EXPERIMENT 7 – GENE EXPRESSION STUDY IN *ARABIDOPSIS THALIANA* (GENE TWO)

Purpose: To determine mRNA accumulation patterns of genes encoding transcription factors in Arabidopsis leaves and siliques/seeds.

Attention: *In the previous* ***Experiment 3****, you had isolated total RNA from leaves and siliques of wild type Arabidopsis plants and synthesized cDNA templates using Reverse transcriptase for these RNA samples. The cDNA (or RT) solutions have been stored in the –20^{o}C freezer and still good for the PCR amplification of the SECOND gene. In this experiment, you, therefore, do* ***NOT*** *need to carry out* ***steps I and II****, but* ***perform step III****.*

OVERVIEW OF RT-PCR

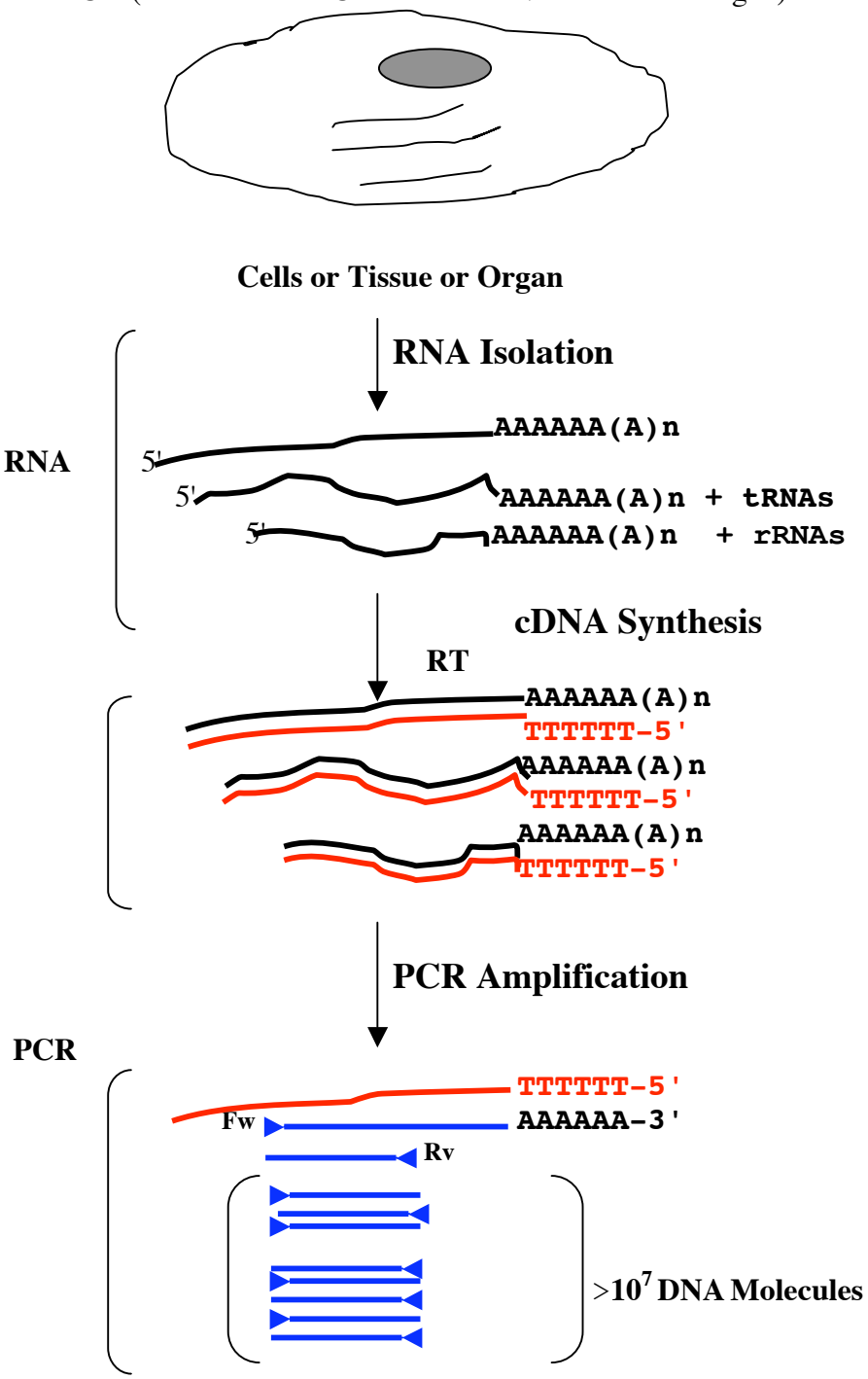
STRATEGY

I. ISOLATION OF TOTAL RNA USING QIAGEN RNEASY PLANT MINI KIT (SEE EXPERIMENT 3)

II. SYNTHESIZING FIRST STRAND cDNA USING REVERSE TRANSCRIPTASE (REVERSE TRANSCRIPTION or RT) (SEE EXPERIMENT 3)

III. CARRYING OUT PCR AMPLIFICATION ANALYSIS

OVERVIEW OF RT-PCR (Based on RT-PCR Technical Note from Invitrogen)



I. ISOLATION OF TOTAL RNA USING QIAGEN RNEASY PLANT MINI KIT (SEE EXPERIMENT 3)

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III. CARRYING OUT PCR AMPLIFICATION ANALYSIS

Purpose: To amplify DNA template corresponding to mRNA that is either absent or present at different levels in different plant organs throughout plant development.

Materials and Reagents Needed:

- Reverse transcription (+RT & -RT) reactions
- 10X Ex-Taq buffer (Takara Mirus Bio)
- dNTP mix (Takara Mirus Bio)
- **12 μM** Gene-specific **RT-PCR Fw** primers
- **12 μM** Gene-specific **RT-PCR Rv** primers
- **12 μM Tubulin Fw** primers
- **12 μM Tubulin Rv** primers
- Sterile water
- Ex-Taq DNA polymerase (Takara Mirus Bio, 5 U/μL)
- Agarose
- 1X TAE buffer
- 1 KB DNA ladder (Invitrogen)
- 6X Loading buffer
- 0.2 mL PCR tubes
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube racks
- PCR Machine (Bio-Rad MyCycler or PE 9700)
- Gel apparatus
- Power supplies

PROCEDURE

1. Get ice from the icemaker in room 2911 or 3906.
2. Determine **how many RT reactions**, including **+RT's** and **-RT's**, will be amplified.
3. Make a **Table** with information sush as **tube #**, **plant organ(s)**, and **+RT's/-RT's** (see the **example** Table below)

Tube #	**1**	**2**	**3**	**4**	**5 (Positive)**	**6 (Negative)**
Organ & RT	Leaf +RT	Leaf -RT	Silique +RT	Silique -RT	Genomic DNA	Sterile Water

4. Label on the **lids** and **sides of FIVE 0.2 mL PCR tubes** with **Number** and **your initial**.
5. Put the labeled tubes on a PCR rack sitting **on ice**.
6. Prepare a **master mix** in a **1.5-mL microcentrifuge tube** for the **number** of **PCR solutions** being carried out **plus** 1 **extra solution volume** as followings: **(How many reactions are carried out?)**

	Mmix for 1 Reaction	**Mmix** for **7** Reactions
10X Ex-Taq buffer	5.0 µL	35.0 µL
dNTP mix	4.0 µL	28.0 µL
12 µM RT-PCR Gene-specific **Fw** primer	1.0 µL	7.0 µL
12 µM RT-PCR Gene-specific **Rv** primer	1.0 µL	7.0 µL
***12 µM Control** (**Tubulin**) **Fw** primer	1.0 µL	7.0 µL
***12 µM Control** (**Tubulin**) **Rv** primer	1.0 µL	7.0 µL
Sterile water	34.5 µL	241.5 µL
Ex-Taq DNA Polymerase (5 U/µL)	0.5 µL	3.5 µL
Total Volume	**48.0** µL	**336.0 µL**

* ***Control primers*** *are used to ensure that the absence of Gene-specific PCR product in* ***+RT samples*** *is NOT due to technical mistakes. The* ***expected size*** *of the* ***Tubulin PCR product*** *is* ***~0.45 kb****.*

16. Pipet **48 µL** of the **master mix** to the labeled tubes and **2 µL** of **appropriate RT** to each of the tubes shown on the table below. Mix the contents by pipetting **gently** up and down for five times.

Tube #	**1**	**2**	**3**	**4**	**5 (Positive)**	**6 (Negative)**
Mmix	**48 µL**	**48 µL**	**48 µL**	**48 µL**	**48 µL**	**48 µL**
Leaf +RT	**2 µL**	---- µL	---- µL	---- µL	---- µL	---- µL
Leaf -RT	---- µL	**2 µL**	---- µL	---- µL	---- µL	---- µL
Silique +RT	---- µL	---- µL	**2 µL**	---- µL	---- µL	---- µL
Silique -RT	---- µL	---- µL	---- µL	**2 µL**	---- µL	---- µL
0.2 ng/µL Genomic DNA	---- µL	---- µL	---- µL	---- µL	**2 µL**	---- µL
Water	---- µL	---- µL	---- µL	---- µL	---- µL	**2 µL**
Total Volume	**50 µL**	**50 µL**	**50 µL**	**50 µL**	**50 µL**	**50 µL**

17. Carry out PCR reactions with the **RT-PCR program** containing the following profile: 1 cycle of 96 ºC, 3 min. → 40 cycles of 94 ºC, 10 sec./60 ºC, 30 sec./72 ºC, 45 sec. → 1 cycle of 72 ºC, 4 min. → 4 ºC, ∞.
18. Prepare **100 mL** of **1.5% agarose** gel in **1X TAE** buffer as usual (Use a **20-tooth** comb).

    <u>Note:</u> *The **percentage** of **agarose gels** depends on the **difference** in **size** of **two PCR products**. If there is **at least 100-bp difference** between two PCR products, then use a **1% agarose gel**. However, if there is **50-100 bp difference** between two PCR products, then use **1.5-2% agarose gel**. For example, the size of PCR products is 0.6 kb and 0.55 kb for the control and gene A, respectively. The **2.0%** agarose gel resolves these two PCR products as **two** discreet DNA bands whereas the **1.0%** agarose gel shows these two PCR products as a **single** DNA band.*
19. Label 1.5 mL microcentrifuge tubes according to the PCR solutions being performed.

20. Add to the labeled 1.5 mL microcentrifuge tubes **20 µL** of **PCR solution** and **3 µL** of **6X loading dye**. *Note: 20 µL of PCR solution was loaded so that you can see the PCR products clearly.*
21. Load samples on a 1.5% agarose gel along with **20 µL** of **50 ng/µL 1-Kb ladder** solution. Record RNA loading pattern.

—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**

22. Run the gel at **105 volts** for **1-2 hours** or until the front dye (bromophenol blue) is about two-thirds of the gel.
    Starting time:
    Ending time:
23. Take a picture of the gel.
24. Analyze the data.
    How many DNA fragments do you see on the gel?
    Is there different brightness between the fragments from one organ to the other?
    What are the sizes of DNA fragments?
    *Note: The expected size of the Tubulin PCR product is **~0.45 kb**.*
    What is the size of the PCR fragment corresponding to mRNA of the gene of interest?
    What is a conclusion on gene expression of the gene of interest for the tested plant organs, leaves and siliques?
    Are the RT-PCR results correlated to the GeneChip data?