1. Practical 1 – Genotype to Phenotype: case studies of PIN1 and MONOPTEROS

You will analyze the relationship between phenotype and genotype using two mutant lines in auxin transport and signalling. PIN1 is one auxin efflux carrier and MONOPTEROS (MP) is a transcription factor in the signalling pathway.

Experimental objective
This experiment will give you an understanding of how mutation affects plant development and how to detect such mutation, using a model plant *Arabidopsis thaliana* (Arabidopsis).

1.1. Materials

1.1.1. Seeds

Seeds were sterilized for you by chlorine gas method
- *pin1 (salk line salk_047613)*
- *mp (mp-B4149)*
- Col wild type

1.1.2. Material

Petri dishes
Micropore tape
Aluminum foil
Stereomicroscope
Toothpicks
Marker pen
Tubes with the seeds
Tips
Tubes
Blue pestles
PCR strips
Electrophoresis material
Gloves
Pipettes (1000, 200, 10, 2ul)
Thermomixer

1.1.3. Solutions

1/2 strength MS for growing the seeds (will be prepared for you)
for 1 Liter
10 g Sucrose
2,3 g MS Salts
0,5 g MES
adjust pH to 5,9 (KOH)
8 g Agar (add separately to each bottle, 4 g per 500 ml bottle!)
Edward's buffer (DNA extraction) (will be prepared for you)

200 mM Tris HCL pH 7.5
25 mM EDTA
250 mM NaCl
0.5% SDS
Autoclave it!

isopropanol
70% ethanol
MQ
primers

MP_1498S CTCTCAGCGGATAGTATGCACATCGG
MP_2082AS ATGGATGGAGCTGACGTTTGAGTTC
pin1-201 RP AATCATCACAGCCACTGATCC
pin1-201 LP CAAAAACACCCCCAAAATTTC
LBaI TGGTTCACGTAGTGGGCCATCG

enzymes

MseI (with reaction buffer)
Taq polymerase (with reaction buffer)

dNTP mix
Agarose
TAE buffer
Ethidium bromide

1.2. Experiment

1.2.1. Timetable

This experiment is designed to take ±fifteen days. We will start the experiment on Friday 9th, October and then remove the petri dishes from cold treatment on Monday 12th. The seeds will grow for 7 days. Phenotype will be scored. Individual seedling will be collected, and its genomic DNA extracted by the CTAB method. Genotype will be performed by PCR (and restriction for *mp*) and analyzed by gel electrophoresis. The two mutants have different types of mutations (point mutation induced by EMS, T-DNA insertion). You will learn how to genotype both and how they are different. The seedlings of the *pin1* mutant will be transfer to soil to observe the shoot phenotype.

Timetable

mutants	Prep Day 1	Prep Day 2-4	Day 1	Day 2	Day 3	Day 4
pin1	Sow the seeds	Cold treatment	Score the phenotypes; DNA extraction	PCR	Gel electrophoresis	Analysis
mp	Sow the seeds	Cold treatment	Score the phenotypes; DNA extraction	PCR	Restriction Gel electrophoresis	Analysis

1.2.2. **Sowing**

All this work is done in sterile conditions under flow benches in room 1S26

- You will be given 3 plates of MS medium
- Label your plates with the name of the seeds that will be sown (Col, *mp*, *pin1*)
- You may draw two lines with the marker pen at the back of the plate
- Take a toothpick and wet its tip by tapping it in the corner of the plate. 1 Toothpick per genotype to avoid mixing the seeds.
- Using the wet tip of the toothpick pick up one seed at a time from the tube and put it in its corresponding place on the Petri dish.
- Place 20 seeds for each genotype on the Petri dishes, following the lines you draw. Separate nicely the seeds along the line.
- Place the lid on the Petri dishes and wrap the edges of each Petri dish with Micropore tape completely sealing them shut.
- Wrap their stack completely with aluminum foil so that no light reaches the seeds.
- Label the wrapped Petri dishes using marker tape, with your name and date.
- Put the wrapped Petri dishes in the fridge (room 1S16) at 4°C-6°C. This process is called stratification (or vernalization). Stratification is important for breaking seed dormancy and synchronizing the germination of the seeds. Be extremely careful when preparing, transferring or working on Petri dishes with seeds. **Rough handling can cause seeds to shift from their intended position and ruin the experiment.**
- All Petri dishes should be kept in cold treatment until Monday 14th, October.

1.2.3. **Data collection**

- After the cold treatment is complete, remove Petri dishes from the fridge, remove the aluminum foil
- Place these dishes upright on one shelf on the cultivation room
- On Tuesday 22nd, score the phenotype of the seedlings. You will scan the plates for record in room 1S29. You may use the binocular microscopes in room A26, 2.08 for counting and observing the phenotypes.

1.2.4. **DNA extraction**

- Use a full seedling as starting material and transfer it to a 1.5 ml eppis
- Use 20 seedlings of *mp*, 20 seedlings of *pin1*, and 4 seedlings of Col. Distribute the samples among you.
- Grind the seedlings with a blue pestle. Change of pestle between each sample. DON'T TRASH THEM. They are washed and reused.
- Add 400 µl of the extraction buffer. Vortex briefly and spin down.
- The samples are left at room temperature until all the samples have been extracted.
- Centrifuge for 1 minutes at 13 000 rpm.
- Transfer 300 µl of the supernatant into a new tube.
- Add 300 µl isopropanol and mix well. Incubate for 2 minutes at room temperature.
- Centrifuge for 5 minutes at 13,000 rpm.

- Discard the liquid.
- Add 200 µl 70% ethanol
- Centrifuge for 5 minutes at 13,000 rpm.
- Discard the liquid.
- Air dry the pellet for at least 30 minutes (usually at 37°C or higher).
- Add 100 µl MQ water

1.2.5. **Genotyping**

The mp mutant

The *mp* mutant has a point mutation induced by EMS. The *mp-B4149* allele has a G to A mutation in its coding sequence at the splice acceptor site of the tenth exon. This mutation creates a T/TAA restriction site that can be cut by MseI. We will prepare one type of PCR reaction. The DNA fragment amplified by PCR will then be cut by the MseI restriction enzyme. MseI will cut the mutant allele but not the wild-type one. Because you have 20 + 4 samples. You will prepare 1 master mixes for 30 samples. You do more volume to compensate for pipetting inaccuracy.

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You need:
- PCR buffer (10x concentrated)
- 10 mM dNTPs
- 10 uM oligo MP_1498S CTCTCAGCGGATAGTATGCACATCGG
- 10 uM oligo MP_2082AS ATGGATGGAGCTGACGTTTGAGTTC
- Taq polymerase (take out of the freezer at the last moment and keep it cold in one of the cold block)
- Genomic DNA

The master mix will be:

reagents/ Nr. of samples	**1**	**10**	**30**
MQ	18,5	185	555
10x Reaction Buffer	2,5	25	75
10mM dNTPs	0,5	5	15
10uM F Primer	0,5	5	15
10uM R Primer	0,5	5	15
***Taq* Polymerase**	0,12	1,2	3,6
total	25	250	750

- Mark the PCR strip at the beginning to recognize where the sample 1 will be. You need three strips, labeled with 1, 9, 17 in one extremity.
- The master mix is distributed in PCR strips, 22,5 ul per tube. Then add 2,5 ul of DNA, one sample

per tube.

- DNA and mix are well mixed, and tubes are spin down.
- In room 2.21, there are the PCR machines. One machine has been booked for you. Put the three strips in the block of the machine. Start the machine.
- The program will be: 1x 95°C, 3'; 30x (95°C, 20"; 55°C, 20"; 72°C, 45"); 72°C, 5'
- We will stop the reactions for you and keep the samples in the fridge.
- The PCR reactions will be used as template for a restriction analysis with MseI. Each of you will prepare a master mix for one strip as follow:
    - MQ – 20 ul
    - 10x CutSmart buffer – 2.5 ul
    - MseI – 2.5 ul
- Mix 3 ul of the RE is added to 5 ul of PCR product in new PCR strips. Incubation for 30 minutes at 37°C in a PCR machine
- During the reaction time, you will prepare an electrophoresis gel. You wish to differentiate PCR fragments of 585 bp (uncut, wild type) and of 379 + 207 bp (*mp*). You need to prepare a 2% agarose gel.
- When working in the elfo room (2.18/2.19), wear lab coat and gloves
- Weight 4g of agarose and pour it an Erlenmeyer/bottle containing 200 ml TAE buffer. Pour the TAE from the barrel and measure it with one of the available cylinders.
- The agarose is melt using the micro-wave. Stay put to avoid over boiling. Be careful to not burn yourself (use the carry-over glove). All the agarose should be melted.
- Work in the chemical bench, prepare a chamber (you will be provided the one, the combs and the casting chamber). When the agarose cools a bit, add 3 ul of ethidium bromide, mix well and pour the agarose in the chamber. Let it solidify under the chemical flow.
- In the meantime, the restriction reaction finished. Get the strips from the machine, switch off properly according to instructions.
- To load the samples in the elfo gel, you will need to add loading buffer. It has two purposes: the blue color helps to visualize the progress of the migration; the glycerol renders the solution heavier and helps the solution to go down the wells. The loading buffer is 6x concentrated, meaning 1 ul for 5 ul of solution. Add 2 ul per sample.
- In the elfo room, place the gel in the appropriate running chamber. Make sure the buffer is clean and in the proper amount. You are ready to load the samples in the gel. One sample per well. Keep one well at one extremity to load a ladder. The ladder will indicate the size of DNA bands in the gel.
- Plug the cap, switch on the power supply. Set the voltage to 100, the timer to 30 minutes. Start.
- After 30 minutes, you collect the gel to take a picture using the BioRad machine. You will be instructed how to proceed.
- Print the gel picture.
- Analyze the size of the DNA fragments for each sample and interpret the results – what seedling is wild-type, heterozygous or homozygous for the mutation.

The pin1 mutant

The *pin1* mutant has a T-DNA inserted in his coding sequence. To detect it, we will prepare two types of PCR reactions, one to amplify the wild-type allele, one to amplify the mutant allele from the T-DNA border. Because you have 20 + 4 samples, you will prepare 2 master mixes for 30 samples.

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You need:
- PCR buffer (10x concentrated)
- 10 mM dNTPs
- 10 uM oligo pin1-201 RP AATCATCACAGCCACTGATCC
- 10 uM oligo pin1-201 LP CAAAAACACCCCCAAAATTTC
- 10 uM oligo LBaI TGGTTCACGTAGTGGGCCATCG
- Taq polymerase
- Genomic DNA

Each of the master mixes will be

reagents/ Nr. of samples	**1**	**30**
MQ	18,5	555
10x Reaction Buffer	2,5	75
10mM dNTPs	0,5	15
10uM RP Primer	0,5	15
10uM LP or LB Primer	0,5	15
***Taq* Polymerase**	0,12	3,6
total	25	750

The WT mix is with pin1-201 RP and pin1-201 LP primers.
The T-DNA mix is with pin1-201 RP and LBaI primers.

- Mark the PCR strip at the beginning to recognize where the sample 1 will be. You need four strips, labeled with 1, 9, 17, 25 in one extremity (1, 9 for WT mix, 17, 25 for TDNA mix)
- The master mixes are distributed in PCR strips, 7,5 ul per tube. Then add 5 ul of DNA, one sample per tube.
- DNA and mix are well mixed, and tubes are spin down.
- In room 2.21, there are the PCR machines. One machine has been booked for you. Put the three strips in the block of the machine. Start the machine.
- The program will be: 1x 95°C, 3'; 30x (95°C, 20"; 55°C, 20"; 72°C, 1'); 72°C, 5'
- We will stop the reactions for you and keep the samples in the fridge overnight.
- You will prepare an electrophoresis gel. We wish to differentiate PCR fragments of ±1200 bp. We need to prepare a 1.2% agarose gel.
- When working in the elfo room (2.18/2.19), wear lab coat and gloves
- Weight 2.4g of agarose and pour it an Erlenmeyer/bottle containing 200 ml TAE buffer. Pour the TAE from the barrel and measure it with one of the available cylinders.
- The agarose is melt using the micro-wave. Stay put to avoid over boiling. Be careful to not burn yourself (use the carry-over glove). All the agarose should be melted.

- Work in the chemical bench, prepare a chamber (you will be provided the one, the combs and the casting chamber). When the agarose cools a bit, add 3 ul of ethidium bromide, mix well and pour the agarose in the chamber. Let it solidify under the chemical flow.
- To load the samples in the elfo gel, you will need to add loading buffer. It has two purposes: the blue color helps to visualize the progress of the migration; the glycerol renders the solution heavier and helps the solution to go down the wells. The loading buffer is 6x concentrated, meaning 1 ul for 5 ul of solution. Add 3 ul per sample.
- In the elfo room, place the gel in the appropriate running chamber. Make sure the buffer is clean and in the proper amount. You are ready to load the samples in the gel. One sample per well. Keep one well at one extremity to load a ladder. The ladder will indicate the size of DNA bands in the gel.
- Plug the cap, switch on the power supply. Set the voltage to 100, the timer to 30 minutes. Start.
- After 30 minutes, you collect the gel to take a picture using the BioRad machine. You will be instructed how to proceed.
- Print the gel picture.
- Analyze the size of the DNA fragments for each sample and interpret the results – what seedling is wild-type, heterozygous or homozygous for the mutation.

1.2.6. **Observation of the shoot *pin1* phenotypes**

- The *pin1* seedlings remaining on plates will be transferred to soil. Done by us after the collection of samples for genotyping on 19/10.
- Plants are growing in the phytotron, room 1S08/1S09. We will perform the phenotyping of *pin1* mutant plants around the first week of December as you need flowering plants to assess the phenotype.
- Follow carefully the instructions for manipulating the plants. Wear only the lab coat available in the phytotron corridor (not yours). The plants will be available on the tables in the corridor.
- Score the phenotype (eventually take a picture for your record) of each plant.

1.2.7. **Analysis of the results**

Report in the following table the results of the scoring and of the genotyping to help you interpret your data and conclude on the status of the plant.

Are all the plants with phenotypes, homozygous? Are all the homozygous plants with a phenotypes?

For the mp mutant

Sample number	**1**	**2**	**3**	**4**	**5**	**6**
Phenotype						
Nb of bands						
Genotype						
Sample number	7	8	9	10	11	12
Phenotype						
Nb of bands						
Genotype						
Sample number	13	14	15	16	17	18
Phenotype						
Nb of bands						
Genotype						
Sample number	19	20	C1	C2	C3	C4
Phenotype						
Nb of bands						
Genotype						

For the pin1 mutant

Sample number	1	2	3	4	5	6
Phenotype						
Band LP+RP						
Band LB+RP						
Genotype						
Sample number	7	8	9	10	11	12
Phenotype						
Band LP+RP						
Band LB+RP						
Genotype						
Sample number	13	14	15	16	17	18
Phenotype						
Band LP+RP						
Band LB+RP						
Genotype						
Sample number	19	20	C1	C2	C3	C4
Phenotype						
Band LP+RP						
Band LB+RP						
Genotype						