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Evo-Devo II: Does Seedling Protein Biochemistry Reflect Plant Phylogeny And Development?

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Biochemistry of plant seeds and seedlings may provide insights into both evolution and development. The present research compared protein banding from seedling extracts in three plant families (Brassicaceae, Fabaceae and Curcubitaceae). Seeds were allowed to germinate for up to three weeks. Cell lysates were prepared over ice and frozen at -80°C. Before electrophoresis (SDS-PAGE), samples were quantified to assure equal protein loading. Stained gels were scanned and quantified to determine banding profile. This exercise enables an undergraduate class to understand whether seedling protein chemistry reflects unique taxonomic characteristics of its plant family.

Keywords: Seedling protein, plant classification, developmental biology

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Introduction

Previous work (Blando-Hoegler and Hoegler, 2013) has demonstrated that seed extracts could be used in a college laboratory to teach protein biochemistry and reveal relationships between evolution and taxonomy. Plants are inexpensive, readily available and not likely to generate ethical conflicts. This present paper proposes the use of seedlings available through a short period of germination. The seedling consists of an easily-procured embryo, together with a separate food source called the endosperm. The purpose of this study is to reexamine the relationship between evolution and taxonomy (in this case using seedlings), and to use this plant embryo model to engage students in the biochemical foundations of development. For example, do changes in protein biochemistry accompany plant ontogeny?

This paper is focused on embryonic development of members of the Brassicaceae. The aim is to find out whether there exist proteins which appear to be conserved across members of the same family as the embryo differentiates into a mature seedling. This approach simplifies an understanding of the role of protein in the development of stems, leaves and roots. Depending on academic background, student teams are encouraged to use basic observation skills to begin the study of plant anatomy. The challenge of this approach is to determine whether protein biochemistry reinforces and correlates with the phenotypic traits that are traditionally used to support phylogenetic classification. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) is used to differentiate and characterize various protein bands into exact discernible patterns (Luthe, 1992). For students with little or no background in biology or plant science, the use of this technique can introduce them to the rudiments of biochemical analysis as an end in itself.

The presentation of these lab exercises also gives latitude to the instructor to pursue the use of plants to explore more complex avenues of research that can be applied to fields, like ecology. For example, modifying environmental components may also trigger developmental adaptations in the biochemistry of plants (Russo and Biles, 1995).

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Student Outline

Evo-Devo is an acronym which suggests that development of the organism reveals a genetic timetable somewhat related to taxonomic classification of that organism. The concept is complex and involves an understanding of early gene expression and how this shapes the morphogenesis and structure of the whole organism. As development continues, changes start to manifest themselves in a way which reveals new traits that will distinguish one organism from another. The underlying basis for these changes is again explained by gene expression.

Evo-Devo is a challenging concept. In order to demystify this concept, we propose a set of exercises using plant model organisms. Plants are inexpensive, readily available and their use is unlikely to trigger ethical conflicts. This study will use plant seedlings to explore developmental dynamics and evolution-based relationships (Ladizinsky and Hymowitz, 1979). The research suggested by this lab exercise will focus on the early development of members of the Brassicaceae (e.g. cabbage and broccoli).

The aim is three-fold: (1) to use polyacrylamide gel electrophoresis (PAGE) to measure total protein banding profiles of members of these plant families; (2) to determine whether the banding profiles reflect taxonomic relationships in these families and (3) to investigate whether these banding profiles change as a function of the time course of early development.

It is the objective of these investigation to help understand whether the biochemical profile (as a function of protein banding) relates to the taxonomy of the particular plant family.

Material and Methods

Sample Preparation

Seeds from different plant families are washed in dilute solution (10%) of bleach for ten minutes. These are rinsed and placed on moist filter paper in sterile Petri dishes, covered with distilled water-saturated Kimwipes® (with lid) and placed within a drawer. Seeds should be checked for moisture at least once every two days. These seedlings are harvested after each week over a three week period. Testa (seed coat) and cotyledons are removed with a scissors and forceps and the remaining whole seedlings are weighed at 0.16-0.17 g and placed in microfuge tubes (Fig. 1). Tubes are frozen at -80° C (at a minimum they were frozen overnight to disrupt cell membranes).

Preparation of the Seedling Homogenate

After thawing, distilled water is added in proportion to the weight of pooled sample (e.g. 160 µL for a 0.16 g sample). The samples are held over ice and ground using Bio-Rad mini-pulverizing tool to make a paste (Fig 2). Depending on the nature of the embryos, one might increase the total volume of water to create a visible slurry. This is briefly vortexed and centrifuged at 10,000 g (4˚C, 2 X 10 min). Supernatant aliquots are delivered into separate microfuge tubes by decanting or using a loading-pipet tip. This point can be considered a "stop point" and the samples may be frozen for later analysis.

Figure 1. Whole seedlings of cabbage, showing green epicotyl and white hypocotyl.

Protein Calibration Assay

Total protein of each sample should be determined to equilibrate samples before loading each of the wells of the precast PAGE gel. A procedure to establish this is the Bio-Rad micro-assay technique (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/LIT33.pdf). This assay is needed to construct a standard curve graphing five different concentrations of a known protein (0.1% bovine serum albumin-BSA) against optical density (Fig.3). This information will help determine the volume of sample needed to deliver an equivalent of protein (in the range of 10-50 µg) to each of the wells on the gel during loading. Constructing an Excel file with the variables of protein (BSA) concentration and optical density are used to graph the standard curve. This curve is used to determine the protein content of your unknown samples. One can then calculate the volume (µL) of the experimental sample needed to load the equivalent amount of protein for each well.

Figure 2. Seedling preparation procedure showing the use of plastic pestle to grind thawed seedlings within the microfuge tube.

Electrophoresis

Prior to electrophoresis, the stored frozen samples in labeled microfuge tubes are thawed in a beaker of tap water. The samples are then mixed under icy conditions with a thin glass pestle (engineered from melting the end of a Pasteur pipette). They are then vortexed and centrifuged for 5 minutes in a Serofuge centrifuge (3400 rpm). After calculating the volume of homogenate needed to load each well, an equal volume of modified 2X Laemmli buffer (5µL mercaptoethanol for each 95µL of this buffer) is added. The mixed sample is then delivered into separate wells on the gel; remember that each well should contain the same amount of protein. At least one lane should contain a marker, with 10 pre-stained known proteins (Bio-Rad Precision Plus Protein™ Dual Color Standards). Two SDS-PAGE protocols explaining details of the process can be found in publications from previous ABLE Conferences (Racusen and Thompson, 1996; Frame, 2000). The process of electrophoresis should be run at 150V for 40 minutes.

Data Analysis

After the electrophoretic run, gels are rinsed three times, each 15 minutes in distilled water on a rocking platform, stained with Bio-Rad Coomassie Blue Safe stain and then destained in distilled water over 2 days. Gels can be scanned (Bio-Rad ChemiDoc™ XRS scanner) and the image will reveal the position and intensity of the protein bands in each of the lanes (Fig. 5). The image can also be labeled (Fig. 4). Other scanners will perform similarly but remember that all images (sometimes stored using a different extension) must be exported to the .jpg format before presentation. Since scans of the band intensity are often quantified using software from the scanner, this data can be exported to Microsoft Excel. Once exported, the data is plotted with Excel software (Fig. 6). The values are then graphed using software which is available through Excel.

Lab Results

Afterward, each student or student team should submit the following to the lab instructor: (1) A labelled photograph (image file) of the protein banding profile of members of a particular plant family; (2) A scan of the gel lanes, showing at least one member of a family with a reference marker at the bottom; and (3) A graph of the optical densities of each band in the lane representing the proteins of one sample.

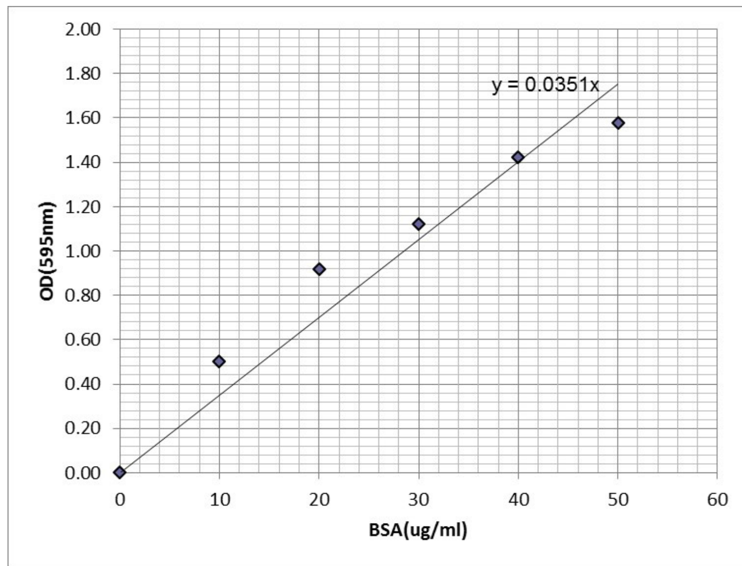

Figure 3. Standard Curve showing the relationship between bovine serum albumin (BSA concentration and optical density.

Notes for the Instructor

Who Should Use This Exercise?

This exercise can be used by students at different levels of sophistication. On an introductory level, students having little familiarity with biochemical techniques could use this exercise as a learning tool to practice electrophoresis. The exercise will introduce them to basic strategies involving tissue preparation, the importance of the standard curve, and the value of markers in establishing some protein characteristics. The lab instructor might work with the students in developing an Excel worksheet for calculating the loading volumes of each sample.

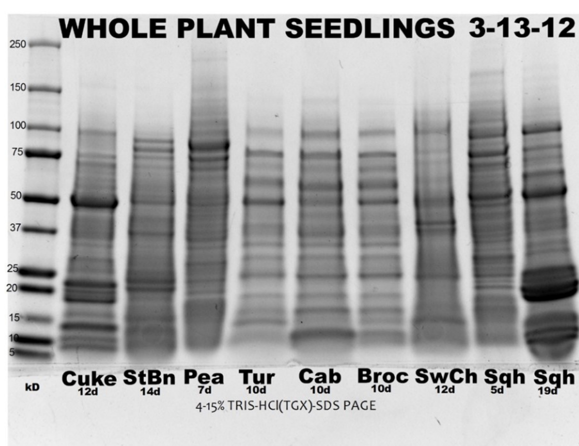

Figure 4. Protein banding of seedlings (at different ages) in members of four plant families. Note the apparent coincidental banding within the Brassicaceae (Lanes 5, 6, 7).

Students taking upper level courses in evolution and molecular biology could also use this exercise to pose questions relating protein banding profiles and taxonomic relationships. In this regard, it might be useful to divide the class into teams of 4-5, and have each team investigate members of a particular plant family. They could then quantify bands that are comparable between seedling members to see if there is more or less coincidence. Similarity coefficients (Khurshid and Rabbani, 2012) could be used to calculate the correlation of banding profiles of one sample against the others. This could provide for a lively discussion.

Questions about the Student Procedure (Student Notes)

One question which often surfaces is whether students should be involved in preparing solutions and gels. There are certainly advantages, since this training can translate into a number of different and unrelated methodologies. The other consideration deals with safety and time management.

It is important that students also be familiar with technical issues of software programs that will help in the presentation of their data in a clear, honest and unambiguous manner. For example, are the student capable of graphing the data, so that two-dimensional multi-plots can be represented one any graph? Are students familiar with tools that help to represent standard errors of the mean? It might also be useful to demonstrate scanner applications which quantify bands of particular lane scans and show how data can be imported into Excel files. One can then use the graphing feature in this program to do multi-plots.

One tool that has proved itself helpful is the "snipping tool" (provided in Microsoft Office). With the Bio-Rad ChemiDoc™ XRS scanner, some displays are not easily copied and pasted into another document. For example, if one lane scan needs to be compared to other lane scans on the same page, the tool allows highlighting each scan separately so each can be copied and pasted into the same document.

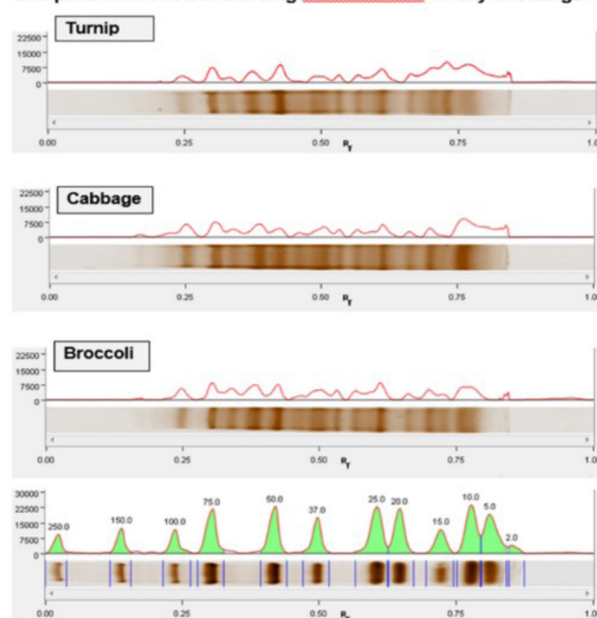

Figure 5. A comparison of total protein banding of 10-day seedlings of different members of the Brassicaceae. Standard molecular weight designations (kD) are listed at bottom.

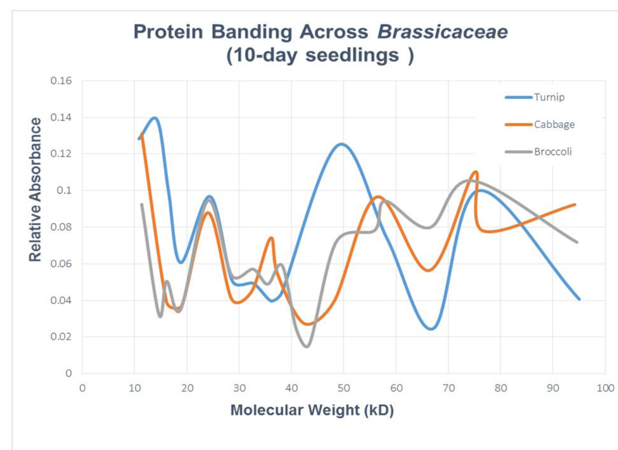

Figure 6. Graphic representation of the protein banding pattern (vs. molecular weight) of 10-day seedlings of members of the Brassicaceae. Data from Fig. 5 was used to create this graph.

Some Examples of Analyzed Data

This exercise compares the banding profiles among members of the Brassicaceae. For example, protein bands among three members of the Brassicaceae are remarkably similar. In 10-day old seedlings of turnip, cabbage and broccoli clear homologies in five molecular weight regions (5-10, 23, 37, 50 and 75 kD) (Fig.4); a scan and graph show this relationship (Figs. 5 and 6). Of course, a quantitative representation of the banding profile (similarity coefficient) might present a more convincing argument.

Acknowledgements

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