Title: Analysis of Soybean Tissue Culture Protein Dynamics Using
Difference Gel Electrophoresis

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Running title: Soybean suspension culture

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 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.

Keywords: DIGE, *Glycine max*, protein dynamics, secretome, tandem mass spectrometry, tryptic peptides

Abbreviations: ABC, ammonium bicarbonate; FA, formic acid

1 Introduction

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

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

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

28 There is an increasing interest in study of the extracellular proteins that
29 suspension cultures of plant cells release into the medium (the secretome) [18,27,28].
30 Initially there did not seem to be any broad-scale consistency in the composition of the

1 plant secretome [1], but more recently it has been proposed that there is a common
2 core of proteins secreted by all plants [29]. This implies functional importance for this
3 cadre of proteins.

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

11 **Materials and methods**

13 **Reagents**

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

20 **Suspension cultures**

22 Embryogenic explants from developing soybean (*Glycine max* (L.) merr. cv. Jack)
23 seeds were provided by Professor J. Widholm, Department of Crop Sciences,
24 University of Illinois, Urbana-Champaign, IL 61801. The explants were incubated on
25 agar-solidified medium [33]. Subsequently, the callus that formed at the base of the
26 explants was transferred to agar-solidified medium containing 30 g/L sucrose, 4.3 g/L
27 Murashige-Skoog basal salt mixture, 1 g/L myo-inositol, 180 mg/L KH_2PO_4 , 5 mg/L
28 nicotinic acid, 1 mg/L thiamine-HCl, 0.5 mg/L pyridoxine, and 0.2 mg/L 2,4-D, pH 5.7.
29 After an extended period of culture, small clumps of cells dissociated from the callus
30 and were transferred to fresh medium using sterile 10 mL plastic pipettes. Following a

1 period of approximately 6 months of uneven growth, the cells assumed a stabilized
2 growth rate (Figure 1), and 5 mL of cells were transferred weekly to 100 mL of fresh
3 medium. The 7-day transfer schedule was used in order to maintain a high growth-rate,
4 and these cells have now been in continuous culture for more than two years. Early
5 log-phase (5 days after transfer to fresh medium) and late-log phase (8 days after
6 transfer) cultures were selected for comparative analyses.

7 8 Microscopy

9
10 Four hundred μ L of cells 5 days after transfer were incubated with 5 μ M SYTO 16 cell
11 permeant fluorescent nucleic acid stain, then observed using a Zeiss Axiovert 200M
12 Fluorescence/Live cell Imaging Microscope. Images were recorded with a Zeiss
13 AxioCam MRC digital camera. After staining all cells display a nucleus containing a
14 prominent nucleolus (Figure 2). During late-log phase (2 to 3 days after transfer) the
15 cells are dividing and have a characteristically dense, granular cytoplasm. After the
16 transition to early-log phase (4 to 5 days after transfer) the cells are expanding and
17 contain prominent lytic vacuoles with transvacuolar cytoplasmic strands. This
18 appearance is maintained to stationary phase.

19 20 Experimental design

21
22 The intent of the analyses described herein was to describe the soybean suspension
23 culture secretome, in order to see if the population of proteins that comprise the
24 secretome undergoes dynamic changes during the culture cycle, to compare the
25 intracellular and extracellular proteomes, and to test the potential for using the
26 suspension cultures as an adjunct for our ongoing studies of soybean seed
27 development.

28 The strategy used in our studies of protein dynamics incorporates 2D DIGE
29 (Figure 3). This method was chosen because it is reproducible, quick, and relatively
30 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 (Figure 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-metacaproethanol (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 x 1-minute cycles with a Kinematica bench-top Polytron homogenizer at a setting of 4. Homogenates were incubated on ice for 5 minutes between each cycle. Final homogenates were transferred to 15 mL glass Corex tubes and centrifuged at 4°C for 10 minutes at 5000 x 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 hours. Precipitates were collected by centrifugation at 4°C for 20 minutes at 10,000 x g. Pellets were washed twice with ice-cold 0.1M ammonium acetate/methanol containing 10 mM DTT, twice with ice-cold 80% acetone containing 10 mM DTT, and finally with ice-cold 80% acetone. The washed pellets were either used immediately or stored at -20°C until analyzed.

For analysis of extracellular proteins, the filtrate was centrifuged at 100,000 x g for 2 h, in order to remove extracellular polysaccharides [34], dialyzed overnight against 400 volumes of deionized H₂O, and lyophilized. Samples of lyophilized medium were re-hydrated in 4 mL MS-grade water. Proteins were precipitated by adding 5 volumes of cold 100% acetone containing 10 mM DTT, followed by incubation at -20°C for 1 hour. The precipitates were collected by centrifugation for 20 minutes at 10,000 x g and 4°C. Pellets were washed by re-suspension in 80 % acetone followed by re-centrifugation. The washed pellets were stored at -20°C until analyzed.

Electrophoretic analyses

Both cellular and extracellular proteins were analyzed by 2D-DIGE [35] at the Charles W. Gehrke Proteomics Center of the University of Missouri (<http://proteomics.missouri.edu/>). Protein pellets were dissolved in 0.5 mL of IEF loading solution (8 M urea, 2 M thiourea, 0.2 % CHAPS, and 0.2 % C7BZO). Solubilized proteins were quantified using an EZQ Protein Quantitation Kit from Life Technologies. For each gel a 24 cm Immobiline DryStrip, pH 3-10, IPG strip (GE Healthcare) was rehydrated at 50 V for 2 hours using the IEF loading solution. Fifty µg of protein from day 5 were labeled with 400 pmol of Cy3 and 50 µg of protein from day 8 were labeled with 400 pmol of Cy5 (Figure 3). The labeled samples were combined 250 µg each of unlabeled proteins and the total sample of 600 µg was added to the IPG strips. Samples were focused at 250 V for 500 Vhr followed by 1,000 V for 1,000 Vhr, and then 8,000 V for a total of 80,000 Vhr. When IEF was completed, samples were held at 500 V.

After IEF, the IPG strips were incubated in 4 mL of SDS equilibration solution (0.5 M Tris, pH 8.8, 30% glycerol, 6 M urea, and 2% SDS) plus 2% (w/v) DTT for 15 min, followed by incubation for an additional 15 min in SDS equilibration solution containing 2.5% iodoacetamide. The IPG strips were then rinsed in gel running buffer (25 mM Tris, 0.1% (w/v) SDS, and 192 mM glycine), placed onto a 12% SDS gel overlaid with 0.5% (v/w) agarose in running buffer (with addition of 0.002% bromphenol blue as the tracking dye). Electrophoresis was performed using an Ettan DALTsix electrophoresis unit (Amersham Biosciences), at 10 mA current overnight or until the tracking dye migrated out of the gel.

Post-electrophoresis the gels were transferred to 24 cm-plate reading cartridges and loaded into the Ettan DIGE Imager (GE Healthcare). After fluorescent imaging, the gels were washed 3 times for 10 minutes each with 500 mL of 18 mOhm deionized water. The sensitivity of the CyDyes makes it impractical to pick spots directly from the DIGE images so the gels were then post-stained in 500 mL of colloidal Coomassie G-250 stain (0.08% (w/v) Coomassie Brilliant Blue G-250 in 20% ethanol containing 1.6% (v/v) phosphoric acid and 8% (w/v) ammonium sulfate, overnight (Figure 3). The Coomassie-stained gels were destained in 18mOhm Milli-Q water for up to 4 hours prior to spot picking.

Mass spectroscopy of tryptic peptides

Spots were selected for subsequent MS-analysis if they were visually scored as red or green in all of the DIGE analyses conducted. Matching of selected spots among the population of DIGE images and Coomassie post-stained images was always verified using the Delta2D software (DECODON; <https://www.decodon.com/delta2d.html>). The selected spots were excised from gels using a 1.5 mm spot picker (Gel company, San Francisco CA) and the gel plugs transferred to 1.5 mL microcentrifuge tubes. Plugs were washed at room temp for 20 minutes with 500 μ L of 50% acetonitrile (ACN) containing 50 mM ammonium bicarbonate (ABC). Washing was repeated until gel plugs were visibly destained. The plugs were dehydrated by washing twice for 15 minutes with 500 μ L of 100% ACN and air-dried. Thirty μ L of a 20 ng/ μ L solution of trypsin (Princeton Separations) in 50 mM ABC were added to each gel plug which was then incubated on ice for 1 hour. The trypsin solution was replaced with 30 μ L of 50 mM ABC, and the gel plugs incubated for 16 hours at 37°C. The digestion solution was transferred to a 1.5 mL microcentrifuge tube and the gel plugs were washed with 30 μ L of 60 % ACN containing 1% formic acid for 10 min with gentle agitation. The combined digestion solution plus wash was stored at -20°C.

Tryptic peptides were purified by ZipTip, and loaded onto a C8 trap column. Bound peptides were eluted onto a 10.5 cm x 150 μ pulled-needle analytical column packed with magic C18 reversed phase matrix. Peptides were separated and eluted from the analytical column with a continuous gradient of ACN from 5 to 45% in 0.1% formic acid over 120 min. The Proxeon Easy nLC-II HPLC system is attached to an LTQ Orbitrap MS. Following a high-resolution scan of the eluting peptides, each second the 9 most abundant peptides were fragmented by CID in the ion-trap. The Phytozome G. max DB version 1.1 (<http://www.phytozome.net/soybean>) has been used, plus a decoy DB generated by reversing the target sequences. Spectra were searched against the combined target-decoy DB using the Sorcerer 2 IDA Sequest-based search algorithm. Identities were verified using the Scaffold 3 program, and the

1 false discovery rate (FDR) was estimated as (Ndecoy/Ntarget) [36,37]. For the 186,364
2 peptide spectra examined from the DIGE analysis, the FDR was between 5.18 and
3 5.26%.

4 For both cellular and extracellular samples, all DIGE spots contained more than
5 one protein. A winner-takes-all strategy was used for protein/spot assignments; only
6 the protein with the largest number of identified peptides has been reported (Tables 1,
7 2). Samples were analyzed in biological triplicate; proteins were included in the results
8 only if they were identified in at least two of three biological replicates. The patterns of
9 spots/colors were remarkably consistent, a property we attribute to the consistency of
10 the suspension cultures, the nature of DIGE, and the use of the immobilized pH
11 gradient strips [38]. Visual spot assignments were always verified using the Delta2D
12 image analysis software (<http://www.decodon.com/>).

13 Shot-gun gel-free analyses of total cellular proteins were conducted exactly as
14 previously described [10]. For gel-free analysis of extracellular proteins, the acetone-
15 precipitates were dissolved in 50 mM ABC, then treated just as describe for the DIGE
16 samples. The MS proteomics data have been deposited with the ProteomeXchange
17 Consortium [[proteomecentral.proteom exchange.org](http://proteomecentral.proteomexchange.org)] via the PRIDE partner repository
18 [39] with the dataset identifiers PXD001172 and 10.6019/PXD001172 for the DIGE
19 analyses, and PXD001895 and 10.6019/PXD001895 for the shot-gun analyses.

20 21 Analysis of posttranslational modifications

22
23 The extracellular protein fraction was subjected to a suite of preliminary analyses for
24 PTM. Analysis for N-glycosylation included both liquid-phase chromatography using
25 con A-Sepharose [40] and post-electrophoretic staining with the glycan-specific
26 fluorescent dye Pro-Q Emerald [41]. Phosphorylation was evaluated by post-
27 electrophoretic staining with the O-phosphorylation-specific fluorescent dye pro-Q
28 Diamond [42], and by neutral loss scanning for H₃PO₄ from CID of phosphorylated
29 precursor ions by tandem MS [43]. Lys-N^ε-acetylation was probed by immunoblotting
30 using specific antibodies [44].

Bioinformatic analyses

Identified proteins were sorted into 11 functional groups: 1, Primary Metabolism; 2, Secondary Metabolism; 3, Cellular Structure; 4, Stress Responses; 5, Nucleic Acid metabolism; 6, Protein Synthesis; 7, Protein Folding; 8, Protein Targeting; 9, Hormones and Signaling; 10, Seed Storage Proteins, and 11, Proteins of Unknown Function [5].

For in silico predictions of targeting, the proteins identified by searching Phytozome were used as queries for a PSI-BLAST search of the NCBI database (<http://www.ncbi.nlm.nih.gov/>). If the tblastn search returned a consensus localization notation, this was accepted. If there was either no consensus or no localization notation, then the protein sequences were used as the query terms to successively search TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) and PSORT (<http://psort.hgc.jp/form.html>). A consensus localization prediction from the two sites was accepted. If there was no consensus between the TargetP and PSORT results, further searches were conducted using both general (e.g., PredSL; <http://hannibal.biol.uoa.gr/PredSL/index.html>) and specialized (e.g., PredPlantPTS1; <http://ppp.gobics.de/submission>) algorithms until a consensus was achieved.

Results

After an extended period in culture, the cells dedifferentiated to callus, and were no longer capable of regenerating whole plants (not shown). The transfer regime employed resulted in a classical sigmoidal growth aspect for the *G. max* cv. Jack suspension cultures (Figure 1.). During the linear growth phase the fresh weight doubling time was approximately 24 h. In appearance, the cultures were finely-divided clumps of 2-4 roughly isodiametric cells (Figure 2.). Nuclei were well-defined, and a relatively dense cytoplasm was maintained throughout most of the culture cycle.

Our experimental design (Figure 3) was set up so that spots containing proteins decreasing in abundance between day 5 and day 8 appear Green, while spots

1 containing proteins increasing in abundance during the same period appear red. Spots
2 containing proteins of approximately equal abundance appear yellow. While the
3 majority of cellular protein spots are yellow, there are many red or green spots (Figure
4 4). Twenty-seven red, green or yellow spots were chosen for further analysis.

5 The same experimental strategy was used to compare early and late log phase
6 extracellular proteins (Figure 5). The pattern of extracellular proteins is much simpler
7 than that of cellular proteins. Unexpectedly, the DIGE results seem to indicate that the
8 extracellular protein population is also dynamic and the majority of spots are either red
9 or green. Fifteen red, green, or yellow spots were selected for further analysis.

10 While the majority of spots visible from 2D-DIGE of cellular proteins appear
11 yellow, there are many individual spots that appear green or red (Figure 4). Most of the
12 27 spots selected for MS analysis are associated with primary metabolism, response to
13 stress, and protein synthesis or folding (Table 1).

14 The 2D-DIGE patterns of extracellular (Figure 5) proteins are much less complex
15 than those of cellular proteins (Figure 4). As a group, the extracellular proteins appear
16 more dynamic than the cellular proteins, with a distinct overall “Christmas tree”
17 appearance. The majority of extracellular proteins selected from DIGE analysis for
18 identification by MS are annotated as functioning in response to stress (Table 2). All of
19 the selected extracellular proteins are predicted to traverse the secretory pathway, and
20 the genome-encoded amino acid sequences of all 15 proteins identified by MS analysis
21 include an easily identified N-terminal signal sequence (Figure S1).

22 N-glycosylation is a common PTM decorating proteins that traverse the classical
23 ER/Golgi apparatus-based secretory pathway [30], and our previous analysis of the
24 secretome of suspension-cultured maize endosperm cells revealed that all of the
25 proteins include at least one high-mannose type glycan [34]. It was thus unexpected
26 when we observed no evidence for N-glycosylation of the selected soybean
27 extracellular proteins. We were unable to detect any extracellular N-glycanase activity
28 [45] when assayed using ovalbumin as the substrate (data not presented), so we
29 consider it unlikely that the soybean proteins were secreted into the medium as
30 glycoproteins and then deglycosylated.

O-phosphorylation and Lys-N^ε-acetylation are less common PTM of secretory proteins, and were not detected among the soybean extracellular proteins (data not shown). Nonetheless, it remains possible that PTM are responsible for some of our DIGE results. Two PTM that we did not measure directly are proteolytic and redox-modifications. Proteolysis would likely be manifest by a vertical “chain” of spots with decreasing Mr, something that we did not observe (Figure 5). Additionally assays of general proteolytic activity in culture filtrates using FTC-Casein (Pierce Thermo Scientific) as the substrate did not reveal any significant activity (data not presented). This does not, of course, rule out the possibility of sequence- or client-specific proteolysis, although this seems unlikely.

Oxidation of amino acid sidechains might lead to a horizontal chain of spots with the same Mr but changing pI. We did not observe this sort of result during our analysis of the maize endosperm-cell derived secretome [1], but it is clearly a possibility with the soybean suspension cells (Figures 4,5). The selected soybean protein sequences are not notably Cys or Met-rich, but many other side-chains are potentially susceptible to oxidation [46].

Results from gel-free analysis of total cellular proteins can be found in Table S2. A total of 367 proteins were identified. Of the 367 extracellular proteins 113 (31%) are annotated as being involved with central metabolism [5]. Unexpectedly, the second and third most populated categories are cell structure (59/16%) and protein synthesis (58/16%). Results from gel-free proteomic analysis of total extracellular proteins can be found in Table S3. A total of 188 proteins were identified. The total extracellular protein sample includes all of the proteins selected for analysis based upon the DIGE results, as well as all nine core secretome proteins described in [29]. It is noteworthy that 68 of the 190 extracellular proteins (36%) have an annotated function related to synthesis or remodeling of the extracellular matrix [47].

A total of 14 proteins were detected in both the cellular and extracellular fractions: three members of the aspartyl-protease family (Glyma19g44540, Glyma07g06100, and Glyma04g17600; a β -1,3-glucanase, Glyma18g52860; EF-1-alpha, Glyma17g23900; two members of the SKU5 family proteins, Glyma17g14730

1 and Glyma04g02140, an extensin family protein, Glyma13g24720; peroxidase,
2 Glyma13g04590, the cyclophilin ROC3; Glyma12g02790; a glycosyl hydrolase family 3
3 protein, Glyma10g15980; a glycosyl hydrolase family 81 protein, Glyma08g27000; a
4 subtilase family serine protease, Glyma09g08120; and a protein of unknown function,
5 Glyma10g11620. Members of the *A. thaliana* aspartyl-protease family are involved with
6 disease resistance signaling [48]. The SKU5 proteins participate in
7 glycosylphosphatidylinositol-anchored glycoprotein synthesis [49].

9 **Discussion**

11 Unlike their animal counterparts, embryogenic plant cells are totipotent, able to divide
12 and produce all of the differentiated cells of an organism. More than a century has
13 passed since the concept and practice of plant cell culture was first introduced [50].
14 Unlike most animal cells, plant cells can be manipulated to change from one
15 differentiated state, expressing a committed developmental program, to a different
16 program by transition through a state of dedifferentiation typically referred to as callus
17 [51]. This switch is accompanied by changes in the relative levels of the auxin and
18 cytokinin plant growth regulators [52]. Under appropriate conditions, callus cells can
19 become immortalized in suspension culture, and can be maintained indefinitely without
20 differentiation. This property is likely the result of long-term genetic and epigenetic
21 instability when cultured in vitro [53].

22 In liquid suspension, calli grow as small clumps of up to 10-12 uniform cells [].
23 As a result, all cells have direct contact with the liquid culture medium and have a
24 uniform metabolic capability. This is in marked contrast to native plants where
25 differentiation of cells into specialized tissues and organs is supported by an underlying
26 functional specialization of the proteome. In part because of their functional uniformity,
27 cell cultures have become an important tool for study of basic plant biology and
28 biochemistry, and have achieved a level of major importance in biotechnology [50].

29 Even in the dedifferentiated callus state, cultured plant cells retain the ability to
30 respond to environmental cues through changes in gene expression. This is

1 particularly evident in terms of plant pathogen interactions [54], a generalized stress
2 response [55], and nutrition [56].

3 Typically, by the end of a culture cycle the cells are starved for reduced carbon
4 and nitrogen [57], and these nutrients are rapidly taken up after the cells are transferred
5 to fresh medium [58]. After uptake, sucrose must be converted to fructose plus UDP-
6 glucose by sucrose synthase (SuSy) [59] before further metabolism. It is then not
7 surprising that three of the DIGE-selected cellular protein spots contained SuSy.
8 Analysis of spots 9 and 10 show SuSy decreasing while spot 8 has it increasing. This
9 seeming contradiction is likely because SuSy is subjected to a suite of PTM [60] that
10 would alter electrophoretic mobility.

11 Several of the DIGE-selected spots contained proteins associated with protein
12 folding (Figure 4, Table 1; spots 1, 2, 4, 13). Each of these spots is green, indicating a
13 decrease in protein abundance. These results are likely because there is an initial
14 wave of protein synthesis and folding upon nutrient repletion, followed by a longer
15 period of cellular growth and expansion characterized by protein homeostasis [14,61].

16 Other green spots contain a member of the annexin protein superfamily [62].
17 The annexins are proteins that are associated with membranes and the cytoskeleton.
18 Proteins present in spots 18, 19, 22, and 23 are involved in cellular signaling [5]. The
19 ribosomal S5 protein was found in two spots, one red and the other green (Figure 4,
20 Table 1). Again, the most likely explanation for the seeming dichotomy is PTM, and this
21 protein has been reported to be O-phosphorylated in bacteria and N-methylated in
22 cellular slime molds [63].

23 The secretion by plant cells of a large number of proteins into the extracellular
24 space has reviewed recently [27,29], and it was suggested that these extracellular
25 proteins play crucial roles in stress responses, communication, and development.
26 Gupta et al. [29] compared the secretome of chickpea (773 proteins) with the previously
27 described secretome of Medicago (50), Arabidopsis (172), and rice (228). Only nine
28 proteins were identified in the secretome of all four plants (3 peroxidases, BG454205,
29 TC106564, TC107670; thaumatin-like protein E22, TC94274; basic 7S globulin low-kDa
30 subunit, TC94309; endo-1,3-beta-glucosidase, TC94670; subtilisin-like protease,

1 TC94936; polygalacturonase inhibitor, TC95395; aspartic proteinase nepenthesin-1,
2 TC96274). The ubiquity of these nine core proteins suggests a conserved common
3 albeit obscure role. Most of the secretome composition was species specific; 94% of
4 proteins were unique to chickpea, followed by 63% to rice, 57% to Arabidopsis and 8%
5 to Medicago. It has been suggested that differences in extracellular protein profile
6 result from a combination of the cellular environment, and the environmental niche
7 occupied by the parental plants [29]. The identification of 145 rice-specific extracellular
8 proteins suggests the possibility of monocot-specificity. Without question the small
9 number of extracellular proteins common to “all” plants is at least partially because
10 none of the extant secretomes has yet been totally defined.

11 In a recent review article, it was reported that more than 50% of extracellular
12 plant proteins lack an identifiable functional signal peptide [27]. In some instances this
13 has led to the assumption that the proteins lacking an obvious signal sequence were
14 contaminants resulting from cell death or simple breakage during culture of
15 manipulation. While this remains a possibility, it must also be noted that a system
16 which allows the unconventional secretion of proteins lacking a signal sequence, the
17 exocyst positive organelle [EXPO; 64] is also a possibility. In the case of our DIGE-
18 designated proteins this point is moot. All of these proteins include a classical N-
19 terminal signal sequence (Figure S1).

20 If PTM are discounted as the basis of our DIGE results would seem to be the
21 developmental or physiological-based secretion of specific proteins. How this might be
22 related to normal soybean cellular protein trafficking or beneficial to the suspension-
23 cultured cells remains obscure, but it is not without precedent. It is well-known that
24 phosphate starvation leads to secretion of phosphatases from both intact [65, 66] and
25 suspension-cultured [18] plant cells, and it has also been shown that starvation for
26 reduced nitrogen can lead to secretion of proteases by maize endosperm suspension
27 cultures [19]. Furthermore, exposure of *A. thaliana* suspension cells to extracellular
28 proteins from *Pseudomonas syringae* led to changes in the plant secretome in
29 response to exposure to proteins from a virulent pathovar of the bacterium [22].
30 Several of the extracellular soybean proteins that changed in abundance during the

1 culture cycle are annotated as pathogen-induced (Table 2), but this might instead be a
2 response to a generalized rather than specific stress [23].

3 4 **Conclusions**

5
6 The study described herein was initially undertaken to evaluate the soybean axis-
7 derived suspension cultures as an easily manipulated platform for analysis of growth,
8 development, and various aspects of control. The results of our proteomic analyses
9 indicate that the cultured cells differ substantially from previously-studied soybean
10 organs, tissues, and cells [10,67-69], which would seem to limit their utility. Per contra,
11 the suspension cells seem to be an excellent system with which to study induction of
12 carbohydrate metabolism, signaling, response to certain abiotic stresses, and protein
13 targeting and sorting. The absence of the typically abundant seed storage proteins
14 [5,70] and chloroplast proteins [71] should increase their utility for study of the latter
15 topics, and they would clearly be useful in analysis of many aspects of the classical ER
16 to Golgi to plasma membrane trafficking of non-glycosylated secretory proteins.

17 18 **Acknowledgements**

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22 23 **Contributions**

24
25 JAM planned the research, assisted with data interpretation, and drafted the
26 manuscript, AAJ and MLJ conducted the research and interpreted the data, all authors
27 read and approved the submitted manuscript.

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Figure legends

Figure 1. Growth of soybean (*Glycine max* (L.) merr. cv. Jack) cells in suspension culture. Five mL of cells were 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 discs of Whatman No 1 filter paper. After removal of culture medium, Fresh weight values were recorded. Data points are means $\pm$ SEM for at least 24 biological replicates.

Figure 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 epifluorescence image of the same cells incubated with 5 μ m 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.

Figure 3. Experimental design. Cells were harvested by filtration either in late lag phase (Day 5) or late log phase (Day 8). Total cellular or extracellular proteins were isolated, and labeled with either Cy3 (Day 5) or Cy5 (Day 8). Fifty μ g each of the labeled proteins were added to 250 μ g each of unlabeled proteins and the 600 μ g of total protein were analyzed by 2D DIGE. Gels were subjected to fluorescent image analysis and spots scored visually as red, green, or yellow. The positions of all spots were determined using the Delta2D software. Gels were then stained with colloidal Coomassie Blue. Spots were manually picked from the Coomassie-stained gels and subjected to in gel tryptic digestion. The resultant tryptic peptides were analyzed by nLC-MS/MS. Cells and culture medium were analyzed in biological triplicate (3 separate flasks of suspension cells).

Figure 4. Analysis of *G. max* suspension culture cellular proteins by Difference Gel Electrophoresis (DIGE) in order to select targets for further analysis. Samples from the early log phase were labeled with Cy3 while samples from the late log phase were labeled with Cy5. As a result, proteins decreasing in abundance from day 5 to day 8 appear as green spots, while proteins increasing in abundance during the same period appear as red spots. Proteins that are of approximately the same abundance in the two samples appear as yellow spots. Proteins in selected spots were subjected to in gel tryptic-digestion followed by nLC-MS/MS. The positions of marker protein migration are indicated to the left of the Y-axis; an estimate of the pI is

1 indicated above the upper X-axis.

2
3 **Figure 5.** Analysis of *G. max* suspension culture extracellular proteins by DIGE in order to
4 select targets for further analysis. Samples from the early log phase were labeled with Cy3 while
5 samples from the late log phase were labeled with Cy5. As a result, proteins decreasing in
6 abundance from day 5 to day 8 appear as green spots, while proteins increasing in abundance
7 during the same period appear as red spots. Proteins that are of approximately the same
8 abundance in the two samples appear as yellow spots. Proteins in selected spots were
9 subjected to in gel tryptic-digestion followed by nLC-MS/MS. The positions of marker protein
10 migration are indicated to the left of the Y-axis; an estimate of the pI is indicated above the upper
11 X-axis.

Figure 1.

Figure 2.

Figure 3.

Figure 4. Two-dimensional DIGE analysis of soybean suspension culture cellular proteins.

Figure 5. Two-dimensional DIGE analysis of soybean suspension culture extracellular proteins.

Table 1. Two-dimensional DIGE analysis of soybean suspension culture cellular proteins.

Spot number	Spot color	Protein name	Accession number	Unique peptides	% Sequence coverage	Predicted localization	Annotated function ²
1	G	PDI-like	Glyma12g07260.1	13	27.0	SP ¹	7
2	G	plHSP70-1	Glyma16g00410.1	32	43.5	P	7
3	G	DC1-domain protein	Glyma08g39390.1	17	36.0	SP	7
4	G	PDI-like	Glyma04g42690.1	20	41.9	C	4
5	R	aconitase	Glyma13g38480.1	20	22.3	M	1
6	R	ribosomal S5 protein	Glyma08g18110.1	46	50.8	C	6
7	G	ribosomal S5 protein	Glyma08g18110.1	27	31.6	C	6
8	R	SUS4, sucrose synthase	Glyma09g08550.1	41	42.9	C	1
9	G	SUS4, sucrose synthase	Glyma09g08550.1	41	47.3	C	1
10	G	SUS4, sucrose synthase	Glyma09g08550.1	36	37.6	C	1
11	R	ATMS1, Met synthase	Glyma16g04240.1	27	35.4	C	1
12	R	ATMS1, Met synthase	Glyma19g29180.1	30	35.5	C	1
13	G	Hsp90	Glyma02g47210.2	13	22.1	C	7
14	Y	aminopeptidase family	Glyma02g36530.1	23	48.2	P	8
15	Y	enolase	Glyma09g28100.1	7	24.9	C	1
16	Y	EF-1 α	Glyma05g24110.1	5	13.6	C	6
17	Y	EF-1 α	Glyma01g04640.1	5	13.6	C	6
18	R	ACC-oxidase	Glyma09g01110.1	15	53.1	C	9
19	R	receptor for CK1C	Glyma08g05610.1	15	55.7	C	9
20	G	annexin	Glyma15g38040.1	21	52.7	C	3
21	G	annexin	Glyma13g34520.1	29	62.3	C	3
22	G	VDAC1, anion channel	Glyma18g16260.1	10	32.6	M	9
23	G	VDAC1, anion channel	Glyma18g16260.1	11	34.8	M	9
24	Y	GRF2, regulatory factor	Glyma02g37120.1	17	57.6	C	4
25	R	TiF 5A3	Glyma04g32950.3	7	45.0	C	6
26	G	universal stress protein	Glyma03g16510.2	10	36.5	C	4
27	R	ROC3, prolyl-isomerase	Glyma12g02790.1	8	65.1	C	7

Predicted localization; SP, secretory pathway; P, plastidial; M, mitochondrial; C, cytoplasmic. Annotated function: 1, Primary Metabolism; 2, Secondary Metabolism; 3, Cellular Structure; 4, Stress Responses; 5, Nucleic Acid metabolism; 6, Protein Synthesis; 7, Protein Folding; 8, Protein Targeting; 9, Hormones and Signaling; 10, Seed Storage Proteins, and 11, Proteins of Unknown Function [5].

Table 2. Two-dimensional DIGE analysis of soybean suspension culture extracellular proteins.

Spot number	Spot color	Protein name	Accession number	Unique Peptides	% sequence coverage	Predicted localization	Annotated function ²
1	G	RLK-like	Glyma01g04640.1	6	19.8	SP ¹	9
2	R	acid phosphatase	Glyma09g35880.1	3	10.2	SP	1
3	G	subtilisin-like protease	Glyma17g17850.1	10	14.6	SP	4
4	G	subtilisin-like protease	Glyma17g17850.1	11	24.2	SP	4
5	R	mannose-specific lectin	Glyma13g34520.1	11	39.2	SP	4
6	G	endo-1,3- β -glucosidase	Glyma14g08200.1	6	24.4	SP	4
7	G	uncharacterized protein	Glyma14g02350.1	8	22.8	SP	11
8	Y	endo-1,3- β -glucosidase	Glyma14g08200.1	4	12.6	SP	4
9	Y	uncharacterized protein	Glyma14g02350.1	8	26.0	SP	11
10	Y	uncharacterized protein	Glyma14g02350.1	5	27.5	SP	11
11	G	pectate lyase	Glyma04g36520.1	3	18.4	SP	4
12	R	peroxidase	Glyma13g23620.1	3	11.9	SP	4
13	Y	peroxidase	Glyma13g23620.1	9	48.4	SP	4
14	R	endo-xylanase	Glyma13g00280.1	7	36.7	SP	4
15	G	β -expansin	Glyma11g17160.1	4	14.5	SP	3

Abbreviations are as defined for Table 1.

Graphical Abstract