this stain is routinely used to assess cell death and/or membrane integrity (Baker and Mock, 1994; Chivasa et al., 2005), the inverse of which is regarded as cell viability in the present study. Using the Evans blue test, sorghum cell suspension cultures were observed to maintain cell viability of over 60% throughout the culture period (Figure 1B).

The pH of the culture medium of sorghum suspension cells was also monitored in order to gain insight on the range of this parameter during the cell growth cycle (Figure 2A). We observed that after 3 days in culture, the pH of the medium drastically decreased from pH 5.0 to 3.8. Thereafter, the pH gradually increased to pH 4.7 on day 12 and was maintained at that level until the end of the culture period. The pH of a cell suspension culture medium changes due to oxidation as well as the uptake and secretion of substances by the growing cells (Beyl, 1999). The observed drop in pH at the beginning of the culture cycle (Figure 2A) is known to occur in plant cell suspension cultures (Evans et al., 2003) and had been observed between day 0 and 4 in the growth cycle of *Psychotria carthagenensis* (Lopes et al., 2000). This possibly indicates a time during which cells adapt to their new culture environment, differentially absorbing nutrients

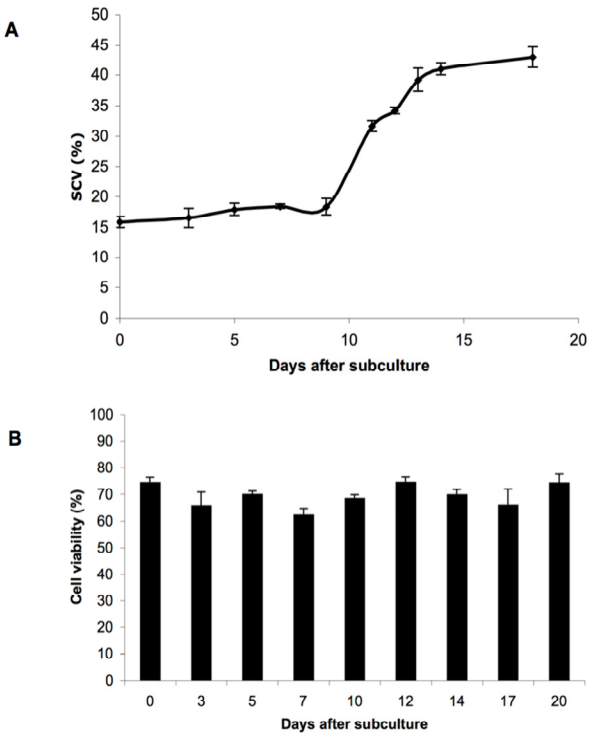

Figure 1: Growth parameters of a sorghum cell suspension culture system over 20 days in culture. (A) Growth curve measurements estimated using the settled cell volume (SCV) method and (B) cell viability measurements (%) estimated using the Evans blue test. Both growth parameters were estimated from three independently established sorghum cell cultures. Vertical bars indicate standard deviation.

from the medium and/or secreting other substances into the culture medium. For example, the drastic drop in pH in the early days of culture may indicaten proton production during metabolic activities in the cell and their transportation into the ECM. In addition, cells may rapidly absorb nutrients such as phosphates, which act as buffering substances in the culture medium (Evans et al., 2003).

Electrical conductivity (ECe), a measure of the amount of soluble salts in a solution (Flowers and Yeo, 1986; Hale and Orcutt, 1987) was also determined during the cell growth cycle. In our sorghum cell suspension culture system, the conductivity of the medium gradually declined from the day of subculture till the end of the culture period (Figure 2B). The conductivity of the cell culture medium decreases with time in culture indicating a depletion of nutrients from the medium and their subsequent utilization by the growing cells (Evans et al., 2003).

Confirming the lack of cytoplasmic contaminants in the culture filtrate protein extracts

Our sorghum cell suspension culture system contains unsynchronised cells at different stages within the growth cycle as evidenced by changes in cell viability readings throughout the culture period (Figure 1B). For this reason, CF protein extracts may have invariable amounts of cytoplasmic contaminants from cytoplasmic leak-ages of dead cells with compromised membrane integrity. To assess the presence of such contaminants in our sorghum CF protein extracts, we performed western blotting analysis against two cytoplasmic proteins, β-tubulin (Zhang et al., 2009) and Hsp70 (Cho et al., 2009). Our results show that none of our independent CF protein extracts contained detectable amounts of either β-tubulin (Figure 3) or Hsp70 (results not shown), while these marker proteins were detected in the TSP fractions.

These observations indicate that the CF proteins extracts are free of cytoplasmic contaminants and therefore validates the use of our cell culture system in secretome analysis.

Protein separation and identification by 2DE and MALDI-TOF-TOF MS

Sorghum CF proteins extracts were separated by 2DE and visualised after CBB R-250 staining (Figure 4). Approximately, 25 CBB stainable protein spots with varying degrees of abundance were visualised on 2DE gels. The molecular weights (Mr) of the CF proteins ranged between 25 and 100 kDa, the majority of which are acidic, having isoelectric points (pI) between the range of 4 and 6. The dominance of acidic proteins in the secreted proteome fractions of plants has been reported in other proteomic studies. About 50% of proteins secreted into the culture medium of A. thaliana seedlings had acidic pI (Charmont et al., 2005). Furthermore, Jamet et al. (2006) reported that the majority of secreted proteins found in the culture medium of cell suspensions or hydroponically grown seedlings are acidic, with isoelectric point ranges between 2 and 6. These reports are in agreement with observations in our current study.
Of the observed CBB stained proteins, a total of 15 highly abundant, well-resolved and reproducible spots (numbered protein spots Figure 4) were selected for identification by MALDI-TOF-TOF MS. Of these 15 trypsinised protein spots, 14 were positively identified, matching protein identities from other grasses such as rice (O. sativa), maize (Zea mays) and barley (Hordeum vulgare) (significance threshold of $p < 0.05$) (Table 1). Although the sorghum genome has been fully sequenced (Paterson et al., 2009) and is available on the Phytozome v5.0 (http://www.phytozome.net/), there is still limited sorghum sequence data on databases that can be search via the mass spectrometry data search engine MASCOT (http://matrixscience.com/). For this reason, using sequence data on NCBI, protein identification in this study was mainly based on the homology between sorghum and other grasses. Despite this technical drawback, the use of a MALDI-TOF-TOF mass spectro-meter in our experimental procedure, which allows for the

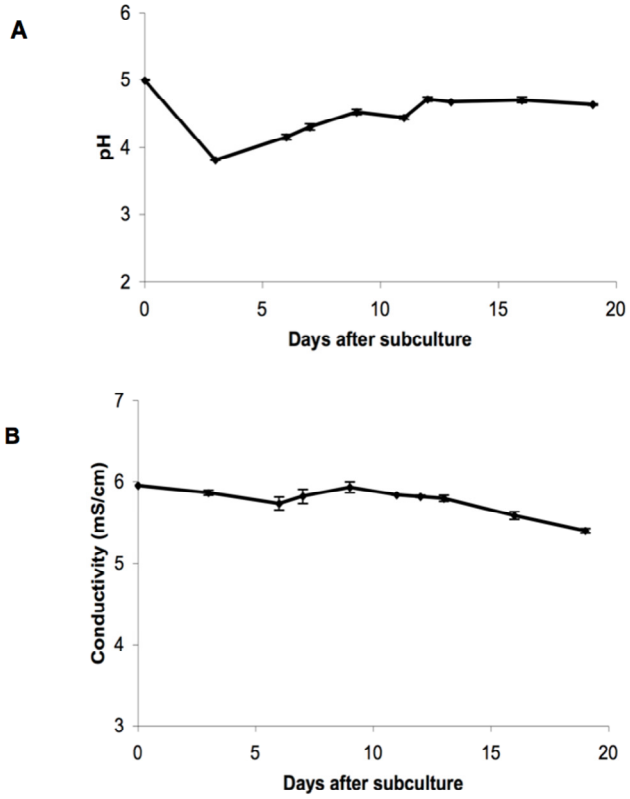

Figure 2: Changes in (A) pH and (B) conductivity measurements of a sorghum cell suspension culture system over 20 days in culture. The measurements were taken from three independently established sorghum cell cultures. Vertical bars indicate standard deviation.

generation of amino acid sequences of peptides, resulted in a 93% protein identification success rate with high confidence (Table 1). Nevertheless, the observed difference between the theoretical and experi-mental M_r and pI values of the respective proteins (Table 1) further indicates the probable species to species variations in the amino acid sequences of these proteins.

The protein identities represented four different classes of proteins, namely: Peroxidases (spots 1 - 7), α-galactosidases (spots 8 - 9), oxalate oxidases (spots 11 - 12) and germin proteins (spots 13 - 15), while spot 10 remained unidentified after database searching (Table 1). It was observed that all protein classes were represented in multiple spots, which differed in M_r, pI or both, anobservation that has also been reported in other studies (Chivasa et al., 2002; Ndimba et al., 2005; Oh etal., 2005). The occurrence of multiple spots for a single protein may be due to post translational modifications, the presence of dimeric and monomeric forms of a protein on the same gel, the presence of proteolytic degradation by endogenous proteases, the presence of different protein isoforms from multigene families and/or the chemical modification of proteins during sample preparation (Albertin et al., 2009).

To establish if the NCBI protein accessions (Table 1) have published homologs in sorghum, we performed Basic Local Alignment Search Tool (BLAST)p searches against the *S. bicolor* proteome found on the Phytozome v5.0 database. For all searches, query sequences were amino acid sequences of the respective NCBI accessions given in Table 1. The BLASTp results showed that all 14 proteins have sorghum homologs (Phytozome gene accessions; Table 1). With the exception of spots 11 and 12 (NCBI protein accession: XP_469351), which matched a putative uncharacterised protein (Phytozome gene accession: Sb01g011370.1), all the other 12 proteins matched similar protein names in both databases.

Identification of classical secretory proteins in the culture filtrate

Generally, classical extracellular matrix proteins have a signal peptide at their N-termini, which targets them for the secretory pathway via the endoplasmic reticulum in eukaryotes (Nielsen et al., 1997). The N-terminal signal peptide is approximately 15 - 30 amino acids long and is cleaved off during protein translocation across the membrane. Signal peptides are composed of three structural regions: a positively charged N-terminal region (n-region), followed by a hydrophobic region (h-region) in the middle and a polar c-region at the C-terminal end. For cleavage to occur correctly, the amino acid residues at positions -3 and -1 relative to the cleavage sites, should be small and neutral, and are highly conserved among proteins (Emanuelsson et al., 2007; Guo et al., 2008; Nielsen et al., 1997). Since the signal peptide is cleaved off during protein secretion, its presence in the pre-protein can be predicted by bioinformatics tools (Jamet et al., 2006).

To verify if the 14 positively identified sorghum CF proteins (Table 1) possess features of proteins destined for the ECM, we used the SignalP 3.0 prediction server (http://www.cbs.dtu.dk/services/SignalP/) to investigate for the presence of a predicted cleavable N-terminal signal peptide domain (Bendtsen et al., 2004; Guo et al., 2008). With the exception of two proteins, α-galacto-sidase (spots 8 and 9) and germin E (spots 13 and 14), the predictions on the presence and location of signal peptide cleavage sites of all the other CF proteins were carried out using full protein sequences obtained from the NCBI database. The signal peptide prediction results are summarised in Table 2.

The amino acid sequences of protein spots 8 and 9 matched a fragment of α-galactosidase (NCBI protein accession: 1UAS_A; Chain A, crystal structure of rice α-galactosidase; Table 1). Since the published protein sequence of 1UAS_A is a partial sequence (Fujimoto et al., 2003) of the α galactosidase pro-protein (Kim et al., 2002), the sequence could not be used for the signal peptide prediction analysis. For this reason, protein

Table 1. Identification of sorghum culture filtrate proteins using MALDI-TOF-TOF mass spectrometry.

Spot[a]	Best Match Protein	NCBI Accession[b]	Species	MOWSE score[c]	Theor.[d] M_r/*pI*	Exp.[e] M_r/*pI*	Matching peptides[f]	Expect value	Sequences of MS and MS/MS matched peptides	Phytozome Accession[g]
1	Putative peroxidase	NP_908708	*O. sativa*	84	34.6/5.3	60.0/5.1	3	0.01	RGFQVIDAAKA RLHFHDCFVRG KAAVEQSCARTVSCADIVAFAA RD	Sb09g004650.1
2	Peroxidase	AAS75402	*Z. mays*	210	36.1/5.7	50.0/5.3	6	2.4e-015	KAYAFLLKS KALVDSFVRS KDAPPNNPSLRF KYYVGLTNNLGLFKS KMGQIEVLTGTQGEIRR RDSVVLSGGLGYQVPAGRR	Sb09g004660.1
3	Peroxidase	AAS75402	*Z. mays*	205	36.1/5.7	50.0/5.5	6	7.5e-015	KAYAFLLKS KALVDSFVRS KDAPPNNPSLRF KYYVGLTNNLGLFKS KMGQIEVLTGTQGEIRR RDSVVLSGGLGYQVPAGRR	Sb09g004660.1
4	Peroxidase	AAS75402	*Z. mays*	350	36.1/5.7	50.0/5.8	8	2.4e-029	KAYAFLLKS KALVDSFVRS KDAPPNNPSLRF RMHFHDCFVRG KYYVGLTNNLGLFKS KALVDSFVRSEATFRT KMGQIEVLTGTQGEIRR RDSVVLSGGLGYQVPAGRR	Sb09g004660.1
5	Peroxidase	AAC49818	*O. sativa*	113	30.5/6.1	39.0/4.6	2	1.2e-005	RDSVVALGGPSWTVLLGRR RKGLDATDMVALSGAHTIGQAQ CQNFRD	Sb02g042850.1
6	Putative peroxidase	NP_908705	*O. sativa*	77	35.2/6.0	36.0/5.3	3	0.043	RGFEVIDAAKA RGCDASVLIFSPNGTAERD MASCSKWLAGLMLLAAALACSL PAASRA	Sb03g013210.1

Table 1. Contd.

Spot[a]	Best Match Protein	NCBI Accession[b]	Species	MOWSE score[c]	Theor.[d] M_r/*pI*	Exp.[e] M_r/*pI*	Matching peptides[f]	Expect value	Sequences of MS and MS/MS matched peptides	Phytozome Accession[g]
7	Putative peroxidase	NP_908705	*O. sativa*	79	35.2/6.0	36.0/5.3	4	0.033	RGFEVIDAAKA KVGFYNKTCPSAERL RGCDASVLIDGNDTEKT RLHFHDCFVRGCDASVLIDGNDTEKT	Sb03g013210.1
8	α-Galactosidase	1UAS_A	*O. sativa*	80	40.0/5.9	40.0/6.1	6	0.025	FENGLGRT KAVVLWNRQ RKAVVLWNRQ KALADYVHAKG RSHFSIWALAKA KMPGSLDHEEQDVKT	Sb01g018400.1
9	α-Galactosidase	1UAS_A	*O. sativa*	86	40.0/5.9	40.0/6.3	6	0.0054	FENGLGRT KAVVLWNRQ RKAVVLWNRQ KALADYVHAKG RSHFSIWALAKA KMPGSLDHEEQDVKT	Sb01g018400.1
10	No Match	-	-	-	-	50/8.8	-	-	-	-
11	Putative oxalate oxidase	XP_469351	*O. sativa*	192	20.9/5.6	26.5/6.4	3	1.5e-013	RAGETFVIPRG RGLMHFQFNVGKT RVDFAPGGTNPPHVHPRA	Sb01g011370.1
12	Putative oxalate oxidase	XP_469351	*O. sativa*	197	20.9/5.6	26.5/6.8	3	4.7e-014	RAGETFVIPRG RGLMHFQFNVGKT RVDFAPGGTNPPHVHPRA	Sb01g011370.1
13	Germin E	AAG00429	*H. vulgare*	79	20.3/7.7	26.5/7.8	3	0.029	KGDVFVFPKA KKGDVFVFPKA RATELLTVLEGTLYLGFVTSNPNRL	Sb06g001690.1

Spot[a]	Best Match Protein	NCBI Accession b	*Species*	MOWSE scorec	Theor.d Mr/pI	Exp.e Mr/pI	Matching peptides f	Expect value	Sequences of MS and MS/MS matched peptides	Phytozome Accessiong
14	Germin E	AAG00429	*H. vulgare*	108	20.3/7.7	26.5/8.5	5	3.8e-005	KGDVFVFPKA KKGDVFVFPKA RIDYGPLGVNTPHIHPRA RATELLTVLEGTLYLGFVTSNPNRL RVNGFPCKDPMAVNTEDFFNPAMLD QPRD	Sb06g001690.1
15	Germin protein type 1	XP_480453	*O. sativa*	104	22.2/8.2	26.5/9.2	2	9.4e-005	KAFQVEKKV RIDYAPLGVNPPHIHPRA	Sb06g001690.1

[a]Spot number as indicated on the 2DE gel images (Figure 4); [b]National Center for Biotechnology Information (NCBI) protein accession numbers: http://www.ncbi.nlm.nih.gov ; [c]combined MOWSE score from MALDI-TOF and MALDI-TOF-TOF MS analyses; [d]Theoretical molecular weight (M_r) and isoelectric points (pI) were calculated on the mature peptide using the programme on http://isoelectric.ovh.org; [e]Experimental M_r and pI were estimated from the 2DE gels (Figure 4); [f]Number of matching peptides analysed by MALDI-TOF-TOF MS; [g]Phytozome (http://www.phytozome.net/) gene accession numbers of *S. bicolor* homologs of the NCBI protein accessions after BLASTp searches.

BLAST searches using the NCBI BLASTp 2.2 program-me were carried out to obtain a full sequence of the rice α-galactosidase. The protein with the highest similarity to IUAS_A on the BLASTp search results was of a 417 amino acid long, full α-galactosidase protein (Swiss-prot protein accession: Q9FXT4). These two proteins showed a 100% sequence alignment homology between the entire 362 amino acid sequence of the NCBI protein accession: 1 UAS_A and the region between 56 - 417 amino acids of the Swiss-prot protein accession: Q9FXT4. The latter region constitutes the 362-amino acid mature α-galactosidase, while the 1 - 55 amino acid region is the signal sequence (Kim et al., 2002). Consequently, signal peptide predictions of spots 8 and 9 were carried out using the full protein sequence of α-galactosidase (Swiss-prot protein: Q9FXT4). Using this sequence, a cleavable signal peptide was predicted on the rice α-galactosidase protein with the most probable cleavage site being positioned between amino acid 33 and 34: AAA-LG (Table 2).

Using the SignalP prediction server, no signal peptides were predicted for the two germin E proteins (spots 13 and 14), possibly because the published protein sequence on NCBI is a partial sequence of the protein. However, the same protein sequence was published as a known apoplastic protein (Wu et al., 2000). For this reason, we considered the germin E proteins to be *bona fide* secretory proteins in sorghum, as observed in other plant systems.

Putative functional characterisation of the identified proteins

Peroxidases

Peroxidases formed the largest group of proteins identified in the secretome of our sorghum cell suspen-sion cultures system (spots 1-7; Figure 4; Table 1). Extracellular peroxidases have also been identified in other proteomics studies, for example, they were shown to be secreted by tobacco BY-2 cell suspension cultures (Okushima et al., 2000), *A. thaliana* seedlings (Charmont et al., 2005) and *A. thaliana* cell suspension cultures in response to fungal elicitors (Ndimba et al., 2003). In plants, extracellular peroxidases are bound to cell wall polymers by ionic or covalent bonds (Minibaeva and Gordon, 2003) and can be released into the apoplastic fluids of plant cells or secreted into the culture medium of cell suspension cultures or hydroponically grown seed-lings. Therefore, this study shows that these enzymes are *bona fide* residents of the sorghum ECM.

Furthermore, peroxidases are heme-containing glyco-proteins, encoded for by a large multigene family and thus exist as isoenzymes in individual plant species. The isoenzymes have variable amino acid sequences and show a diverse expression profile (Hiraga et al., 2001). The well-documented existence of a diverse population of peroxidase isoenzymes in literature further reinforces our observation that of the 14 identified CF proteins, 50% were peroxidases with a diverse M_r and pI range. However, these observations would have to be verified on whether they are physiological or just mere cell suspension culture artefacts.

Functionally, peroxidases are known to catalyse the oxidoreduction between H_2O_2 and a variety of

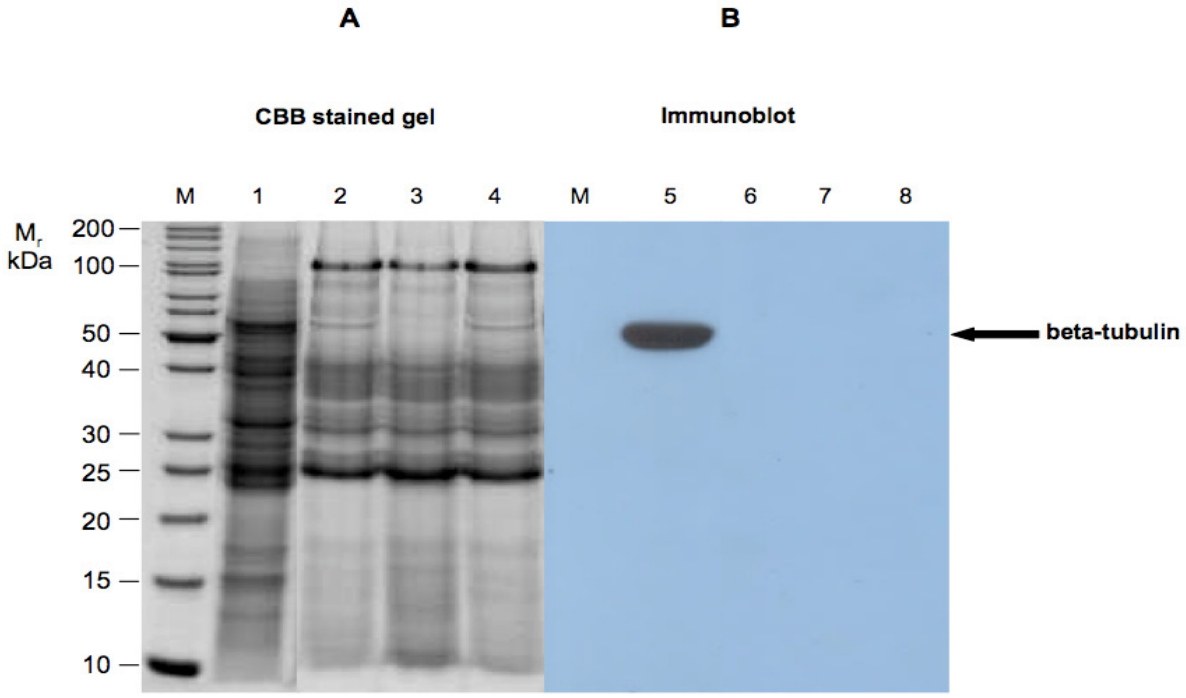

Figure 3: Western blotting analysis and evaluation of the level of cytoplasmic protein contaminants in sorghum culture filtrate (CF) protein extracts. Total soluble protein (TSP; Lane 1) and proteins secreted into the culture medium of 10-day old sorghum cell suspension cultures (CF proteins; Lanes 2 - 4) were extracted, separated on 12% SDS-PAGE gels (protein loading of approximately 20 µg per well) and either (A) stained with Coomassie brilliant blue R-250 or (B) transferred onto polyvinylidene difluoride membrane before probing with anti-β-tubulin monoclonal antibody, diluted 1:2000. β-Tubulin was only detected in the TSP fraction (Lane 5) at approximately 55 kDa, while all three biological replicates of CF protein extracts (Lanes 6 - 8) were free of this cytoplasmic marker protein. Lane M is the molecular weight marker protein in kDa.

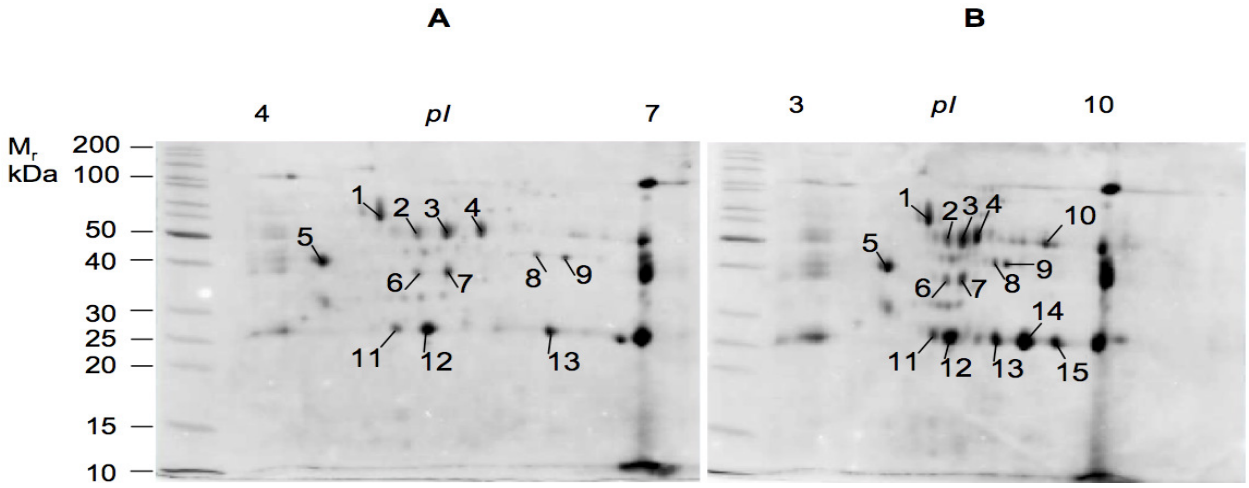

Figure 4. Representative Coomassie brilliant blue R-250 stained 2-dimensional gels of proteins secreted into the culture medium of 10-day old sorghum cell suspension cultures. Approximately, 100 µg of the culture filtrate proteins were each separated by isoelectric focusing on 7 cm linear immobilised pH gradient strips of pH range (A) 4 - 7 and (B) 3 - 10 in the first dimension. In the second dimension, proteins were separated on 12% SDS-PAGE gels. The most abundant, well-resolved and reproducible protein spots were numbered and identified using MALDI-TOF-TOF MS. The experimental molecular weight (M_r) in kDa and isoelectric points (pI) of the proteins were estimated from the gels and tabulated in Table 1.

Table 2. Signal peptide predictions of sorghum culture filtrate proteins using the SignalP prediction server.

Protein class	Spot[a]	NCBI accession[b]	Signal peptide[c]	Cleavage site[d]
Peroxidases	1	NP_908708	1-27 (0.999)	27 and 28:SRG-QL
	2, 3 and 4	AAS75402	1-21 (0.983)	21 and 22:ATA-AC
	5	AAC49818	1-25 (1.000)	25 and 26:ASA-QL
	6 and 7	NP_908705	1-19 (1.000)	19 and 20:ALA-CS
α-Galactosidases	8 and 9	1UAS_A	1-33 (0.998)*	33 and 34:AAA-LG
Oxalate oxidases	11 and 12	XP_469351	1-29 (1.000)	29 and 30:TDA-DP
Germin proteins	13 and 14	AAG00429	-	-
	15	XP_480453	1-23 (1.000)	23 and 24:AIA-FD

[a]Spot number as indicated on the 2DE gel images (Figure 4); [b]National Center for Biotechnology Information (NCBI) protein accession number: http://www.ncbi.nlm.nih.gov; [c]Length of signal peptide predicted using the SignalP 3.0 prediction server (http://www.cbs.dtu.dk/services/SignalP/). Both the neutral networks (NNs) and the hidden Markov models (HMMs) were used (Emanuelsson et al., 2007). Numbers in brackets indicate the probability of having a signal peptide; [d]most likely cleavage site position and corresponding amino acid sequence; *signal peptide predicted on the full protein sequence Swiss-prot accession: Q9FXT4; - No signal peptide predicted using SignalP because the published amino acid sequence of NCBI protein accession: AAG00429 is incomplete. However, the same protein is a known apoplastic protein (Wu et al., 2000).

organic and inorganic substances (Chittoor et al., 1997; Hiraga et al., 2001). Plant peroxidases are thus involved in a number of normal physiological functions including cell wall metabolism (lignification and suberization), auxin metabolism and defence mechanisms against wounding and pathogenic attack (Hiraga et al., 2001).

Germins and oxalate oxidases

Germins and oxalate oxidases are discussed together because of their common functional features. Collectively, these two classes of proteins form the second largest protein group identified (36%) in the current study with a total of three (germins; spots 13 - 15; Figure 4 and Table 1) and two (oxalate oxidases; spots 11 - 12; Figure 4 and Table 1) isoforms of each protein type, respectively (Table 1). Various isoforms of germin proteins have also been identified in both the apoplastic fluids of *A. thaliana* rosettes (Boudart et al., 2005) and secreted proteins from *A. thaliana* seedlings grown in liquid culture (Charmont et al., 2005). Furthermore, a total of four germin-like proteins were found to dominate the apoplastic proteins of rice (*O. sativa*) leaves (Haslam et al., 2003), a result which is consistent with the findings of the current study. Germins were first discovered in germinating wheat embryos and therefore can be regarded as protein markers of early plant development. Germins are apoplastic and multimetric glycosylated proteins, which are resistant to both heat and protease activities (Lane et al., 1993). These proteins have been shown to have both oxalate oxidase (Lane, 1994) and superoxide dismutase activity (Woo et al., 2000) and are therefore, implicated in defence roles against pathogenic attack (Borderies et al., 2003).

Oxalate oxidases and germins with oxalate oxidase activity degrade oxalate in the presence of dioxygen to form CO_2 and H_2O_2 (Lane, 1994). The H_2O_2 produced may (i) be used in the peroxidase-catalysed cross linking reactions of the cell wall thereby initiating and terminating the expansion of the cell wall; (ii) function as a powerful oxidizing agent for the direct destruction of pathogens; and/or (iii) act as a signal transduction molecule during pathogenic infection (Lane, 1994; Low and Merida, 1996). Therefore, both germin and oxalate oxidase proteins may have roles in cell wall biosynthesis and defence mechanisms (Hurkman and Tanaka, 1996) as well as other roles in plant growth and development.

α-Galactosidases

Two isoforms of α-galactosidases (spot 8 and 9) (Figure 4 and Table 1) were identified in this study. This class of protein thus constituted approximately 14% of the identified sorghum CF proteins. α-Galactosidases are *bona fide* ECM proteins (Boudart et al., 2005) and have also been identified in the apoplast proteome of tobacco leaf (Dani et al., 2005). They catalyse the hydrolysis of α-1,6-linked galactosyl residues from galactooligosaccharides and polymeric galacto-(gluco) mannas (Fujimoto et al., 2003). As such, they are classified into the glycoside hydrolase family, a group of proteins that is involved in the modification of cell walls.

The sorghum cell culture medium contains known secretory proteins

Collectively, proteins identified in the sorghum culture medium have functions in defence mechanisms against

pathogenic attack, signal transduction processes as well as cell wall metabolism. Although all these functions are commonly known among secreted proteins of cultured cells and the apoplast of whole plants, the apparent dominance of oxidoreductases (peroxidases) and defence proteins (germins and oxalate oxidases) in our sorghum secretome is striking. Indeed, it is accepted that cell suspension cultures are non-physiological systems that exert both mechanical and oxidative stresses during continuous agitation in liquid culture medium (Isaacson and Rose, 2006; Jamet et al., 2006). For this reason, the presence of peroxidases and germins in the culture medium might be a reflection of responses towards stresses inherent to this culture system. Alternatively, this proteome composition might also reflect *bona fide* constituents of the sorghum secretome, a virtually unknown field at present. This rationale is supported in part by (i) results from a comparative proteomic study of apoplast proteins of *A. thaliana* and two grasses, rice (*O. sativa*) and wheat (*Triticum aestivum*) conducted by Haslam et al. (2003). They identified germin-like proteins to dominate the proteomes of both rice and wheat. Since Haslam and co-workers conducted their study using the apoplast of leaf material from whole plant systems, the dominance of germins in our study might be a true reflection of the ECM constituents of grasses; (ii) the presence of H_2O_2 producing enzymes (oxalate oxidases and germins with oxalate oxidase activity) as well as those that utilise H_2O_2 (peroxidases) may be a reflection of a naturally balanced biological system in sorghum; and (iii) since germin protein synthesis is upregulated by auxins (Bernier and Berna, 2001; Caliskan, 2000), the high abundance of these proteins in our sorghum cell culture system could possibly be due to the presence of two auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthalene acetic acid (NAA), in the formulated sorghum callus and cell suspension MS medium (Ngara et al., 2008).

However, not all classes of the expected secretory proteins were identified in this study. This may be due to the limitations of the gel-based 2DE proteomics approach. These limitations are related to protein solubility, protein abundance and the under representation of proteins with extreme M_r and/or pI values (Lin et al., 2003). Firstly, secretomics offers technical challenges relating to protein solubility as some proteins are embedded in an insoluble polysaccharide matrix (Rose et al., 2004). The solubility of these proteins is thus drastically reduced in most of the commonly used 2DE solubilizing buffers. As a result, only a partial fraction of the soluble secretome is represented on the 2DE gels for further analysis by mass spectrometry and database searches. This reasoning could possibly explain the low total number of visible CBB stained proteins observed on sorghum secretome in this study (Figure 4). Secondly, since only the highly abundant protein spots were selected for MALDI-TOF-TOF MS analysis, low abundant spots and/or poorly resolved ones are not represented in our annotated 2DE proteome map. The dynamic range of protein expression in biological systems is estimated to be about 10^5 to 10^6 for eukaryotic cells (Corthals et al., 2000; Patterson and Aebersold, 2003; Rabilloud, 2002). This high dynamic range makes visualisation and quantification of the entire proteome rather difficult (Carpentier et al., 2008; Park, 2004; Rabilloud, 2002). Low copy number proteins with about 10 - 100 copies per cell such as most signal transduction proteins, regulatory proteins or receptors become masked by the more highly expressed, 'housekeeping' proteins with over 10,000 copies per cell (Blackstock and Weir, 1999; Rose et al., 2004; Xi et al., 2006). Since no polymerase chain reaction (PCR) equivalent exists for the amplification of proteins (Blackstock and Weir, 1999; Carpentier et al., 2008), low abundant proteins have a higher chance of not being detected in 2DE gels and are therefore not identified in downstream protein identifycation procedures (Wilkins et al., 1998). With the limited loading capacities of IPG strips, the bulk of the protein load would therefore be made up of the highly abundant proteins, thus limiting the lower abundant ones from being absorbed by the strips (Kim et al., 2002; Xi et al., 2006). This effectively results in only a fraction of the total proteome being analysed and identified (Patterson, 2004). Therefore, the dominance of peroxidases, germins and oxalate oxidases in the sorghum secretome (Figure 4) could have possibly reduced the visualisation of other low abundant proteins.

Thirdly, it was observed that the CBB R-250 stainable sorghum secretome was limited to the M_r range of 25 - 100 kDa and pI range of 3 - 10. Higher and lower M_r proteins outside this range are not represented in this proteome. Furthermore, the more basic proteins with pI greater that 10 remained unresolved because of the limited resolving capabilities of the IPG strips used in the study.

Conclusion

This study is the first proteomics analysis of proteins secreted in the culture medium of sorghum cell suspensions. All the identified proteins are known secretory proteins, with predicted signal peptides, targeting proteins for secretion. The identified proteins participate in cell wall metabolism, signalling and defence related processes as well as in normal physiological processes during plant growth and development. The absence of any unexpected cytoplasmic protein contaminants in the CF protein extracts as shown by the western blotting results against known cytoplasmic marker proteins, β-tubulin and Hsp70, validates the reliability of our experimental system for future use in secretomics. The findings of this study are thus a step in the process of bridging the gap that currently exists in sorghum proteomics and also provides a foundation for future studies in the same crop. We are currently using this secretome map to landmark abiotic stress responsive proteins of sorghum in a bid to understand the role(s) of secreted proteins in stress adaptation of this crop, a potential model for cereals.

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