#### Plant Growth and Development, 15p

#### Swedish University of Agricultural Sciences

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#### LAB2: Growth & Defense trade-off in *Nicotiana benthamiana* and Turnip Mosaic Virus interaction

Schedule:

2024-11-25 Day 1 9:15-12:00 Infection of seedlings

2024-12-10 Day 15 09:15-12:00 Symptom scoring and samples harvesting

2024-12-11 Day16 09:15-16:45 RNA extraction and cDNA synthesis

2024-12-12 Day17 09:15-14:00 qPCR

2024-12-13 Day18 09:15-12:00 Discussion of qPCR results

**Day 16: RNA extraction and cDNA synthesis**

Today, we will finalize the RNA extraction, synthetize from it the cDNA and set up the qRT-PCR to measure the viral accumulation in your leaf disk. qRT-PCR is a standard method in molecular biology that is used to quantify the amount of nucleic acids (genomic DNA or cDNA from transcripts) in a given sample compared to a known standard (housekeeping gene).

**Materials**

* Gloves
* Goggles
* Tube stand in Zippered bags
* Pipettes + tips
* RNeasy kit
* Maxima First Strand cDNA Synthesis kit
* ICE box
* Thermocycler
* Centrifuge
* 100% ice-cold EtOH

**RNA extraction**

1. Prepare a ICE box.
2. Collect the tissue in RLT buffer (Note: The RLT buffer will protect the sample from RNases).
3. Label three set of tubes, the QIAshredder spin column (lilac), 1.5mL collection tubes and the RNeasy spin column (pink).
4. Transfer the lysate to a **QIAshredder spin column (lilac)** placed in a 2 ml collection tube, and centrifuge for **2 min** at full speed (**13,000 rpm**).

**OBS!** Take care that the centrifuge is balanced! Wait until a few groups are ready to start the centrifuge. Do not start without checking in with course assistant.

1. Carefully transfer the **supernatant** of the flow through to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube.
2. Add **0.5 volumes of ethanol (96–100%)** to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.
3. Transfer the sample (usually **650 μl,** but could also be less than this), including any precipitate that may have formed, to an **RNeasy spin column (pink)** placed in a 2 ml collection tube. Close the lid gently, and centrifuge for **15 s at 10,000 rpm**. Discard the flow-through.
4. Add **700 μl Buffer RW1** to the RNeasy spin column. Close the lid gently, and centrifuge for **15 seconds at 8,000 rpm.** (This will wash the spin column membrane.) Discard the flow-through. Reuse the collection tube the next step.

**Note:** After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

1. Add **500 μl Buffer RPE** to the RNeasy spin column. Close the lid gently, and centrifuge for **15 seconds at 10,000 rpm** to wash the spin column membrane. Discard the flow-through and reuse the collection tube in step the next step.
2. Again, add **500 μl Buffer RPE** to the RNeasy spin column. Close the lid gently, and centrifuge for **2 min at 10,000 rpm** to wash the spin column membrane.

**Note**: This long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

1. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied**). Add 30 μl RNase-free water** directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at **10,000 rpm** to elute the RNA. Discard the column and **keep the flow-through**! The flow-through contains the RNA, so **keep it on ICE**!

**Checking yield and quality for RNA extracts**

1. Measure the concentration of the RNA with a NanoDrop spectrophotometer. 2 μl of each sample will be used. The lab assistants will show you how to do this.

**cDNA Synthesis or Reverse Transcription**

1. Prepare the following reaction mixture in a tube on ice:

x μl RNA (equivalent to 1 μg RNA)

**4µL** 5X Reaction Mix

**2µL Maxima Enzyme Mix**

y μl nuclease-free water to **15 µl** total volume

Mix the samples and centrifuge shortly (a few seconds) to collect everything at the bottom of the tube

1. Incubate the mixture in a thermocycler for 10min at 25˚C followed by 15min at 50˚C, and then terminate the reaction at 70°C for 5 min.
2. Put the samples back on ice.
3. Take **10µl** of your resuspended sample and add it into 190 µl of water. **This is your DNA ready for qPCR**

Viral DNA is highly abundant in infected plants, so we need to dilute the sample a lot for the highly sensitive qRT-PCR to be able to quantify it. qRT-PCR are generally done in 96-well plates, meaning that 96 reactions can be analyzed at the same time. Just as any other PCR, this method works with the constant duplication of the DNA with complementarity to the primers of interest. The difference, however, is that, the qRT-PCR method measures the amount of fluorescent dye incorporated into DNA after every duplication event and by this, can show the accumulation of signal for any given time point. By comparing our three region of interest (in this case GFP primers, TuMV Capsid protein and TuMV 6K2 with a GFP chimeric region) to a stable housekeeping gene (in this case: PP2A) we can calculate the amount of virus present our samples and enable comparison between samples.

**Day 17: Quantitative real-time PCR (qRT-PCR)**

Today, we will set up qRT-PCR to measure the viral accumulation in your plants.

**Material**

1ml pipettes + tips

10 µl pipettes + tips

Gloves

qRT-PCR plates and sealers

1. Prepare four qPCR master mixes (on ice):

**OBS!** The SYBR dye is light sensitive, keep your sample in ice and away from direct light

MM1: Housekeeping gene with the set of primers:

**PP2A\_F**: TAACGTGGCCAAAATGATGC

**PP2A\_R**: GTTCTCCACAACCGCTTGGT

MM2: GFP with the set of primers:

**GFP\_F**: TCCATGGCCAACACTTGTCA

**GFP\_R**: GGCATGGCACTCTTGAAAAAG

MM3: TuMV CP with the set of primers:

**TuMV\_CP\_F**: CACGCCAAACCCACATTTAGG

**TuMV\_CP\_R**: GCTTTCATCTGGATGTGTGCC

MM4: TuMV 6K2-GFP with the set of primers:

**TuMV\_6K2GFP\_F**: CCGAACCCGTAACCCATG

**TuMV\_6K2GFP\_R**: TGACAAGTGTTGGCCATGGA

Regions amplified by the three last set of primers:

A group of purple rectangular objects with black text with Seawise Giant in the background

Description automatically generated with medium confidence

|  |  |  |
| --- | --- | --- |
|  | **1 reaction [µl]:** | **14 (12+2) [µl]:** |
| **Nuclease-free water** | 5,5 | 77 |
| **Forward Primer** | 1,5 | 21 |
| **Reverse Primer** | 1,5 | 21 |
| **SYBR qPCR MM (2x)** | 12,5 | 175 |
| **MM no cDNA** | **21** | **294** |

Once your Master mixes are ready and your cDNA is diluted, we can start to prepare the qRT-PCR plates. To account for pipetting errors each sample will be run 3 times in technical replicates that will be averaged in the later analysis. Two groups will share one plate and each plate will prepare 48 wells (see attached plate scheme).

1. Pipette 4 µl of DNA sample at the bottom of each well

**OBS!** Take care not to touch the sides and be precise!

**OBS!** Take your time and take care - which samples goes in which well? Use the scheme or make your own before starting! This is an essential step!

**OBS!** You can pipet the three technical replicates with one tip, but always change tips between samples

1. Add 21 µl of MM in each well that that was assigned for this MM (12 wells for MM\_PP2A, 12 wells for MM\_GFP, 12 wells for MM\_CP and 12 for 6K2GFP)

**OBS!** This time, pipette at the side of the well so you can use one tip for all wells, this will reduce your differences due to different tips!

1. When both groups have pipetted their plates. Seal them tight with a plate seal and leave them on ice. The qRT-PCR will be run over night, and we will discuss the results the next day.

**Suggested qPCR Layout:**

A table with green and white text

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**qRT-PCR Program:**

1. 95.0 C 15:00 min Denaturation

2. 95.0 C 00:15 min Amplification

3. 60.0 C 00:30 min

4. 95.0 C 01:00 min Melting curve

Repeat steps 2-3 40x

**Day 18: Discussion of qRT-PCR results**

On the last day of this practical we will analyze your Fluorescence intensity values and qRT-PCR results and discuss the outcomes of TuMV disease in *Nicotiana benthamiana*. **Please bring your laptop for this exercise.**

You will have to write a report in the form of a scientific paper (see *Lab Reports - Instructions* on Canvas) for this practical. **Your individual reports should be uploaded on CANVAS (Deadline X December 2024, 6 pm).**