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Analysis of soybean tissue culture protein dynamics using difference gel electrophoresis

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ABSTRACT

Excised hypocotyls from developing soybean (*Glycine max* (L.) merr. cv. Jack) were cultivated on agar-solidified medium until callus formed. The calli were then propagated in liquid medium until stable, relatively uniform, finely-divided suspension cultures were obtained. Cells were typically transferred to fresh medium at 7-day intervals. Cultures were harvested by filtration five days (early log phase) or eight days (late log phase) after transfer. In order to evaluate dynamic changes, both intracellular and extracellular proteins were analyzed by 2-dimensional difference gel electrophoresis. Selected spots were subjected to in-gel tryptic-digestion and the resultant peptides were analyzed by nLC-MS/MS. In follow-up studies gel-free shot-gun analyses led to identification of 367 intracellular proteins and 188 extracellular proteins.

Significance: The significance of the described research is two-fold. First a gel-based proteomics method was applied to the study of the dynamics of the secretome (extracellular proteins). Second, results of a shot-gun non-gel based proteomic survey of both cellular and extracellular proteins are presented.

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1. Introduction

Plant cell suspension cultures have been used for decades as a tool for the study of basic physiological, biochemical, and molecular processes. Stable, terminally-differentiated cell cultures have been useful for analysis of protein targeting and localization [1,2], posttranslational modifications (PTM) [3,4], the packaging of storage proteins [5–7], and analysis of developmental regulation [8]. Often, however, cells dedifferentiate to callus during long-term culture [9]. While the dedifferentiated callus cultures no longer faithfully recapitulate developmental sequences, they remain useful as a ready source of relatively homogeneous cells with rapid and uniform access to nutrition, precursors, growth factors, and signaling compounds from the growth medium.

We are developing a proteomic-based platform for system analysis of soybean (*Glycine max* (L.) merr. cv. Jack) seed development. Because staging of seed development based upon chronology is inconsistent, a staging system based upon physiological age has been developed [e.g., [10]]. This system is a modification of the one previously described by Meinke et al. [11]. The staging of developing soybean seeds is invasive and requires harvesting and opening the pods before weighing and measuring the seeds. Most harvested seeds do not fall within the ranges of our staging system, so they are used for non-data generating method-development. Because of the potential utility in our systems analysis and ready availability of material, we have undertaken a preliminary characterization of suspension cultures originally derived from cv. Jack embryos.

The proteome of plant cells is dynamic, changing in response to developmental programs [12–14], environmental stress [15–17], nutrition [18,19], and host:pathogen interactions [20–23]. The changes in response to developmental cues typically involve complex networks mediated by signaling pathways [24–26]. We are unaware of any corresponding studies of the dynamics of extracellular plant proteins.

There is an increasing interest in the study of the extracellular proteins that suspension cultures of plant cells release into the medium (the secretome) [18,27,28]. Initially there did not seem to be any broad-scale consistency in the composition of the plant secretome [1], but more recently it has been proposed that there is a common core of proteins secreted by all plants [29]. This implies functional importance for this cadre of proteins.

Many proteins that traverse the classical ER/Golgi secretory pathway are subjected to a suite of PTM including N-glycosylation [30], disulfide bond formation [31], acylation [32], etc. These PTM might be necessary for secretion, or they might be an inevitable consequence of the pathway. We have additionally undertaken a preliminary analysis of the PTM of proteins secreted by the soybean suspension cells in order to see if this contributes to the dynamics of the secretome.

Abbreviations: ABC, ammonium bicarbonate; FA, formic acid.

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2. Materials and methods

2.1. Reagents

Unless otherwise noted, all reagents were supplied by Sigma-Aldrich. The cell-permeant SYTO 16 green fluorescent nucleic acid stain is from Invitrogen. The CyDyes for Difference Gel Electrophoresis (DIGE) are from GE Healthcare/Amersham. The C7BZO detergent is from Calbiochem/EMD Millipore.

2.2. Suspension cultures

Embryogenic explants from developing soybean (*Glycine max* (L.) merr. cv. Jack) seeds were provided by Professor J. Widholm, Department of Crop Sciences, University of Illinois, Urbana–Champaign, IL 61801. The explants were incubated on agar-solidified medium [33]. Subsequently, the callus that formed at the base of the explants was transferred to agar-solidified medium containing 30 g/L sucrose, 4.3 g/L Murashige–Skoog basal salt mixture, 1 g/L myo-inositol, 180 mg/L KH_2PO_4, 5 mg/L nicotinic acid, 1 mg/L thiamine-HCl, 0.5 mg/L pyridoxine, and 0.2 mg/L 2,4-D, pH 5.7. After an extended period of culture, small clumps of cells dissociated from the callus and were transferred to a fresh medium using sterile 10 mL plastic pipettes. Following a period of approximately 6 months of uneven growth, the cells assumed a stabilized growth rate (Fig. 1), and 5 mL of cells was transferred weekly to 100 mL of fresh medium. The 7-day transfer schedule was used in order to maintain a high growth-rate, and these cells have now been in continuous culture for more than two years. Early log-phase (5 days after transfer to fresh medium) and late-log phase (8 days after transfer) cultures were selected for comparative analyses.

2.3. Microscopy

Four hundred μL of cells 5 days after transfer were incubated with 5 μM SYTO 16 cell permeant fluorescent nucleic acid stain, then observed using a Zeiss Axiovert 200 M Fluorescence/Live cell Imaging Microscope. Images were recorded with a Zeiss Axiocam MRc digital camera. After staining all cells display a nucleus containing a prominent nucleolus (Fig. 2). During late-lag phase (2 to 3 days after transfer) the cells are dividing and have a characteristically dense, granular cytoplasm. After the transition to early-log phase (4 to 5 days after transfer) the cells are expanding and contain prominent lytic vacuoles with transvacuolar cytoplasmic strands. This appearance is maintained to stationary phase.

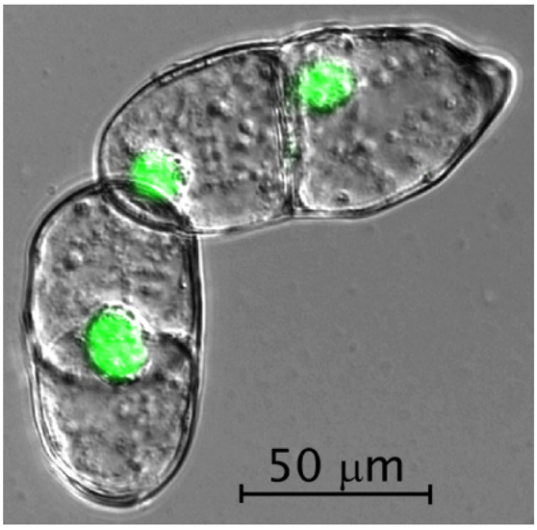

Fig. 2. Image analysis of the suspension-cultured *G. max* cells. An image captured from differential interference contrast microscopy using Nomarski optics was overlaid with an epi-fluorescence image of the same cells incubated with 5 μm SYTO16. Cells are 5 days after transfer to fresh medium. The nuclei are fluorescent as a result of the dye intercalating in the DNA. At this growth stage the cytoplasm is relatively dense.

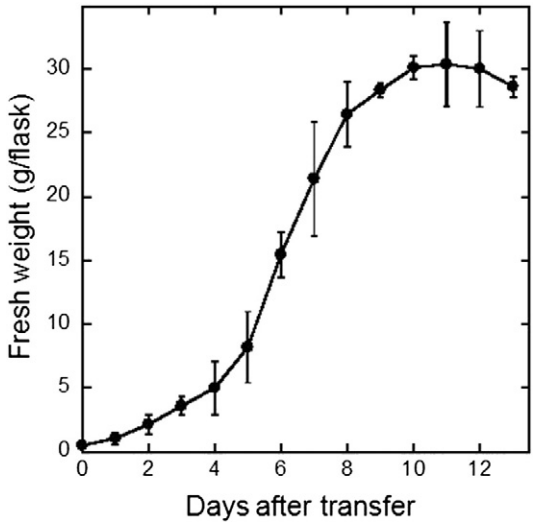

Fig. 1. Growth of soybean (*Glycine max* (L.) merr. cv. Jack) cells in suspension culture. Five mL of cells was transferred to 100 mL of fresh medium using a sterile disposable 10 mL plastic pipette. At 24 h intervals whole cultures were harvested by filtration at reduced pressure onto 2 disks of Whatman No 1 filter paper. After removal of culture medium, fresh weight values were recorded. Data points are means ± SEM for at least 24 biological replicates.

2.4. Experimental design

The intent of the analyses described herein was to describe the soybean suspension culture secretome, in order to see if the population of proteins that comprise the secretome undergoes dynamic changes during the culture cycle, to compare the intracellular and extracellular proteomes, and to test the potential for using the suspension cultures as an adjunct for our ongoing studies of soybean seed development.

The strategy used in our studies of protein dynamics incorporates 2D DIGE (Fig. 3). This method was chosen because it is reproducible, quick, and relatively simple to do, and the results can be easily interpreted by visual examination of the gels.

At the transition stages of the growth curve, either five or eight days after transfer, cells were separated from the medium by filtration through two layers of Whatman no. 1 filter paper (Fig. 3). If not used immediately, the harvested cells were frozen at −20 °C, lyophilized, and then stored at −70 °C until analysis. Samples of 200 mg of lyophilized cells were suspended in 10 mL of a 1:1 mixture of Tris-buffered phenol, pH 8.8, and 0.1 M Tris–HCl, pH 8.8, containing 10 mM EDTA, 0.4% 2-metacaptoethanol (all % solutions are *v*/*v*), 0.9 M sucrose, and 0.1% C7BZO, in a 50 mL plastic Falcon tube. The cells were disrupted by 3 × 1-min cycles with a Kinematica bench-top Polytron homogenizer at a setting of 4. Homogenates were incubated on ice for 5 min between each cycle. Final homogenates were transferred to 15 mL glass Corex tubes and centrifuged at 4 °C for 10 min at 5000 ×*g*. The phenol phase was transferred to clean Corex tubes followed by addition of 5 volumes of 0.1 M ammonium acetate in 100% methanol (pre-chilled to −20 °C) and vortex mixing. Proteins were precipitated by incubation at −20 °C for 2 h. Precipitates were collected by centrifugation at 4 °C for 20 min at 10,000 ×*g*. Pellets were washed twice with ice-cold 0.1 M ammonium acetate/methanol containing 10 mM DTT, twice with ice-cold 80% acetone containing 10 mM DDT, and finally with ice-cold 80% acetone. The washed pellets were either used immediately or stored at −20 °C until analyzed.