Laboratory 3
Spectrophotometric Analysis of Membrane Stability in Beet Root Cells

I. Introduction

All cells are surrounded by a plasma membrane composed of phospholipids and proteins. Additional membranes in eukaryotic cells are used to divide the cytoplasm into discrete functional compartments and to confine various metabolic processes to these compartments. Thus, the genetic material in the nucleus is separated from the cytoplasm by the nuclear envelope, the enzymes needed for respiration, photosynthesis, and oxidative processes are localized to specific organelles by the mitochondrial, chloroplast, and peroxisomal membranes, and the various protein modification and targeting reactions are confined by the membranes of the endoplasmic reticulum and the Golgi complex. In plant cells, there is usually a large central vacuole surrounded by a tonoplast membrane. The purpose of this laboratory session is to study the effects of various physical factors on the stability of these membranes using beet root cells as a model system. Beet root cells contain a pigment called betacyanin in the central vacuole. Damage to the tonoplast membrane and the plasma membrane will lead to the release of betacyanin into the surrounding solution, where it can then be detected quantitatively using a spectrophotometric assay.

As part of this lab, you will:

- prepare a series of beet root tissue segments
- visualize the location of betacyanin in beet root cells by light microscopy
- expose beet root segments to various physical or chemical treatments that can damage cellular membrane
- use a simple colorimeter or spectrophotometer to construct an absorption spectrum for betacyanin
- measure the amount of betacyanin released after each treatment quantitatively using the spectrophotometer
- prepare graphs and tables of different types as a way of summarizing the data.
- learn how to present results in the style of a scientific journal article

As you carry out the experiments, make notes, record your observations, and write down your measurements and calculations. This experiment is adapted from one originally described in Choinski, Jr., J. S. (1992) **Experimental Cell and Molecular Biology**, 2/e. WCB/McGraw Hill, Boston, MA.

II. Pre-Lab Preparation

Read the Introduction, Background Information, and Experimental Procedures for this laboratory session. Also read the sections of your Cell Biology textbook which deal with light microscopy and basic cell structure. After preparing for the lab, you should be able to answer the following questions.

A. What features distinguish a plant cell from an animal cell?

B. What are the functions of the central vacuole of a plant cell?

C. What is betacyanin? Why might some plant cells accumulate this compound?

D. What is the difference between an *in situ* and an *in vitro* assay?

E. List the four major types of biochemical assays.

F. What is the difference between the specificity of an assay and the sensitivity of an assay?

G. What is the difference between absorbance and fluorescence?

H. What is an absorption spectrum?

I. Why are absorbance measurements usually done at the wavelength of maximal absorbance?

J. What is the Beer-Lambert Law?

K. What should be included in the "blank" or reference solution that is used to set a spectrophotometer to zero absorbance?

L. What is the structure of a plasma membrane?

M. Why might heating or freezing damage a plasma membrane?

N. Why would exposure to a detergent or an organic solvent damage a plasma membrane?

O. Give the formulas for calculating the mean, variance, and standard deviation of several replicate values.

III. Background Information

A. Plant Cells

Plants are composed of eukaryotic cells, and so each cell contains a nucleus, an endomembrane system, and various membrane-bound organelles. However, most plant cells differ from other eukaryotic cells in two major ways: 1) they are surrounded by a rigid cell wall composed of cellulose and other polysaccharides; and 2) they contain a large central vacuole (Figure 3.1).

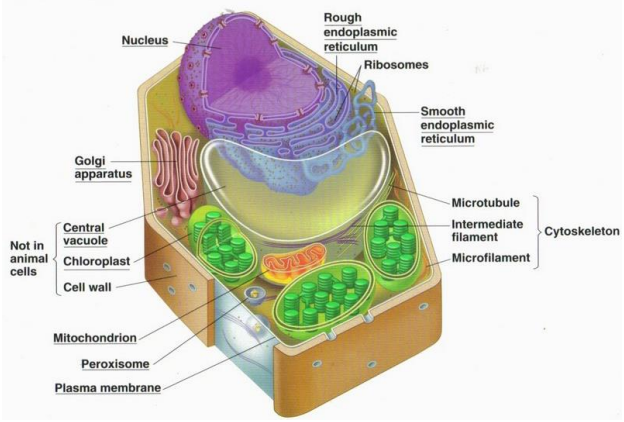


Figure 3.1. A Typical Plant Cell. A schematic drawing of a plant cell. Notice the plant cells are characterized by the absence of lysosomes and the presence of chloroplasts, a cell wall, and a large vacuole.

The central vacuole is a large storage compartment surrounded by a membrane called the tonoplast. Like other membranes, this boundary layer is composed of phospholipids and proteins. Water can move back and forth across the tonoplast membrane by osmosis but other solutes and ions are transported from one side of the membrane to the other by specific carriers. The interior of the central vacuole contains water, relatively high concentrations of ions, and various other water-soluble compounds. The vacuole serves as the primary storage site for many plant pigments including betacyanin. Betacyanin is a reddish-purple colored molecule that gives beet roots their distinctive color. It is commonly added to strawberry ice cream or yogurt to make the product more colorful (Figure 3.2).

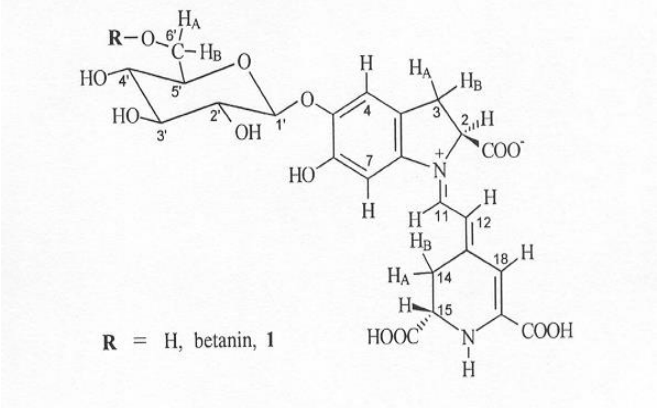


Figure 3.2. Structure of a betacyanin molecule.

The central vacuole also serves as a waste compartment for degraded molecules and cellular debris. The central vacuole is formed by the coalescence of smaller vacuoles and so varies in size as plant cells grow and mature. In young cells that are actively dividing, the central vacuole is relatively small. In older cells, such as those in storage tissues like roots or in photosynthetic tissues like leaves, the central vacuole is much larger. The relative volumes of various plant organelles in young and mature cells are given in Table 3.1.

Table 3.1. The Relative Volumes of Organelles in Plant Cells

Organelle	Young Cell	Mature Cell
Nucleus	32.4	0.23
Vacuole	4.93	83.3
Mitochondria	5.35	1.01
Plastids	3.72 (proplastids)	3.16 (chloroplasts)
Dictyosomes	0.40	0.04
Hyaloplasm	52.9	12.1
Lipid	0.23	0.00

Young cells tend to be small with large nuclei; as they grow and differentiate, the cell volume increases and certain organelles become more prominent, depending on the specialization of the cell. The relative volumes of organelles (the volume of the organelle expressed as a percentage of the volume of the whole cell) are given here for a young cell from a shoot tip and for a mature, photosynthetic cell from the outer part of the stem. The size of the nucleus in both cells is the same, but the mature cell is so large that the nucleus constitutes only a small part of it, whereas the young cell is so small that the nucleus occupies one third of its volume. These values are for a cactus, *Echinocereus*.

B. Biochemical Assays

The molecules found in a living organism, such as the beet root pigment betacyanin, can be analyzed in two general ways. The first is through in situ studies, that is, through the use of whole cells or tissues whose structure has been altered as little as possible by experimental manipulation. Because cells are usually quite small in size, in situ biochemical analysis is often coupled to light or electron microscopy. The second is through in vitro studies, that is, through the use of cells or tissues that have been disrupted and fractionated in one way or another into different chemical classes. This approach is commonly used in cell biology because it permits both the quantitative analysis of biomolecules and their complete purification and characterization. However, in vitro studies must ultimately be coordinated with other types of experiments to give an accurate picture of cell structure and function.

To detect, quantify, or isolate a particular type of molecule, it is necessary to have a method or procedure for measuring it. This procedure is called an assay. Although many different assays are used in biology, they fall into four major categories: 1) spectrophotometric assays, 2) radiochemical assays, 3) activity assays, and 4) immunological assays.

1. **Spectrophotometric assays.** This type of assay involves exposing molecules to light and then measuring either the resulting absorption or fluorescence. Light absorption or fluorescence may result directly from the intrinsic chemical properties of the molecules of interest. Alternatively, they may occur indirectly as a result of treating the molecules of interest with other compounds which react with them to create new chemicals that exhibit absorption or fluorescence.

2. **Radiochemical assays.** This type of assay is based on the incorporation of a radioactive isotope such as ^{3}H, ^{14}C, ^{32}P, or ^{35}S into the molecules of interest. The molecules then can be detected or traced through the release of energy in the form of beta-particles (high-energy electrons) or gamma-radiation.

3. **Activity assays.** This type of assay involves measuring the activity or effect of the molecules of interest. Most enzymes are detected by their ability to catalyze a specific chemical reaction and most transport proteins are detected by their ability to bind a substrate or to translocate it across a cellular membrane. In these cases, the amount of the molecule of interest (the enzyme or binding protein) is inferred from the amounts of other molecules (for example, an enzyme's substrates or products).

4. **Immunological assays.** This type of assay is based on the use of antibody proteins that bind specifically to the molecules of interest. Binding of antibodies may lead to the formation of a visible precipitate, a radioactive complex, or an enzyme-linked complex that can catalyze a chemical reaction.

In using any type of assay, four major factors need to be considered: 1) specificity, 2) sensitivity, 3) quantitative characteristics, and 4) convenience and reproducibility.

1. **Specificity.** The usefulness of an assay depends in part on the number of different biomolecules that give a positive result. The spectrum of reactive molecules may be relatively broad (for example, all phospholipids) or relatively narrow (for example, only proteins that catalyze the oxidation of lactate).

2. **Sensitivity.** The value of an assay also depends on quantitative range within which a positive reaction occurs. Some assays are relatively insensitive and require milligram (10^{-3} g) or

millimole (10^{-3} mole) amounts of material. Other assays are more sensitive and can detect molecules in the microgram (10^{-6} g) or micromole (10^{-6} mole) range, the nanogram (10^{-9} g) or nanomole (10^{-9} mole) range, or even the picogram (10^{-12} g) or picomole (10^{-12} mole) range. The sensitivity of an assay is often affected by other molecules in the solution such as hydrogen ions, salts, or detergents.

3. **Quantitative characteristics.** For an assay to be used quantitatively, there must be a direct relationship between the amount of the molecule being assayed and the reaction or response that is detected. Most assays give a linear response only within a limited range of amounts. Assays that give nonlinear responses are much more difficult to use.

4. **Convenience and reproducibility**. Ideally, an assay should be relatively easy and inexpensive to perform, and should give results that are consistent from experiment to experiment. High costs or complex protocols may make it difficult to carry out large numbers of assays, and poor reproducibility may make the data meaningless.

The choice of assays for a given experiment depends on the questions to be answered and the molecules to be detected. No assay procedure is ever perfect, and even published protocols often must be modified to fit a particular experimental situation.

C. Spectrophotometric Assays

Of the assays used for detecting biomolecules, spectrophotometric assays are the most common. A simple spectrophotometric assay for beet betacyanin will be used in this experiment to measure the integrity of beet root cellular membranes. Spectrophotometric assays are based on the interactions of light with specific molecules. Light is a form of electromagnetic radiation and can interact with matter in a number of different ways, leading to differences between the incident radiation and the transmitted radiation. For spectrophotometric biochemical assays, the most important interactions are absorption, which leads to a change in the intensity or amplitude of the transmitted light, and fluorescence, which leads to a change in the energy or wavelength of the transmitted light so that it has a different color.

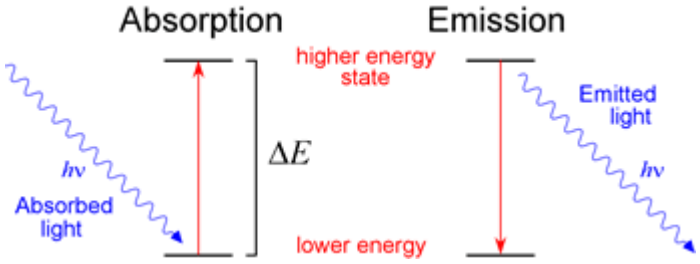


Figure 3.3. Diagram showing the effect of light on a molecule.

Each molecule can be thought of as having a series of alternative electronic states (Low energy and high energy), which are determined by its chemical structure (Figure 3.3). Prior to exposure to light, most molecules will be in their lowest energy or ground state. Upon irradiation with light, some of the molecules may absorb light energy, elevating them to a higher energy or excited state. These excited molecules can then go through a process of relaxation and return to the ground state. Relaxation can occur in two ways: 1) by internal conversion, which involves gradual dissipation of the absorbed energy by transferring it to other molecules or the solvent; and 2) by fluorescence, which involves releasing the energy as a burst of electromagnetic radiation at a lower energy (longer wavelength). Internal conversion is much more common than fluorescence, particularly for those molecules found in living

organisms. Both light absorption and relaxation are very rapid processes whose speed is usually measured in nanoseconds (10^{-9} seconds) or picoseconds (10^{-12} seconds).

Absorption of light can only occur if the energy of the incident radiation exactly matches the energy difference between the ground state of the molecules of interest and one of the excited states. Since the energy of light is related to its wavelength, this means a molecule can only absorb light of certain wavelengths. A graph showing the amount of light absorption as a function of wavelength is called an absorption spectrum. Because there are actually several alternative forms at each electronic state or level, an absorption spectrum usually consists of a series of relatively symmetrical peaks (Figure 3.4).

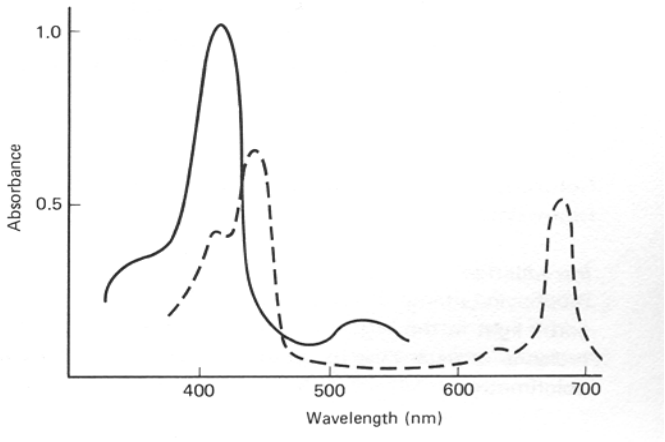


Figure 3.4. The absorption spectra of cytochrome c (solid line) and chlorophyll a (dashed line). Cytochrome c is a protein in mitochondria while chlorophyll a is a pigment in chloroplasts.

The absorption characteristics of a particular molecule can be summarized in terms of the wavelengths at which there is maximal absorption (λ_{max} values). In most cases, quantitative measurements are done at these wavelengths because that is where the assay is most sensitive. Those molecules that exhibit fluorescence following light absorption have both a characteristic absorption spectrum and a characteristic emission spectrum. The latter spectrum indicates the wavelengths of the fluorescent light and usually consists of one or two relatively broad peaks.

D. Quantitation of Absorbance

The total amount of light absorbed at any particular wavelength (the absorbance) is determined by three factors: 1) the absorption characteristics of the molecules of interest; 2) the pathlength or distance through which the light must travel; and 3) the concentration of the absorbing molecule. The absorption of light is usually measured in an aqueous solution, where the intensity of the light passing through the sample decreases exponentially with the thickness of the water layer. The intensity of the light passing though the sample also decreases exponentially with the concentration of the solute. These factors are summarized in the following expression, which is called the Beer-Lambert law:

$$\mathbf{A} = \log_{10} \frac{\mathbf{I_0}}{\mathbf{I}} = \mathbf{E\,c\,l}$$

where **A** is the absorbance of the solution, $\mathbf{I_0}$ is the intensity of the incident light, and **I** is the intensity of the transmitted light; **E** is the molar extinction coefficient**; c** is the concentration of the absorbing solute; and **l** is the pathlength of the light.

The concentration of the solute (**c**) is usually expressed in moles/liter (M) and the pathlength of light (**l**) is expressed in cm. The molar extinction coefficient (**E**) is an intrinsic characteristic of each molecule at a particular wavelength. It is numerically defined as the absorbance of a 1.0 M solution of the molecule of interest in a 1.0 cm light path. Because E has the units of liter cm^{-1} $mole^{-1}$, absorbance itself is a parameter with no units. The larger the value of E, the more a compound absorbs at a particular wavelength.

Because molecules vary in structure and have different electronic states, they have characteristic absorption or emission spectra and unique extinction coefficients. Some biologically-important compounds absorb light in the visible region of the spectrum (400-700 nm), some in the ultraviolet (UV) region of the spectrum (200-400 nm), and some in the infrared (IR) region of the spectrum (700-1000 nm). Fluorescence usually occurs in the visible or infrared region. Biochemical assays for biomolecules are usually based on absorption in the UV or visible region and fluorescence in the visible region.

Quantitative assays based on the absorption or fluorescence of light are usually performed using a spectrophotometer or spectrofluorometer. These instruments usually compare the absorption or fluorescence of a solution containing the compound of interest (the experimental sample) to one that does not contain that compound (the reference sample or blank) (Figure 3.5). The difference in amount of light absorbed or emitted can be expressed as an absorbance value, a percentage of the incident light transmitted, or a relative fluorescence.

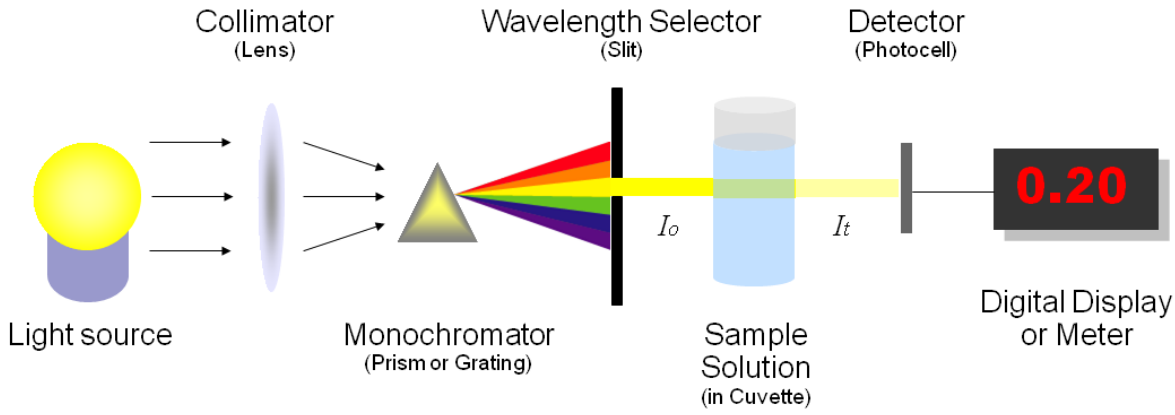


Figure 3.5. The major components of a spectrophotometer.

Light is produced from a lamp, which usually has a tungsten filament bulb for light in the visible region and a deuterium discharge bulb for light in the UV region. The light then enters a monochrometer, which splits the light into different wavelengths using a prism or diffraction grating. Light of a selected wavelength then passes into the sample compartment. Some spectrophotometers are single-beam instruments which have only one light path. The reference and experimental samples are compared by moving first one into the light path and then the other. Other spectrophotometers are double-beam instruments in which the monochromatic light is split into two beams that pass simultaneously through the reference and experimental samples. Light transmitted by a sample then enters a detector, which is usually a photomultiplier tube that converts light energy into an electric current. The current coming from the experimental sample is compared with that coming from the reference sample and the result displayed on a digital or analog meter, a cathode ray tube, or a recorder. Fluorescence measurements are made in a similar way. A spectrofluorometer has the same basic components as a spectrophotometer, but it also has a second monochrometer to select the wavelengths of emitted light that are detected and used in the measurements. In most spectrofluorometers, the detector is positioned at a 90 degree angle from the sample to avoid the effects of normal transmitted light. Many different types of spectrophotometers and spectrofluorometers are commercially available.

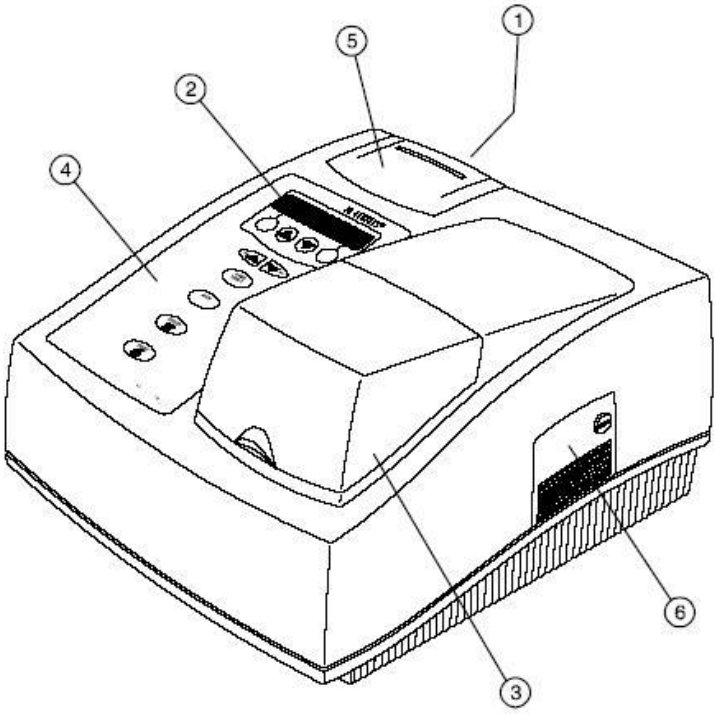


Figure 3.6. Components of a Spectronic 20 Genesys Spectrophotometer. (1) On/Off Switch, (2) LCD Display, (3) Sample Compartment Door, (4) Keyboard, (5) Optional Printer, (6) Lamp Compartment Door.

The instrument should be used in the following way:

1. Be sure the power cord is plugged into a grounded 120 Volt outlet.

2. Turn on the power switch on the back of the instrument. The instrument will go through a short power-up sequence that takes about 2 minutes. Then allow the instrument to warm up for 15 minutes before taking any readings.

3. Press the A/T/C button on the key pad to select absorbance.

4. Press the nm(UP) or nm(DOWN) buttons on the key pad to select the wavelength to be used.

5. Lift up the cover of the sample compartment and insert a test tube or plastic disposable cuvette containing the reference solution. Be sure to wipe the outside of the tube or the cuvette first with a Kim-Wipe to remove any liquid or fingerprints. Be sure to insert the cuvette correctly so that light passes through the clear walls. Close the cover of the sample compartment.

6. Press the 0 ABS/100% T button on the key pad to set the instrument to 0 absorbance. The zero reading will appear on the LCD display.

7. Remove the tube or cuvette with the reference solution and insert another tube or cuvette containing the sample solution. Again, be sure the outside is clean and is oriented correctly.

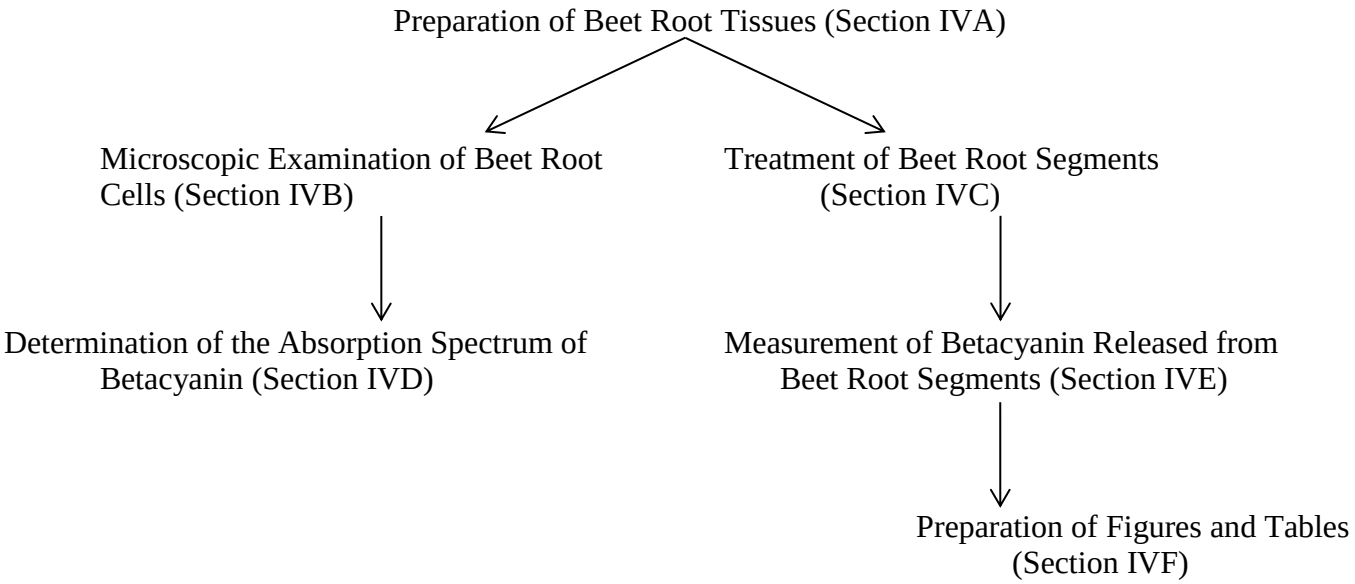
8. The absorbance of the solution will appear on the LCD display. Record the number in your lab notebook.

9. Continue to take readings as necessary.

IV. Experimental Procedures

This experiment involves several parts. To carry out the lab work efficiently, it will be helpful to divide up the tasks within the group and to work on one part of the experiment while you are waiting for something to happen with another part of the experiment. For example, you can look at the slides of the beet root cells microscopically while washing the beet root segments or determining the absorption spectrum of betacyanin. Someone can determine the absorption spectrum of betacyanin while others are doing the beet root treatments.

The following is a flow chart for this laboratory session:



A. Preparation of Beet Root Segments

The objective of this part of the lab is to prepare a series of beet root segments that can be used to study the integrity of the cellular membranes under different conditions. Breakage of the cellular membranes in these segments will lead to the release of betacyanin, which can then be measured in a spectrophotometer.

1. Select a large red beet root and wash it carefully with cold tap water. Rinse the root several times with distilled water and dry it with paper towels.

2. Using a cork borer about 1/4 inch in diameter, remove a series of core samples. You will need enough root tissue to make about 40 cylindrical tissue samples that are **2 cm** in length. Using a ruler and a single-edge razor blade, divide the cored material into a series of **2 cm** segments.

3. Once about 40 segments have been prepared, place them in a 250 ml beaker of tap water and wash the segments under running tap water for about 10 minutes to remove any betacyanin from the cut surfaces.

4. Remove the beet root segments from the beaker and pat them dry with a paper towel.

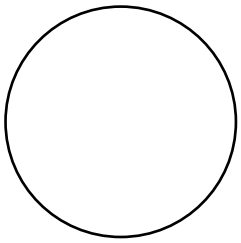
B. Microscopic Observation of Beet Root Cells

The objective of this part of the experiment is to locate betacyanin in beet root cells and to visualize the central vacuole. This will be done as a **demonstration**. Refer back to **Laboratory 2 (Introduction to Microscopy)** for more information about light microscopy.

1. Leica DME microscopes like those you used last week will be set up on the side benches with slides of wet mounts of thin sections of beet root tissue.

2. Look at the slide with the beet root tissue suspended in water at a magnification of 400X. Make a drawing of what you see. **Indicate the total magnification and include a description of your observations next to the drawing.** Where is the reddish-purple betacyanin pigment located?

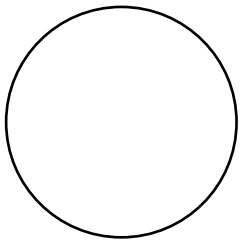
   Distilled Water:

   Total Magnification _____X

3. Now look at the slide with the beet root tissue in a drop of 2.0 M NaCl. **Make a drawing of what you see. Indicate the total magnification and include a description of your observations next to the drawing.** What happened to the cells in the presence of the NaCl? What does this tell you about the location of the betacyanin?

   2.0 M NaCl:

   Total Magnification_____X

4. What is the significance of your observations of beet root tissue in distilled water and 2.0M NaCl?

C. Treatment of Beet Root Segments

The objective of this part of the experiment to treat the beet root segments in various ways that are likely to cause damage to the membranes and result in the release of betacyanin. The treatments will include: 1) freezing and thawing; 2) heating at various temperatures for one minute; 3) exposure to organic solvents; and 4) exposure to detergents. Three replicates of each treatment will be performed in order to test for the consistency of the results.

1. As a control for this experiment, set up a series of three (3) 13 x 100 mm tubes and label them 25-1, 25-2, and 25-3. The samples in these tubes will be held at room temperature (about 25^{o}C) and the absorbance due to any betacyanin in them will be subtracted from the other samples. Using a 5 ml pipet and a green pipet pump, add 5 ml of distilled water to each tube.

2. With forceps, carefully pick up one of the 2 cm beet root segments and transfer it to the tube labeled 25-1. Repeat the process with two more segments and added them to the tubes labeled 25-2 and 25-3. Allow the tubes to sit at room temperature for at least 30 minutes. Periodically, cover the top of each tube with a piece of Parafilm and invert it several times to mix the segment with the solution.

3. For the freezing/thawing (F/T) treatment, transfer some crushed dry ice to a 100 or 150 ml beaker. With a forceps, carefully pick up one of the 2 cm beet root segments and bury it in the dry ice. Repeat the process with two more segments. Allow the segments to sit in the dry ice for 10 minutes.

4. Set up a series of three (3) 13 x 100 mm tubes and label them F/T-1, F/T-2, and F/T-3. Using a 5 ml pipet and a green pipet pump, add 5 ml of distilled water to each tube.

5. Remove one of the frozen segments with a pair of forceps and place it in the tube labeled F/T-1. Then remove the other segments and place them in the tubes labeled F/T-2 and F/T-3. Allow the segments to remain in the tubes at room temperature for at least 30 minutes. Periodically, cover the top of each tube with a piece of Parafilm and invert it several times to mix the segment with the solution.

6. For the heating experiment, set up a new series of 18 13 x 100 mm test tubes. Label the tubes as follows:

75-1	75-2	75-3
70-1	70-2	70-3
65-1	65-2	65-3
60-1	60-2	60-3
55-1	55-2	55-3
50-1	50-2	50-3

   Add 5 ml of distilled water to each tube.

7. Add approximately 900 ml of water and a stir bar to a 1000 ml beaker. Place a thermometer in the beaker and heat the water with continuous stirring on a hotplate until the temperature reaches 75^{o}C. Carefully remove the beaker from the hot plate and set it on the bench top. With a forceps, place three of the 2 cm beet root segments in a tea strainer. Close the strainer and hold it in the hot water for exactly 1 minute. Then open the strainer and place one segment in the tube labeled 75-1, one in the tube labeled 75-2, and one in the tube labeled 75-3.

8. Allow the water to gradually cool on the bench top and check the temperature periodically. When the temperature drops to 70^{o}C, expose three beet root segments to this temperature for 1 minute using the tea strainer and add them to tubes 70-1, 70-2, and 70-3. When the temperature drops to 65^{o}C, expose three beet root segments to this temperature for 1 minute and add them to tubes 65-1, 65-2, and 65-3. Continue the process until you have done all of the heat treatments.

9. Allow all of the tubes to sit at room temperature for at least 30 minutes. Periodically, cover the top of each tube with a piece of Parafilm and invert it several times to mix the segment with the solution.

10. For the organic solvent treatment, set up a new series of six (6) 13 x 100 mm tubes. Label the tubes as follows:

    E-1 E-2 E-3
    I-1 I-2 I-3

    Add 5 ml of 95% ethanol (E) to the first three tubes and 5 ml of 70% isopropanol (I, rubbing alcohol) to the second three tubes.

11. With a forceps, carefully pick up one of the 2 cm beet root segments and transfer it to the tube labeled E-1. Repeat the process with two more segments and add them to tubes E-2 and E-3. In the same way, add 2 cm beet root segments to tubes I-1, I-2, and I-3. Allow the tubes to sit at room temperature for at least 30 minutes. Periodically, cover the top of each tube with a piece of Parafilm and invert it several times to mix the segment with the solution.

12. For the ionic detergent treatment, set up a new series of three (3) 13 x 100 tubes and label them SDS-1, SDS-2, and SDS-3. Add 5 ml of 1% sodium dodecyl sulfate (SDS) to each tube. SDS is an ionic detergent commonly used in cell biology to denature proteins and to disrupt cellular membranes.

13. With forceps, carefully pick up one of the 2 cm beet root segments and transfer it to the tube labeled SDS-1. Repeat the process with two more segments and add them to tubes labeled SDS-2 and SDS-3. Allow the tubes to sit at room temperature for at least 30 minutes. Periodically, cover the top of each tube with a piece of Parafilm and invert it several times to mix the segment with the solution.

14. For the nonionic detergent treatment, set up a new series of three (3) 13 x 100 tubes and label them TX100-1, TX100-2, and TX100-3. Add 5 ml of 1% Triton X-100. Triton X-100 is a nonionic detergent commonly used in cell biology to extract proteins from cellular membranes.

15. With forceps, carefully pick up one of the 2 cm beet root segments and transfer it to the tube labeled TX100-1. Repeat the process with two more segments and add them to tubes labeled TX100-2 and TX100-3. Allow the tubes to sit at room temperature for at least 30 minutes. Periodically, cover the top of each tube with a piece of Parafilm and invert it several times to mix the segment with the solution.

16. The time during which the treated beet root segments sit in water is not too critical, but it should be about the same for all of the treatments.

D. Determination of the Absorption Spectrum of Betacyanin

The exposure of the beet root segments to different treatments will result, in some cases, in the release of betacyanin into the surrounding solution. To measure the amount of this compound in the solution quantitatively with a spectrophotometer, you will need to know what wavelength to use. The objective of this part of the lab is to construct an absorption spectrum of betacyanin and to determine the wavelength of maximal absorbance. You will do this by measuring the absorbance of a betacyanin solution at 20 nm intervals from 440 nm to 620 nm. You should do this part of the experiment while the treated beet root segments are sitting for 45 minutes in water or the other solutions.

1. Turn on a Genesys 20 spectrophotometer and allow it to warm up for 15 minutes. The instructor will demonstrate how to use this type of instrument and show you which cuvettes to use.

2. To determine the absorption spectrum of betacyanin, you will use a beet root extract that was prepared by the instructor. Some chunks of beet root were combined with water and homogenized in a blender to disrupt the cells. The resulting liquid was filtered first through cheesecloth and then through filter paper.

3. Using a plastic transfer pipet, transfer about 3 ml of distilled water to one plastic cuvette and about 3 ml of the beet root extract to another plastic cuvette. The first cuvette will serve as a "blank" or reference solution and the second will be the experimental solution.

4. Set the spectrophotometer to a wavelength of 440 nm. Place the cuvette with the water into the sample compartment and set the instrument to zero absorbance. Remove the cuvette and replace it with the cuvette containing the beet extract. **Measure the absorbance of the solution and record your results in the following table.**

Table ___ Absorption Spectrum of Betacyanin

Wavelenth(nm)	Absorbance (A)
440	
460	
480	
500	
520	
540	
560	
580	
600	
620	

5. Remove the cuvette with the beet extract and reset the wavelength of the instrument to 460 nm. Insert the cuvette with the water and set the absorbance again to zero. Remove the cuvette, replace it with the cuvette with the beet extract, and measure the absorbance at this new wavelength.

6. Repeat the process of measuring the absorbance of the beet extract at **20 nm** intervals until you get to a wavelength of 620 nm. **NOTE: YOU WILL NEED TO RESET THE INSTRUMENT TO ZERO ABSORBANCE WITH THE REFERENCE SOLUTION EACH TIME YOU CHANGE THE WAVELENGTH.**

7. When all of the absorbance values have been determined, use a piece of linear graph paper or Excel to make a graph in which you plot absorbance on the Y axis as a function of wavelength on the X axis. Determine the wavelength of maximal absorbance and use this wavelength for the rest of the experiments today.

   Wavelength of Maximal Absorbance = _____nm

E. Measurement of Betacyanin Released from Beet Root Segments

The objective of this part of the experiment is to measure the amount of betacyanin released from the beet roots after the various treatments. The absorbance values for the replicate samples will be averaged to get a mean. The value for the control samples 25-1, 25-2, and 25-3 will be subtracted from the other values to determine the specific effect of each treatment. The maximum absorbance that can be read accurately with the Genesys 20 spectrophotometers is 2.0. Samples with higher absorbance values will usually cause the instrument to read >2.XXX and to flash on and off. **If you find that a particular sample is too dark to read accurately you will need to make a dilution of it (as described in #4).**

1. Set the Genesys 20 spectrophotometer to the wavelength found to give the maximal absorbance with the beet root extract. Using the reference cuvette that contains just water, reset the instrument to zero absorbance.

2. After each of the treated segments has been allowed to sit in the surrounding aqueous solution for at least 30 minutes, transfer the liquid to a cuvette and read the absorbance. You can either carefully pour the liquid from the tube into the cuvette or transfer the solution to the cuvette with a plastic transfer pipet. It is not necessary to rinse out the cuvette between replicate samples, but do rinse it with water between different samples.

3. Read the 25^{o}C samples first since these are the controls. For each treatment, record the absorbance values for the three replicate beet root segments and calculate the mean. **Then subtract the value for the 25^{o}C control samples from all of the other treatments to get the net absorbance due to each type of treatment.** Then calculate the standard deviation for the replicates of each treatment. Record this information in the following table.

Table _____ Absorbance of Betacyanin Solutions at _____ nm after Various Treatments

Treatment	1	2	3	Mean	Net absorbance	Standard Deviation
25°C					0	
F/T						
75°C						
70°C						
65°C						
60°C						
55°C						
50°C						
Ethanol						
Isopropanol						
SDS						
TX100						

4. The maximum absorbance that can be read accurately with the Genesys 20 spectrophotometers is 2.0. Samples with higher absorbance values will usually cause the instrument to read >2.XXX and to flash on and off. **If you find that a particular sample is too dark to read accurately and give an absorbance >2.0, make a 1/10 dilution of it.** Add 4.5 ml of water to another clean tube and then add 0.5 ml (500 µl) of the sample to it. Mix the solution carefully and then read the absorbance again. Be sure to rinse out the cuvette with water first. To calculate the absorbance of the original solution, multiply the observed absorbance by 10. **If you diluted any of the samples, indicate which were diluted and record the calculated absorbance value for the original solution.**

F. Preparation of Tables and Graphs

The data obtained in an experiment such as this one can be summarized in several ways. For quantitative results, the most useful methods are as tables or figures (graphs). Tables compress data into a compact form while figures express data visually. In a scientific paper, a particular data set might be presented as a table or a figure but never as both. The objective of this part of the experiment is to summarize the results for the release of betacyanin from beet root tissues. The various references in the section on Lab Reports all give good advice on the preparation of tables and graphs. You can either make graphs using photocopies of graph paper or using a software program like Excel that you learned about in **Laboratory 1**. Include a copy of the graph after this page in your manual. However, for your paper you will need to create your graphs in Excel.

1. Since the freeze/thaw, solvent, and detergent treatments are single independent treatments, the best approach to presenting the data is to make either a table or a bar graph. Use the values for the net absorbance after each treatment and fill in the following table. Note that in a table, values for the same property are presented in vertical columns.

   Table _____ (Include a title) _____

Treatment	Net absorbance* (_____ nm) +/- SD
Freeze/thaw	
Ethanol	
Isopropanol	
1% SDS	
1% Triton X-100	

   *n=3

2. An alternative way to presenting the same data might be as a bar graph, in which you plot the net absorbance at a certain wavelength on the Y axis as a function of each treatment on the X axis. Bar graphs work well if you have discrete independent variables such as different treatments. Be sure to include both the mean and the standard deviation for each treatment.

3. To determine which would be better to include in your paper think about what you want to be able to tell your reader based on the data. If there are trends you want to be able to point out a graph may be more appropriate than a table. However, if they need the data itself to make sense of what you plan to describe then it might be better to include a table

4. Since the temperature experiment involved a constant exposure time (1 minute) at different temperatures, the data from this experiment might best be presented as a graph in which you plot net absorbance at a certain wavelength on the Y axis as a function of temperature on the X axis. Using a piece of linear graph paper, create a graph of this type. If you use Excel to make this graph, be sure to use an X-Y scatter plot. Be sure to include both the mean and the standard deviation for each temperature.

5. Based on these summaries of the data, which treatment caused the most damage to the cellular membranes? What were the effects of different temperatures on membrane stability?

G. Post Lab Analysis

In addition to a data sheet for Lab 3, you will **write up the Results section for Lab 3**.

The **Results** section should provide a summary of the results. It should consist of a written text that refers to individual figures and tables where appropriate. This section comprises the crux of the research and so is the heart of the paper. The legends/captions of the figures and tables contain useful information about experimental details and procedures, the number of replicates used, and sometimes, interpretive information. As you gain more experience, this is often the section that you will look at before reading (or not reading) the rest of the paper. To understand a figure you should be able to re-draw it and explain it in words understandable to anybody. This is largely the purpose of the text of the Results section. In a well-written paper, the conceptual flow of the series of experiments will also be included in the text of the Results section. Some papers present Results without extensive discussion; others contain extensive discussion in the results. The Results section is often divided into carefully labeled subsections.

Results Section Checklist

- Body (text) in which you describe the data in each visual:
  - Describe the trend, rather than listing the actual numbers
  - Refer to each table and figure by number in parentheses at the end of the first sentence in which you describe that visual
  - Do not give possible explanations for the results
- Visuals (tables and graphs):
  - Do not include raw data; instead reduce and summarize the data
  - Do not include both a table and a figure for the same data.
  - Position the visual immediately after the paragraph in which you first describe it
  - Give each figure and table a caption that consists of a number and a short, descriptive title. The title should enable the reader to understand the visual without having to refer to the body of the Results section.
    - Figure captions go *below* the figure
    - Table captions go *above* the table

The following results section illustrates several key points to remember when writing a results section. The results are from the following article:

Frost, C.J., Nyamdari, B., Tsai, C.J., and Harding, S.A. 2012. The Tonoplast-Localized Sucrose Transporter in Populus (PtaSUT4) Regulates Whole –Plant Water Relations, Responses to Water Stress, and Photosynthesis. PLOS One. 7(8): e44467.doi: 10.1371/journal.pone.0044467.

The full-text version of this article can be found at:
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0044467#s3

Results are written in past-tense.

(Figure 1C). The SRWC change in plant-free pots was negligible (Figure 1B). Significant changes in growth metrics were not expected to occur as a result of the short, acute treatment. The SUT4-RNAi and wild-type plants used for the experiment exhibited similar average height growth increments of approximately 2.5–3 cm/day, depending on the cohort (Table 1). Total leaf area, leaf:root ratio, and the ratio of stem diameter to height were largest in SUT4-RNAi plants (Table 1). Stem mass was comparable between SUT4-RNAi and wild-type plants, but water comprised a higher percentage of fresh stem weight in SUT4-RNAi plants (Table 1).

For the chronic less severe drought treatment, plants were maintained for two months under well-watered versus water-limiting conditions. In general, RNAi plants were more sensitive than wild-type plants to prolonged differences in SRWC. Leaf

Results

Water Utilization and Biomass Accumulation

In the acute drought experiment, water was withheld from potted plants until the onset of leaf turgor loss. Plant water uptake assessed by the rate of SRWC decrease preceding turgor loss was slower in RNAi plants (Figure 1A–B). In addition, the onset of turgor loss occurred one day later in RNAi than wild-type plants

Results are described in text, not only in figures or tables.

Tables and Figures are referenced in parentheses.

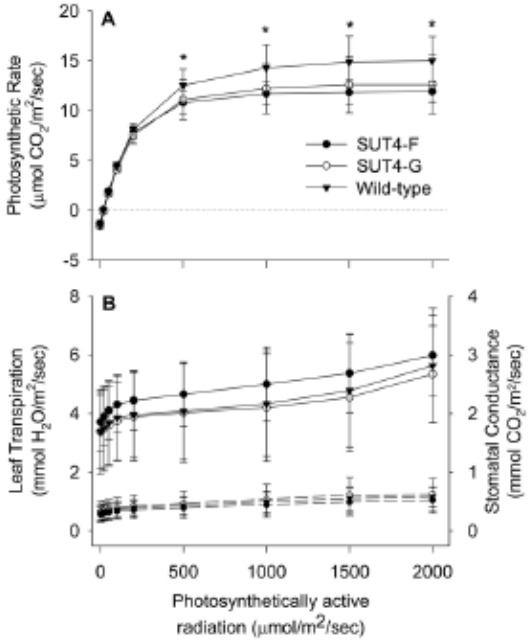


Figure 3. Leaf gas exchange properties of *Populus* wild-type and RNAi plants. Light response curves for (A) Photosynthetic CO_2 fixation, and (B) Leaf transpiration (solid lines) and stomatal conductance (dashed lines). A fully expanded source leaf (LPI-10) was used for the measurements. Data points are means ± SD of 19 WT, 10 line G, and 12 line F plants. * $p \leq 0.05$ for each transgenic line compared to wild type as determined by Student's *t*-test.
doi:10.1371/journal.pone.0044467.g003

areas were higher in SUT4-RNAi than wild-type plants under well-watered conditions, but suffered greater decreases under water-limiting conditions (Table 2). Leaf water concentrations (% dry mass basis) were lower in SUT4-RNAi than wild-type plants with reduced SRWC, but specific leaf dry mass was unaltered by SUT4 perturbation or water regime (Table 2). Furthermore, height growth was most reduced under low SRWC in SUT4-RNAi plants (Figure 2A), while diameter growth rate was reduced under low SRWC in all plants (Figure 2B). Wood and bark water concentrations were higher in well-watered SUT-RNAi plants than in wild-type, but no difference between genotypes was observed under low SRWC (Figure 2C–D).

Leaf Gas Exchange and Chlorophyll Concentration

Basal respiration, quantum yield and light compensation point were similar between well-watered SUT4-RNAi and wild-type plants used in the acute drought experiment, but photosynthesis (A_{max}) was reduced in the SUT4-RNAi plants (Table 3). A_{max} was also lower in well-watered SUT4-RNAi plants than wild-type plants in the chronic drought experiment (Table 4; Figure 3A). A_{max} increased under water-limiting conditions in the SUT4-RNAi plants, but was stable across moisture regimes in wild-type plants (Figure 3A; Table 4). C_i varied as expected in accordance with changes in A_{max} due to carboxylation limitation, and was highest when A_{max} was lowest (Table 4). In contrast, Gs and E were not different between RNAi and wild-type leaves under normal watering conditions, and decreased similarly in all plants during water-limited growth (Figure 3B; Table 4). Concentrations of chlorophyll a, chlorophyll b, and their ratio were also not affected by SUT 4 perturbation, though they all were responsive to soil moisture availability (Table 4, Figure S3).

Foliar Suc and RFO Responses to Contrasting SRWC

Suc concentrations were higher in mature leaves, xylem and phloem/bark tissues of SUT4-RNAi plants than wild type under well-watered conditions (Figure 4A; Table S1). Under chronic water-limiting conditions, leaf expression of *PtaSUT4* was substantially down-regulated in wild-type plants (Figure 4B), and leaf Suc concentrations increased by 59%, nearly matching levels observed in leaves of the RNAi plants (Figure 4A). Fru and Glc also responded strongly to water-limiting conditions independent of SUT4 expression (Figure 4C–D). Ino (an RFO precursor derived from Glc [38]) concentrations were more strongly induced in wild type than SUT4-RNAi plants experiencing water-limitation (Figure 4E). Under the same conditions, xylitol and the RFOs Gol and Raf, were sharply up-regulated in wild type, but not SUT4-RNAi plants (Figure 4F-H). Furthermore, Ino/Glc ratios were not affected by SUT4 perturbation, but Gol/Ino and

Results are subdivided into clearly labeled subsections.

Table 4. Comparison of gas exchange parameters among well-watered wild-type and transgenic *Populus* used in the acute drought experiment.

	WT	SUT4-G	p^1	SUT4-F	p
A_{max} (mmol/m²/s)	**14.62±2.43**	**12.48±2.75**	**0.025**	**11.81±1.11**	**0.001**
Conductance (mmol/m²/s)	0.54±0.20	0.61±0.31	0.360	0.52±0.24	0.673
Transpiration (mmol/m²/s)	4.77±1.34	5.37±1.92	0.241	4.55±1.85	0.653
Respiration (mmol/m²/s)	−1.47±0.31	−1.59±0.24	0.401	−1.31±0.32	0.087
Quantum yield (mol CO_2/mol PAR[2])	0.06±0.00	0.06±0.00	0.316	0.06±0.00	0.378
Light compensation point (mmol/m²/s PAR)	21.98±4.38	24.60±2.72	0.106	20.38±4.00	0.164
Photosynthetic WUE[3] (mmol CO_2/mol H_2O)	3.34±1.08	2.75±1.28	0.173	2.90±1.17	0.321
C_i = Intracellular [CO_2] (ppm)	316.19±34.01	320.69±23.60	0.644	318.73±32.16	0.814

[1]*P*-values as determined by Student's *t*-test represent comparisons between the specific SUT4-RNAi line and the wild type, based on means ± SD of 19 WT, 10 SUT4-G and 12 SUT4-F plants.
[2]PAR = Photosynthetically active radiation.
[3]WUE = Water use efficiency = A_{max}/Respiration.
doi:10.1371/journal.pone.0044467.t004

Table numbers and Titles are ABOVE Tables

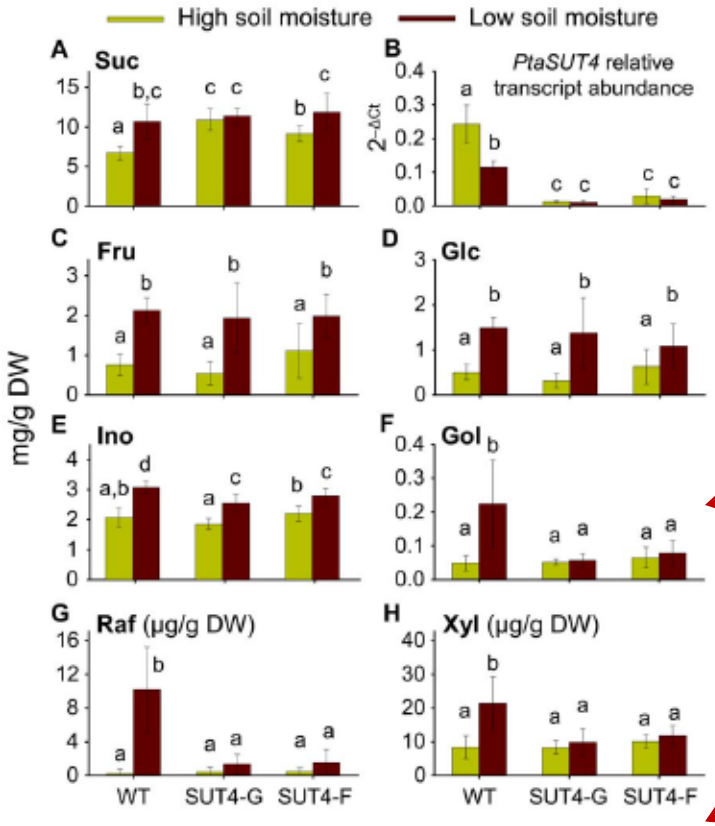


Figure 4. Effects of water availability on sucrose and RFO metabolism in source leaves. Metabolite concentrations (mg/g DW in A-F or µg/g DW in G-H) were determined using standard curves of authentic standards. Light and dark bars represent plants maintained under high or low soil moisture, respectively. Bars are means ± SD of 6–8 replicates each. Bars with different letters are statistically different at $\alpha = 0.05$ based on Student's *t* post-hoc test of LSMeans models using JMP 9.0 (SAS Institute, Cary, NC).
doi:10.1371/journal.pone.0044467.g004

Raf/Gol were both lower in SUT4-RNAi plants relative to wild types under water-limiting conditions (Table 5).

Figures and tables show a summary of the data rather than raw data

Figure numbers and Titles are BELOW Figures

Figures include a caption that describes what is shown in the figure