Stress-Responsive Gene Expression in Poplar Leaves

Plants are sessile organisms that live in a dynamic and frequently changing environment. This has resulted in the evolution of a plastic developmental program that is driven, in part, by the environment in which a plant develops. During the plants' life cycle it will experience environmental stresses that reduce productivity and threaten survival. In the absence of any option to physically remove themselves from a stressful environment plants have learnt to cope with stress at the physiological level. Frequently encountered stresses include: drought, cold or freezing temperatures, excess water (flooding), excess light levels, toxic growing conditions related to salinity or heavy metals, herbivory by insects, grazers and pathogens, mechanical damage, and high temperatures. Furthermore, it is not uncommon for these stresses to be compounded e.g. excess light combined with drought conditions and high air temperatures. Stress physiology is an active and important part of plant physiology that addresses the adaptations present in plants to overcome environmental limitations, and the mechanisms by which plants cope with environmental stress. This field is especially important given the extremely negative impact that environmental stress has on crop yields in agricultural settings.

Many physiological changes (although not all; e.g. stomatal closure) elicited by environmental stress are underpinned by global changes in the pattern of gene expression. Many genes that are regulated by various environmental stresses have been isolated and are known to play a role in a variety of processes that include: cell wall loosening (permits growth), the synthesis of compatible solutes (for osmoregulation), protection from cell damage that arises from the formation of reactive oxygen species or ion toxicity, the synthesis of hormones and other signalling molecules, the synthesis of defense compounds, and the regulation of gene expression etc. At this time numerous stress-responsive genes have been isolated or identified and the focus of ongoing research is to better understand the mechanisms that regulate the plants' response to environmental stress and the signalling pathways involved in detecting stress and translating the signal into an appropriate response (Yamaguchi-Shinozaki and Shinozaki, 2006).

Abscisic acid (ABA) plays a major role in regulating changes in gene expression in the leaves of stressed plants and is known to be required for the expression of many genes. However, ABA is not required for the expression of all genes indicating that other signals play a role to coordinate the molecular response of plants to drought and other stresses (Yamaguchi-Shinozaki and Shinozaki, 2006).

During this lab you will examine the effect of drought stress on the expression of *NCED3*, which encodes the enzyme (9-cis-epoxy carotenoid dioxygenase) that catalyses the rate limiting step in ABA synthesis. As a result, this gene is subject to transcriptional regulation to bring about an increase in the level of the NCED enzyme needed to allow for ABA accumulation in drought stressed plants. To achieve this you will isolate RNA from non-stressed and drought-stressed leaves of **poplar**. The RNA will then be used for

cDNA synthesis, and the cDNA will be used for the assessment of *NCED3* gene expression by real-time quantitative PCR (qPCR). qPCR allows for an accurate assessment of the amount of RNA corresponding to the *NCED3* gene that is present in your RNA samples.

METHODOLOGY:

Material:

1. You will be provided with RNA that has been prepared from drought-stressed and control leaves of poplar. The RNA is not yet pure, you will purify it so that the RNA can be used for cDNA synthesis. In the first week you will complete the RNA preparation and make cDNA with your RNA. In the second week you will use your cDNA for qPCR.
2. 3.3 M sodium acetate
3. Ice in ice buckets
4. Ice-cold 75% (v/v) ethanol
5. Ice-cold isopropanol
6. DEPC-treated sterile distilled water
7. Autoclaved microfuge tubes and pipette tips
8. Gilson pipettes (P10, P200, P1000)
9. Kim wipes
10. Microfuge centrifuge
11. Disposable gloves
12. Fine marker pens
13. Distilled water in bottles
14. Ethanol in bottles
15. Vortex mixer
16. DNase I enzyme
17. DNase I buffer (10 x concentration)
18. Water bath at 37^{o}C
19. Water bath at 65^{o}C
20. Programmable temperature block (2)
21. iScript cDNA synthesis kit (BIORAD)
22. iQ SYBR Green Supermix (BIORAD)
23. qPCR primers for NCED3, UBQ, EFI1A and EFI1b
24. Optical 96-well plate

Isolation of RNA (week one)

The golden rule to making good RNA is to keep everything as cold as possible and as clean as possible to prevent degradation of the RNA by RNases .

Below is the protocol used to isolate high quality RNA from poplar leaves
DAY ONE – *has been done*

1. Partially grind frozen leaf material and add 0.05 g polyvinylpolypyrrolidine (PVPP, an anti-oxidant). Keep everything frozen and, using liquid nitrogen, grind the PVPP into the tissue until the mixture is a fine powder
2. Pour the tissue from the mortar into a separate tube. Keep this tube partially immersed in liquid nitrogen and measure out ~ 0.1 g of the powder into a weigh boat that has been cooled in liq. nitrogen. **Do these steps in a fume hood**.
3. Immediately after you have weighed the correct amount of powder, add 0.5 mL "Plant RNA Isolation Reagent" (Invitrogen) and mix in the weigh boat.
4. Transfer the solution to a labeled RNase-free tube. Leave the tubes on their side to incubate for 5 minutes.
5. Centrifuge the tubes for 10 minutes at 12,000 x g at room temperature. When the supernatant is clear transfer to an RNase free tube.
6. Add 100 μl of 5M NaCl to the supernatant extract and mix. Centrifuge solution for 5 minutes until the supernatant is clear. Transfer the supernatant to an RNase-free tube.
7. Add 300 μl chloroform in fume hood and mix. Centrifuge the tubes at 4^{o}C for 10 minutes at 12,000 x g then transfer aqueous layer to an RNase free tube. (the aqueous layer should be the top layer).
8. Add ¼ volume 10 M LiCl (you will need to know the volume of the aqueous phase) and leave overnight at 4^{o}C.

DAY TWO – *This is where you start*

9. Centrifuge tubes at 4^{o}C for 20 minutes at 12,000 x g.
10. Resuspend the pellet in 133 μL DEPC water. Add 17 μL 3.3 M sodium acetate and precipitate the RNA by adding 150 μL ice-cold isopropanol. Mix by inversion and leave on ice for ten minutes and then centrifuge at 4^{o}C for 10 minutes at 12,000 x g.
11. Carefully pour off the supernatant – make sure the pellet does not move! Wash the pellet with 200 μL 75% ice-cold ethanol as follows: Add 75% ethanol and invert tube then centrifuge as in step 10.
12. Pour off the ethanol, pipette out any remaining ethanol that is around the RNA pellet (carefully!). Allow pellet to air-dry for 5 minutes.
13. Resuspend pellet in 30 μL DEPC water.
14. Determine the amount of RNA using the Nanodrop. The Nanodrop will provide an RNA concentration (ng /μL). The concentration of RNA in your sample is determined based on the absorbance of RNA at 260 nm.

cDNA Synthesis

1. Add the following components to a clean sterile microfuge tube:
    a. 6 to 8 μL RNA (amount should be less than 1μg)
    b. 1μL DNase I
    c. 1μL DNase I buffer
    d. Sterile water (to make volume to 10 μL)

    You should have 10 μL in your tube.
2. Incubate the tube at 37^{o}C for 10 minutes. During this time the DNase I will digest any DNA that is in your RNA sample. Why is this important?
3. Transfer your tube to 65^{o}C for 10 min. This step inactivates the DNase I enzyme

4. Cool on ice for 2 min. then add the following components to your DNase I-treated RNA
    a. 4 µL 5 X iScript reaction mix (BIORAD)
    b. 1 µL iScript Reverse Transciptase (BIORAD)
    c. 5 µL sterile water
    Volume should be 20 µL

    The iScript reaction mix contains oligo (dT) and random hexamer primers.
5. Incubate as follows:
    a. 5 minutes at 25°C
    b. 30 minutes at 42°C
    c. 5 minutes at 85°C
6. Add 100 µL sterile water, mix and store at -20°C until next week

Setting up your qPCR reactions (week two)

Quantitative PCR is a technique to measure the relative or absolute transcript level in a given sample and is now routinely used in place of northern hybridization analyses. qPCR is similar to conventional PCR but the biggest difference is that in qPCR the amount of amplified product is measured after each round of amplification instead of at the end. Amplified products are measured based on the generation of a product-specific fluorescent signal. This can be achieved via a number of different methodologies but one of the most common is to SYBR Green and this is what we will use. SYBR Green is a molecule that intercalates with double-stranded DNA, when this happens the SYBR Green molecules fluoresce. This fluorescence is detected by the qPCR instrument and used to calculate the amount of DNA template present in the sample at the beginning of the reaction. Bear in mind that this DNA template is present in your cDNA and corresponds to the transcript you are measuring. It was amplified due to the specific primers used for PCR. As the template DNA is copied during PCR the amount of amplified product increases exponentially before the level plateaus – see figure below.

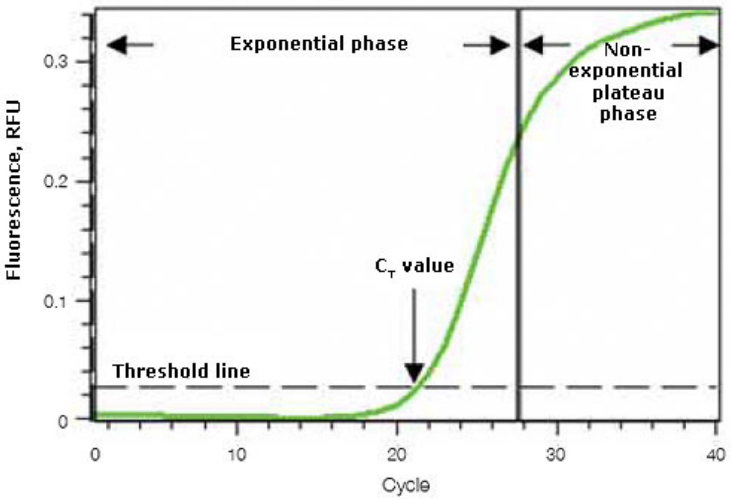

Figure taken from Biorad gene expression gateway

When performing qPCR it is important to set the threshold cycle or C_T cycle. This is the point at which fluorescence is detected as being significantly greater than the background

noise. It is set above the baseline and within the exponential phase of the reaction. The C_T value is inversely correlated to the log of the copy number (amount) of the template DNA in your sample.

You will set up qPCR to assess the amount of the *NCED3* transcript in your control and drought-stressed poplar leaf RNA samples. In addition, you will use three reference genes by which to normalize the *NCED3* transcript level. This is called relative quantification and is necessary to minimize potential variation arising due to sample preparation and handling. A reference gene is one whose expression does not change as a result of the experimental treatment (drought) and they are difficult to find. Therefore, we use three reference genes (*EF1a*, *EF1b*, *UBI*) all of which show minimal fluctuation in expression.

Set up your qPCR as follows:

1. The first thing you need to do is to quantify the amount of cDNA you made last week. You will use the Nanodrop to do this. The concentration of DNA in your sample is determined based on the absorbance of DNA at 260 nm and the Nanodrop provides the DNA concentration as ng / μL.
2. Adjust your cDNA samples so that the concentration is 25 ng/μL
3. Prepare a master mix for qPCR adding the components in the order specified:
    a. Forward primer 5 μL
    b. Reverse primer 5 μL
    c. iQ SYBR Green supermix (BIORAD) 50 μL

    The qPCR supermix is a 2X concentrated mix containing reaction buffer, dNTPs, iTaq DNA polymerase, 6 mM $MgCl_2$ and SYBR Green.

4. Mix and dispense 30 μL master mix into **2** tubes (label one tube **control + your inititals**, label the other **drought + your initials**). Add 20 μL of your **drought cDNA** to the tube labelled "drought" and 20 μL of your **control cDNA** to the tube labelled "control". Mix.

    All together you will set up **4 master mixes**, one for each gene (*NCED3, UBI, EF1a, EF1b*). Each gene has a set of specific primers to amplify it and therefore the 4 mixes you make will differ **according to the primers** that you add. When you are finished you will have **8** tubes, one control and one drought for each of the 4 genes.

5. Your samples are now ready for qPCR. Transfer 20 μL of your master mix / cDNA combination into an individual plate well. Repeat so that you have duplicate samples for each master mix / cDNA combination that you have. You will use **16** wells, **2** control and **2** drought for each of the 4 genes.
6. qPCR samples will be loaded into a single 96-well optical plate as below, do not use the outside wells! For loading order see the diagram on the next page.
7. qPCR will be performed using a BIORAD Opticon 2 qPCR instrument. This will be a demo.

8. After qPCR is complete we will provide you with the C_T values from which you can calculate the drought-induced fold change in the amount of the *NCED3* transcript.

Loading order of samples into 96-well plate

UBI rep 1	UBI rep 2	EFIa rep 1	EFIa rep 2	NCED rep 1	NCED rep 2	EF1b rep 1	EF1b rep 2	
x	x	x	x	x	x	x	x	Control 1
x	x	x	x	x	x	x	x	Drought 1
x	x	x	x	x	x	x	x	Control 2
x	x	x	x	x	x	x	x	Drought 2
x	x	x	x	x	x	x	x	Control 3
x	x	x	x	x	x	x	x	Drought 3
x	x	x	x	x	x	x	x	Control 4
x	x	x	x	x	x	x	x	Drought 4

Relative Quantification of the NCED3 transcript

1. You will use the delta-delta CT method, which is an approximation method to quantify transcript levels. This method assumes that the target (NCED3) and reference genes (housekeeping genes) amplify with similar efficiencies and we are going to assume amplification efficiencies of 100%
2. To calculate $\Delta\Delta C_T$:

$$R = \text{relative gene expression} = 2^{[\Delta C_T \text{ drought} - \Delta C_T \text{ control}]} = 2^{\Delta\Delta C_T}$$

Average C_T values for *NCED3* and reference gene expression in control and drought-stressed *POPULUS* roots

Reference genes (mean)	NCED3	Treatment
18.32	26.67	Control
19.6	23.8	Drought

$\Delta C_{T\ control}$ $= C_{T\ target\ (NCED3)} - C_{T\ reference}$
$= 26.67 - 18.2$
$= 8.34$

$\Delta C_{T\ drought}$ $= C_{T\ target\ (NCED3)} - C_{T\ reference}$
$= 23.8 - 19.6$
$= 4.2$

$\Delta C_{T\ control} - \Delta C_{T\ drought} = 8.32 - 4.2$
$\Delta\Delta C_T = 4.15$

$2^{4.15} = 17.75$

NCED3 was up-regulated 17.75 fold in drought-stressed roots.

3. Use the $\Delta\Delta C_T$ method to calculate the fold up-regulation for NCED3 in your drought-stressed leaves.

References:

Thompson AJ, Jackson AC, Parker RA, Morpeth DR, Burbridge A and Taylor IB (2000) Abscisic acid biosynthesis in tomato: regulation of zeaxanthin epoxidase and 9-cis-epoxycarotenoid dioxygenase mRNAs by light/dark cycles, water stress and abscisic acid. Plant Mol. Biol. 42: 833-845.

Yamaguchi-Shinozaki K and Shinosaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annual Review of Plant Biology 57: 781-803.