Lab manual for the course

Plant Genetics

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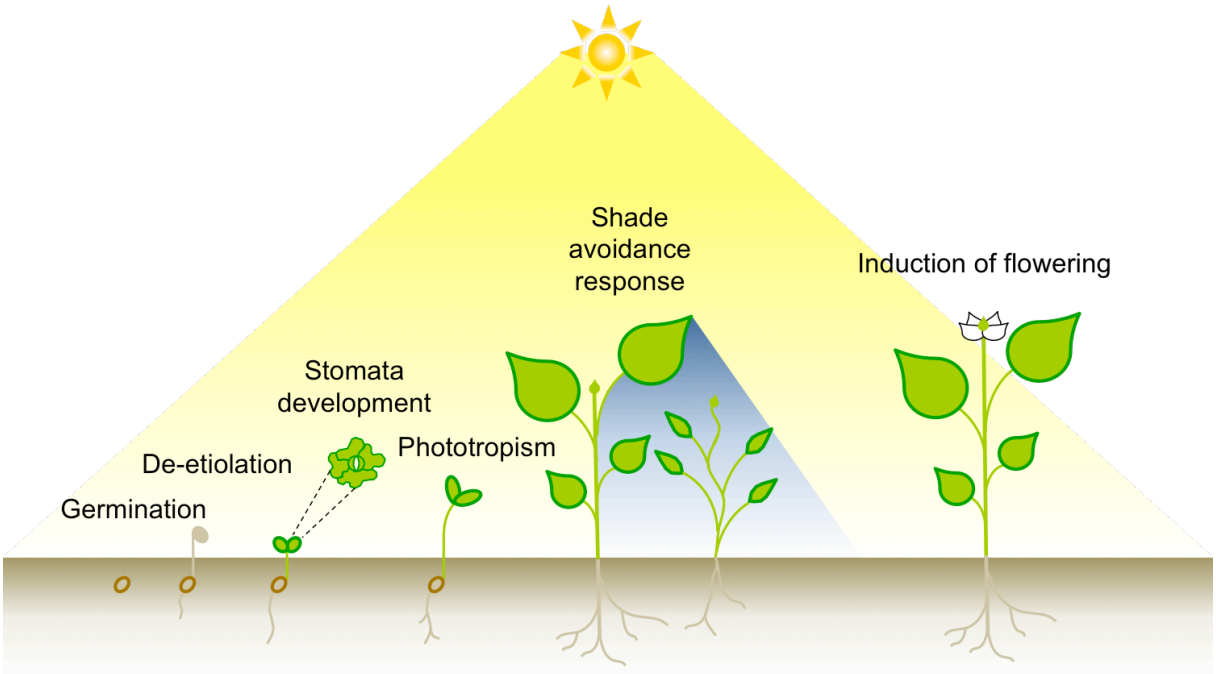
(Biocenter, courseroom 4th floor)



General introduction into light signaling

One area of current research in molecular biology is the elucidation of signalling pathways. A classical signal transduction pathway consists of:

- a receptor perceiving the signal
- components of signal transduction and
- transcription factors which alter gene expression in response to the signal.

The light signal transduction cascade will pose as an example for you to obtain insight into the methods and approaches used for the analysis of a signalling pathway. Most studies have been conducted with the model species *Arabidopsis thaliana* because a great number of molecular and genetic tools have been developed for it. In recent years, however, this knowledge has been applied to crop plants (e.g. rice) in order to optimize plant architecture for growth in a densely planted, monocultured field.

Green plants harvest energy from sunlight through photosynthesis. Optimised use of sunlight is a crucial criterion, which will determine the chances of survival and reproduction for a plant in competition with its environmental surroundings. Since plants cannot change their location, they have evolved responses to adapt their growth and development to the ambient

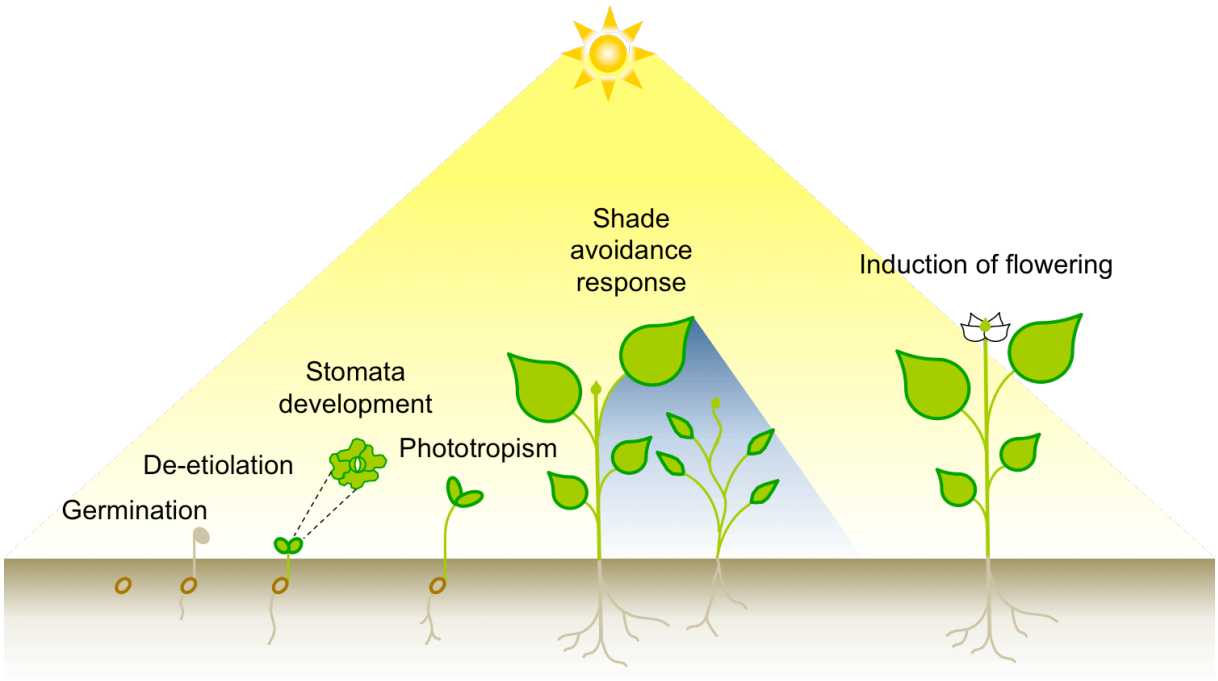


Figure 1 Light controls plant growth and development

light conditions at their specific location. This includes light-induced seed germination (so that seeds only germinate when NOT covered by soil), seedling deetiolation (short hypocotyl, open and green cotyledons in the light), phototropism and the shade-avoidance response (increased elongation growth of plants in the shade). Furthermore, light regulates the induction of flowering via the photoperiod (= duration of daytime; long day plants, short day plants), and thus allows flowering during a climatically favourable season (Figure 1).

Plant photoreceptors and light signal transduction

To perceive ambient light conditions, plants have evolved several classes of photoreceptors which respond to light of specific wavelengths. These include the blue light-sensing cryptochromes and phototropins, and the red and far-red light sensing phytochromes.

The mechanism of signal transfer from photoreceptors to the transduction pathway is still quite poorly understood. Because light causes drastic changes in gene expression (approx. 10% of all genes in *Arabidopsis thaliana* are regulated by light), the light signal transduction pathway needs to "end" in the nucleus by regulating the activity of transcription factors. While cryptochromes are always localized in the nucleus, phytochromes are localized in the cytosol in darkness and transported into the nucleus in the light. Several transcription factors (e.g. HY5) are known to induce the expression of light-regulated genes by directly binding to the promoters of these genes. A mechanism must exist to ensure that these transcription factors are only active in the light and not in darkness. This mechanism involves the COP1/SPA repressor complex (Figure 2) which ensures that these transcription factors are degraded in darkness and, therefore, are only present in a plant that is exposed to light.

The COP1/SPA complex is a tetramer consisting of two COP1 proteins and two SPA proteins. COP1/SPA is an E3 ubiquitin ligase, an enzyme which adds a polyubiquitin chain to its substrates and thereby marks these substrates for degradation in a multi-protein complex called the proteasome. The substrates of COP1/SPA are HY5 and other transcription factors involved in the response to light. The question why these substrates are only degraded in the dark and not in the light might be raised. The answer is, that

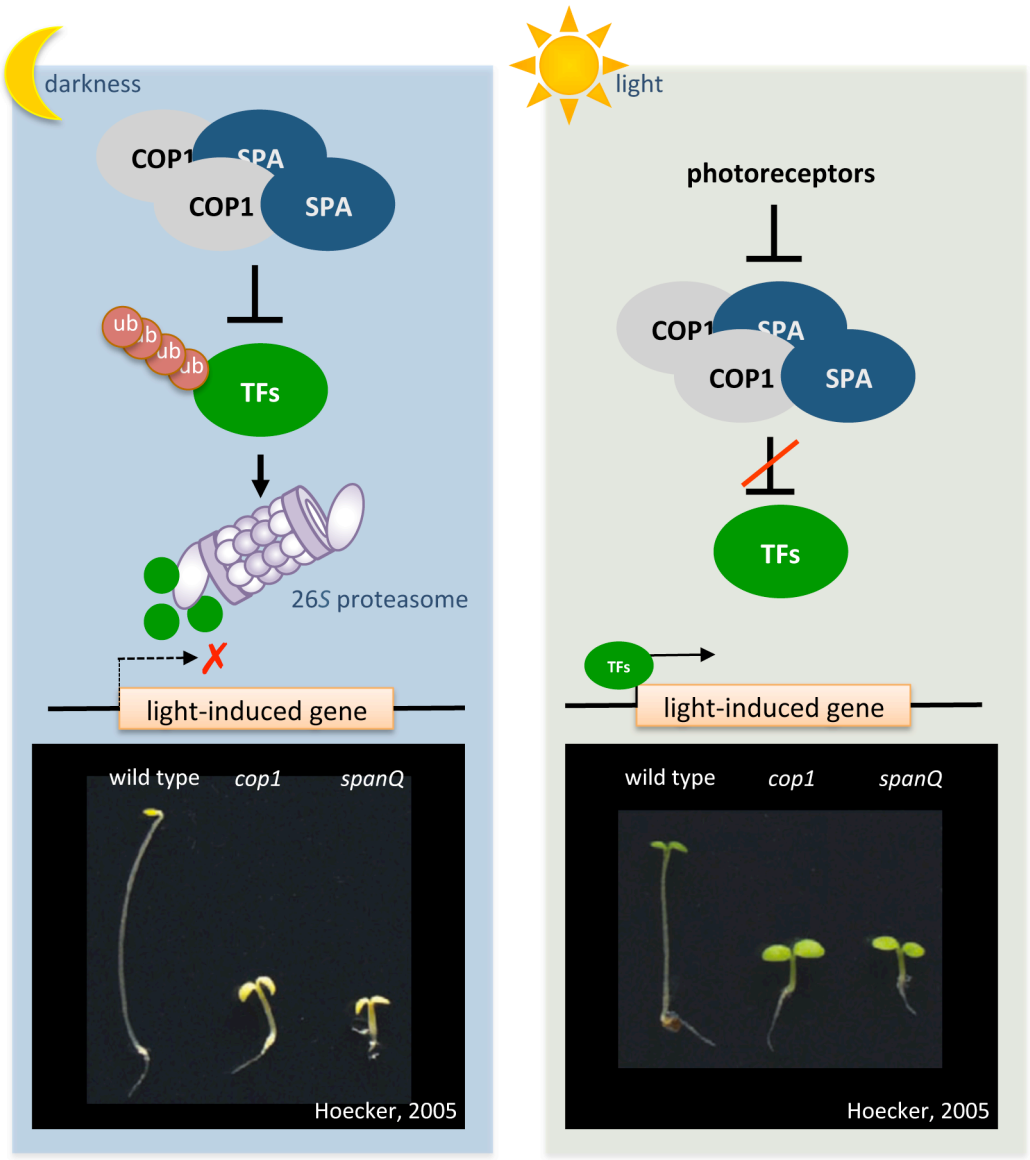


Figure 2 The function of the COP1/SPA repressor complex which prevents the activity of the light signalling components in darkness (for explanations see text)

COP1/SPA is primarily active in darkness because light inhibits COP1/SPA function. As a consequence, in light-exposed plants, COP1/SPA is inactive and these transcription factors are no longer ubiquitinated and degraded and can do their job in regulating gene expression.

Light inhibits COP1/SPA function by several mechanisms: 1) it excludes COP1 from the nucleus (long-term response), 2) it causes SPA protein degradation and 3) it inhibits COP1/SPA ubiquitin ligase activity through a not yet characterized mechanism that involves direct interaction between COP1/SPA and photoreceptors.

SPA proteins are encoded by four genes, called *SPA1*, *SPA2*, *SPA3* and *SPA4*, while COP1 is a single-copy gene. What happens in a *cop1* or in a *spa1 spa2 spa3 spa4* quadruple mutant which lacks all four SPA proteins? In such mutants, HY5 and other transcription factors are

not degraded in darkness and therefore activate (normally) light-induced genes even in complete darkness. As a consequence, *cop1* and *spa* mutants display features of light-grown seedlings even when grown in the dark; they undergo so-called “constitutive photomorphogenesis”, thus they "think" that the light is always on (Figure 3a).

The COP1 protein contains several domains that can interact with other proteins (such domains are generally called "protein-protein interaction domains", Figure 3b):

- a WD-repeat domain which is responsible for binding of ubiquitination substrates such as HY5.
- a coiled-coil domain. Coiled-coil domains are known to interact with other coiled-coil domains. The coiled-coil domain of COP1 can interact with the coiled-coil domain of another molecule of COP1 (homodimerization) and with the coiled-coil domain of SPA. Together, this forms the tetrameric COP1/SPA complex.
- a RING-finger domain, which is a special type of zinc-finger and is typical for many ubiquitin ligases.

COP1 also exists in humans and other animals in which it is a critical regulator of cell division by ubiquitinating transcription factors like the tumor suppressor protein p53 and the proto-oncoprotein c-Jun. COP1 activity is not controlled by light in humans.

SPA proteins contain the following domains:

- a WD-repeat domain which is responsible for binding of ubiquitination substrates such as HY5.
- a coiled-coil domain that can interact with the coiled-coil domain of another molecule of SPA (homodimerization) and with the coiled-coil domain of COP1.
- a kinase-like domain that has weak sequence similarity to kinases but probably no kinase activity.

SPA proteins only occur in plants and not in animals. Hence, SPA proteins may have evolved in plants to place the COP1 ubiquitin ligase under the control of light.

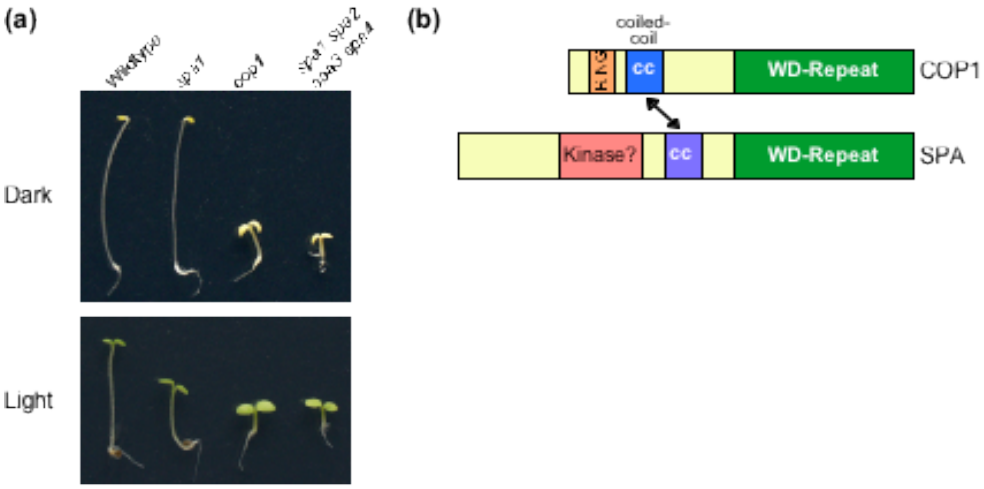


Figure 3 COP1 and SPA proteins suppress photomorphogenesis in dark-grown seedlings
(a) *cop1* mutants and *spa1 spa2 spa3 spa4* quadruple (*spanQ*) mutants exhibit constitutive photomorphogenesis. *spa1* mutants show enhanced responsiveness to light but they grow normally in darkness, suggesting functional redundancy amon SPA family members.
(b) Domain structure of COP1 and members of the SPA protein family.

References:

Reviews

Bae G, Choi G. (2008) Decoding of Light Signals by Plant Phytochromes and Their Interacting Proteins. Annu Rev Plant Biol. 2008 Jan 7

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Selected original publications

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Laubinger, S., Fittinghoff, K. and Hoecker, U. (2004). The SPA-quartet: a family of WD-repeat proteins with a central role in suppression of photomorphogenesis in Arabidopsis. *Plant Cell* 16: 2293-2306.

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Zhu, D., Maier, A., Lee, J.H., Laubinger, S., Saijo, Y., Wang, H., Qu, L.J., Hoecker, U., Deng, X.W. (2008). Biochemical characterization of Arabidopsis complexes containing CONSTITUTIVELY PHOTOMORPHOGENIC1 and SUPPRESSOR OF PHYA proteins in light control of plant development. Plant Cell 2008 20: 2307-2323.

Experiment 1: Do COP1 and SPA-proteins interact *in vivo*?

Introduction

There are a number of methods one can use to investigate whether two proteins are interacting with each other. The following review provides an excellent overview of these methods:

Lalonde S, Ehrhardt DW, Loqué D, Chen J, Rhee SY, Frommer WB. (2008) Molecular and cellular approaches for the detection of protein-protein interactions: latest techniques and current limitations. Plant J. 2008 Feb;53(4):610-635.

One can examine protein-protein interactions *in vitro*, *in vivo* and in yeast:
In vitro methods use recombinant proteins in a cell-free system. These in vitro methods are suitable to ask whether two proteins are directly interacting (in contrast to being part of a larger multi-protein complex, bridging by a third protein). Also, they allow easy mapping of the protein domains responsible for the interaction of two proteins. However, two proteins interacting *in vitro* might not necessarily interact in the organism under investigation. For example, if two proteins are expressed in different tissues of the organism they are clearly unable to interact. Hence, *in vitro* interaction results should be confirmed by *in vivo* methods. *In vitro* methods include co-immunoprecipitation and GST-pulldown.

In vivo methods ask whether two proteins interact in the living cell of the respective organism, i.e. Arabidopsis. Generally, they are much harder to do than *in vitro* methods. *In vivo* methods include co-immunoprecipitation from plant extracts (which you will do here), fluorescence energy resonance transfer (FRET), bioluminescence resonance energy transfer (BRET) and split YFP.

A third method is the yeast two-hybrid system (Y2H) which examines protein-protein interactions in the yeast cell. Here, yeast is simply used as a tool. A great advantage of the

Y2H system is that it allows high-throughput analysis. One can easily examine thousands of protein-protein interactions.

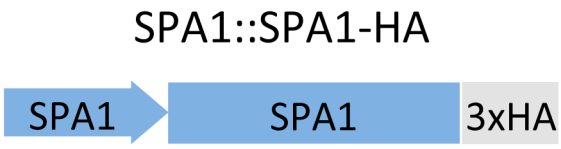


Figure 4 SPA1-HA constructs used to generate transgenic plants expressing HA-tagged SPA1 protein under the control of the SPA promoter

In this lab course you will investigate whether SPA1 can interact with COP1 using *in vivo* co-immunoprecipitation.

For co-immunoprecipitation experiments, highly specific antibodies are necessary for the immunoprecipitation (pull-down). Mostly, highly specific antibodies are not available. Therefore, transgenic plants are generated that express the desired protein as a fusion protein with a "tag" for which commercial antibodies are available (see Fig. 4 for SPA1). These antibodies can then be used to pull down the "transgenic" protein from plant extracts. Normally, one uses short peptides as tags (8-10 amino acids) to reduce the probability that the function of the protein is disturbed by the tag. In order to increase detection sensitivity, one often fuses several copies of the tag to the protein of interest. The following tags and their respective antibodies are frequently used: 3xHA (haemagglutinin), 6xMyc, 6 x histidine).

The principle of a co-immunoprecipitation experiment is shown in Fig. 5.

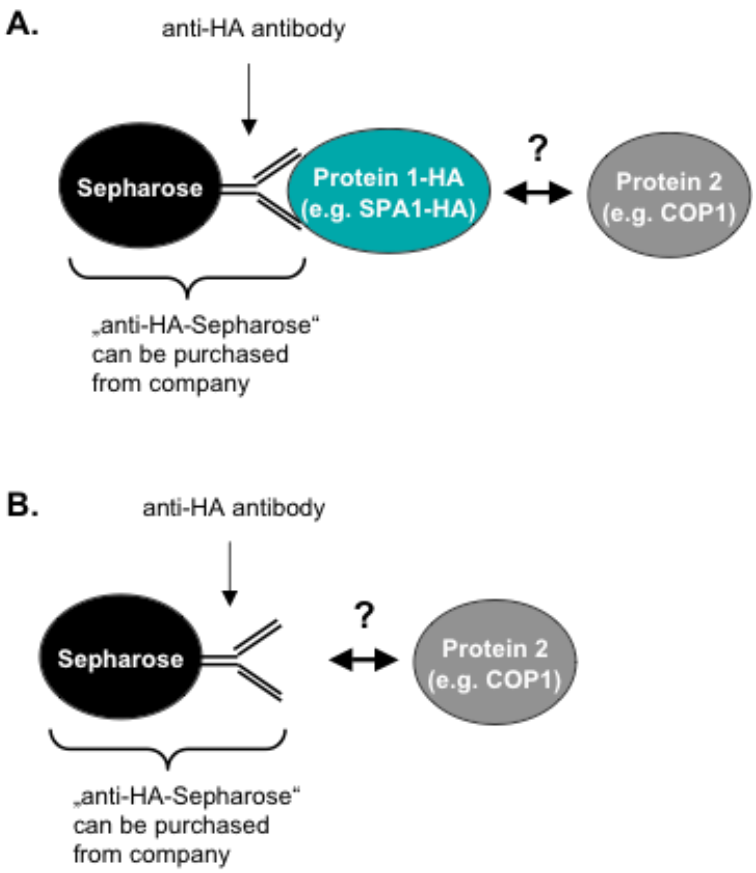


Figure 5 Schematic overview of the co-immunoprecipitation principle
Sepharose coupled with anti-HA antibodies can be obtained from companies (e.g. Roche).

A. This anti-HA-Sepharose is incubated with plant extract. The anti-HA antibody binds to the SPA1-HA protein in the plant extract. Centrifugation "pulls down" (immunoprecipitates) the heavy anti-HA-Sepharose together with the bound SPA1-HA protein. If COP1 binds to SPA1-HA in plant extracts, then COP1 is pulled down ("co-immunoprecipitated") as well. COP1 can then be detected using anti-COP1 antibodies.

B. Negative control: anti-HA Sepharose is incubated with extracts from non-transgenic plants. In this negative control, it is tested whether COP1 can bind non-specifically to the anti-HA-Sepharose. This negative control is very important!!!

Experiment 1: *In vivo* co-immunoprecipitation to investigate whether SPA1 interacts with COP1

Plant material

- transgenic seedlings expressing SPA1-HA, grown for 4 days in darkness and then exposed to red light for 2 hours to boost SPA1-HA expression (the SPA1 promoter is induced by red light), frozen in liquid nitrogen and then stored at –80ºC freezer.
- wild-type seedlings grown as above (=negative control)
- cop1-4 mutant seedlings grown as above (negative control for Western Blot with COP1 antibodies)

Solutions

Solutions used in the course	
Lysis buffer (YODA buffer)	50 mM Tris/HCl pH 7.5 150 mM NaCl 1 mM EDTA 0.1% (v/v) Nonidet-P40 10% (v/v) glycerol 1% Protease Inhibitor Cocktail (PIC), add freshly[1]
Bradford reagent[2]	
α-HA magnetic beads[2]	
TBS-T 0.05% (from 10 x TBS[2])	20 mM Tris/HCl pH 7.5 137 mM NaCl 0.05% (v/v) Tween-20
ddH_2O	
5x Lämmli buffer[2]	310 mM Tris/HCl pH 6.8 10% (w/v) SDS 50% glycerol 0.5% bromphenol blue 500 mM dithiotreitol (DTT), add freshly

[1]: **Caution!** Protease inhibitors are powerful neurotoxins. Wear gloves at all times! As protease inhibitors are highly unstable at room temperature, their activity and toxicity are gone within 30 min at RT. Hence, keep them on ice! Before discarding, leave the aliquot on your bench at RT for a couple of hours to detoxify.
[2]: Will be prepared/provided by the supervisor

Instructions

The two most important factors for a successful extraction of proteins are time and temperature. **Work quickly and always keep your samples on ice!**

Question: Briefly describe the function of all six components of the lysis buffer.

Protein extraction

1. Pre-cool mortar and pestle using liquid nitrogen. Grind SPA1-HA, wild-type and cop1-4 mutant seedlings in liquid nitrogen to a fine powder.

2. Add 300 µl lysis buffer to the powder and transfer the sample into a new reaction tube.

3. Centrifuge at 13.000 rpm for 15 min at 4°C.

4. Transfer the supernatant (approx. 250 µl) to a clean reaction tube.

5. Determine protein concentration using the Bradford assay and keep an aliquot of 20 µg protein as the input sample for all three genotypes.

Bradford Assay

1. Transfer 5 µl of the supernatant into a clean reaction tube. Prepare a second technical replicate. Also prepare a BSA standard (usually 0, 0.25, 0.5, 1 mg/ml).

2. Prepare Bradford buffer (stock is 5x concentrate, use H_2O for dilution).

3. Add 1 ml 1x Bradford buffer to the 5 µl of the supernatant and mix well.

4. Transfer 100 µl to a 96-well-plate (half area, transparent) and measure OD_{595} using the TECAN reader

(backup equation: y = 0.2743x + 0.4157; y = OD_{595}, x = protein abundance in mg/ml)

Preparation of α-HA magnetic beads

1. To ensure homogeneity, thoroughly mix the beads before use by gentle vortexing.

2. Place 20µL of Pierce α-HA Magnetic Beads into a 1.5mL microcentrifuge tube.

3. Add 200µL of 0.05% TBS-T to the beads and gently vortex to mix.

4. Place the tube on a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant.

5. Add 1mL of TBS-T to the tube. Invert the tube several times or gently vortex to mix for 1 min. Collect beads with a magnetic stand. Remove and discard the supernatant.

Binding of target protein

1. Add sample (200 µg -1 mg of total protein) containing HA-tagged protein to the pre-washed magnetic beads. Incubate the protein sample with α -HA Magnetic beads at 4°C for 30 min- 1 h.

2. Collect the beads with a magnetic stand and save an aliquot as unbound fraction sample (20 µl) and remove the rest of the unbound fraction.

Washing of α-HA magnetic beads

1. Wash the beads 3 times by adding 1 ml of ice-cold TBS-T to the tube and mixing gently. Collect the beads and transfer the supernatants of the first washing step into new 1.5 ml tubes: Take aliquots of wash 1 for SDS-PAGE.

2. Add 1 ml of ice-cold ddH_2O to the tube and mix gently. Collect the beads on a magnetic stand and make sure to remove as much liquid as possible.

Elution of proteins

1. Add 50 µl of SDS loading buffer to the sample and boil them for 5 min at 95°C. This is your bound fraction. Also boil your other samples at this point.

Question: Briefly describe the function of all five components of the Lämmli buffer. Describe the role of SDS in a a little more details (4-5 sentences).

SDS-PAGE

Solutions

Solutions used in the course	
Acrylamide stock solution (30%)[1,3]	
10% (w/v) ammonium peroxodisulfate (APS)[1,2]	
1 M Tris/HCl, pH 8.8[1]	
1M Tris/HCl, pH 6.8[1]	
10% (w/v) SDS[1]	
TEMED[1]	
10x PAGE running buffer[1]	1.9 M glycine 240 mM Tris 34 mM SDS

[1]: Will be provided/prepared by the supervisor
[2]: Store one ice
3: **Caution!** Unpolymerized acrylamide is highly toxic. Wear gloves at all times!

Instructions

Preparation of 7.5 % acrylamide gels

1. Assemble gel plates with appropriate spacers/spacer plates and combs and place in gel caster; perform a leak test with H_2O. Remove H_2O afterwards.

2. Prepare the separating gel first, then the stacking gel. The following recipe is for 2 small gels. Make sure you add APS just before you pour the gel as it is a source of free radicals catalysing gel polymerization.

	stacking gel (5%)	**separating gel (7.5%)**
gel buffer (ml)	0.625	5.625
H_2O (ml)	3.462	5.343
10% SDS (µl)	50	150
30% acrylamide (ml)	0.833	3.750
TEMED (µl)	5	12
10% APS (µl)	25	120

3. Load samples on gel according to the following loading scheme:

1	2	3	4	5	6	7	8	9	10
M	input	input	input	unbound	unbound	wash	wash	bound	bound
	WT	cop1-4	SPA1-HA	WT	SPA1-HA	WT	SPA1-HA	WT	SPA1-HA
	20µg	20 µg	20µg	20 µg	20µg	max	max	max	max

M= Molecular weight marker (Prestained Protein Ladder, Fermentas), 5 µl

4. Run gel at 20 mA constant current for approx. 2 hours.

Western Blot and antibody incubation

Solutions

Solutions used in the course	
Towbin buffer	96 mM glycine (7.36 g/l) 10 mM Tris (1.21 g/l) 10% MeOH (100 ml/l)
10x TBS	1.37 M NaCl (80 g/l) 100 mM Tris (1.21 g/l) adjust pH to 7.3 (HCl)
1x TBS-T 0.1%	100 ml 10x TBS (for 1 l) 1 ml Tween-20 (for 1 l)
Roti-Block[1]	
Antibody incubation solution	100 ml 10x TBS (for 1 l) 3% skim milk powder
Coomassie staining solution	0.25% (w/v) Coomassie Brilliant Blue 50% (v/v) MeOH 7% (v/v) glacial acetic acid
Coomassie destaining solution	50% (v/v) MeOH 7% (v/v) glacial acetic acid

[1]: Will be provided/prepared by the supervisor

Instructions

Western Blot

1. For each gel cut 8 pieces of Whatman paper and 1 piece of PVDF membrane to the size 4.6 cm x 8.6 cm. Proteins stick to the PVDF membrane very easily, thus wear gloves at all times and use a forceps.

2. To pre-wet PVDF membrane incubate it for 10 sec in 100 % methanol and then in Towbin buffer for additional 15 min. Also incubate the gel in Towbin buffer for 15 min.

3. Assemble Western Blot (Figure 6). Briefly pre-wet Whatman filter paper in Towbin buffer prior to assembly. Avoid trapping air bubbles! Remove air bubbles by rolling a glass tube across the filter paper/membrane/gel each time.

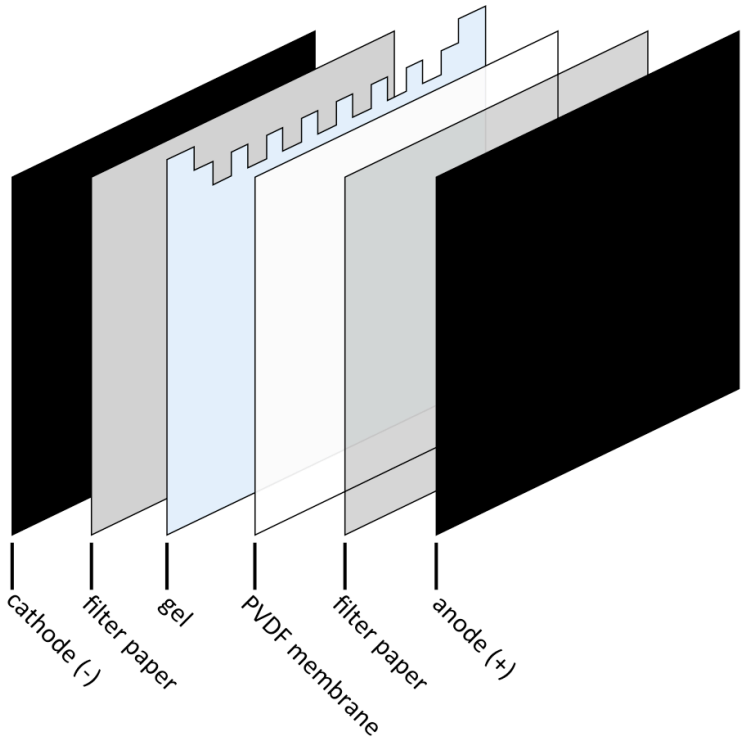


Figure 6 Western Blot Assembly

4. Blot for 2h at 0.35 mA/cm^2 membrane.

5. Block free sites on membrane with 1x Roti-Block (Roth) for 30 min to 1 hour at room temperature. (You can also do that over-night). Rotiblock is a protein-free blocking solution. Hence, it allows you to stain your membrane with Coommassie after the antibody incubation.

6. Carefully cut the membrane at 100 kDa. The upper part will be used to detect SPA1-HA, the lower part will be used to detect COP1.

Incubation of membrane with anti-COP1 and anti-HA antibodies

First the COP1 protein is going to be detected, then the SPA1-HA protein. The COP1 antibody was made in rabbits, therefore anti-rabbit antibody with a coupled horseradish peroxidase (HRP) is used as a secondary antibody for detection. The commercial HA-antibody (Roche) is already coupled to HRP; therefore no secondary antibody is needed.

Question: What are the advantages and disadvantages of using a system that consists of a primary and secondary antibody (e.g. α-COP1 + α-rabbit-HRP) in comparison to a single antibody system (e.g. α-HA-HRP)

COP1:

1. Incubate the blocked membrane with α-COP1 antibody over-night at 4°C at a dilution of 1:200 in 3% skimmed milk powder (in 1xTBS).

2. Wash membrane three times with 1x 0.1% TBS-T (10 min each).

3. Add the secondary antibody (anti-rabbit, coupled with horseradish peroxidase) at a dilution of 1:80.000 in 5 % skimmed milk powder (in 1x TBS) for 1 hour at room temperature.

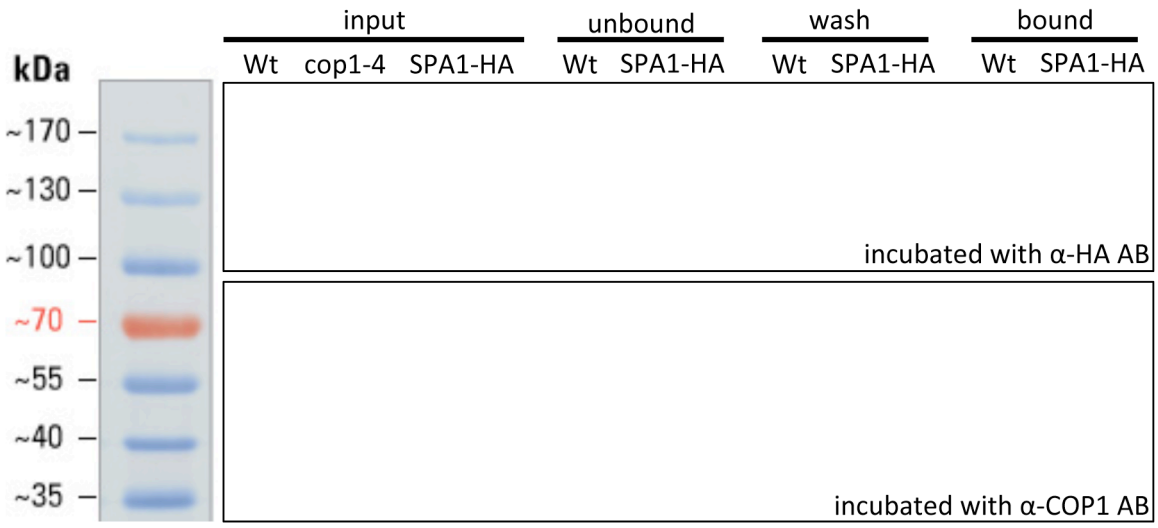
4. Wash three times with 1x 0.1% TBS-T (10 min each).

SPA1-HA:

1. Incubate membrane with α-HA-HRP antibody at room temperature for 1 hour at a dilution of 1:1000 in 5% skimmed milk powder (in 1xTBS).

2. Wash membrane three times with 1xTBS-T (10 min each).

Question: Assuming that COP1 and SPA1 interact under the testes conditions, what will the picture of the membrane look like after detection? Please add the respective bands to the picture below. (Apparent molecular weights on a 7.5% PA-gel: COP1 – 73 kDa, SPA1-HA – 130 kDa)



Detection of the antibody via its coupled HRP activity

The HRP-coupled antibody can now be detected quantitatively. In the presence of H_2O_2, peroxidase catalyzes the oxidation of the substrate luminol to an excited intermediate whose electrons – when returning to the ground state - emit light (=chemiluminescence). This light emission is detected using an X-ray film or a light-sensitive CCD camera (e.g. LAS 4000 Mini from Fuji).

Solutions

ECL Plus™ Western Blotting Reagents (Solutions A&B; provided)

Instructions

1. Place each membrane in between two pieces of plastic foil.

2. Mix 250 µl of Solution A with 250 µl of Solution B per membrane.

3. Pipet the solution directly onto the membrane and incubate.

4. The HRP signal is detected and quantified using a LAS-4000 mini CCD camera and a computer software.

(5. Only if nothing can be detected on the blot. After the antibody detection the membranes are stained with Coomassie staining solution for 1 min and subsequently destained with destaining solution as needed.)

Experiment 2: Light-controlled gene expression

Light, being one of the most important environmental factors for plants, influences the expression of a great number of genes (approx. 10% of all genes in Arabidopsis), such as many photosynthesis-related genes, e.g. genes coding for the chlorophyll a/b binding protein (Cab) or the small subunit of ribulose 1,5-bisphosphate carboxylase (RbcS), but also genes encoding components of light signal transduction (e.g. SPA1). Some genes are induced in response to light, others are repressed.

Tasks

During this part of the course, you are asked to examine the effect of light on the expression of several genes using REAL-Time PCR.

Isolation of total RNA from light- and dark-grown Arabidopsis seedlings

Arabidopsis seedlings are grown in complete darkness for three days and subsequently exposed to light for 1-24 h. This allows us to determine a time-course on the light-induced change in transcript levels. Thus we can distinguish between rapidly and more slowly responding genes. From these seedlings, total RNA is isolated from 100 mg of tissue using the Plant RNeasy Mini Kit (Qiagen). The concentration of the isolated RNA is subsequently determined photometrically. To check for RNA quality (degraded yes/no?), the RNA is separated on an agarose gel.

Caution! RNA is quickly degraded by RNases which are basically everywhere, such as on your skin. Therefore clean your working space before you start the extraction and wear gloves to protect your samples!

Solutions und Materials

solutions and materials used in the course	
RNeasy-Kit (Qiagen)[1]	
1x Electrophoresis buffer[1]	10.8 g/l Tris 5.5 g/l boric acid 0.93 g/l EDTA
agarose	
sample buffer (loading dye)[1]	30% (v/v) glycerol 0.25 %(w/v) bromphenol blue do not autoclave, use destilled water
ethidium bromide (EtBr)[1,2]	5 mg/ml (concentration in gel: 0.5 µg/ml)

[1]: Will be prepared/provided by the supervisor.

[2]: Caution! EtBr might be cancerogenic. Wear gloves at all times!

Plant samples

WT, cop1-4, spa-nq, 5 day dark-and light-grown *A.th* seedlings, two biological replicates

Instructions

1. Use RNeasy kit according to manufacturer´s instructions.

2. To quantify RNA concentration transfer 1 µl of RNA solution to a clean reaction tube containing 99 µl of water. Mix briefly by vortexing.

3. Determine the OD_{260} using a photometer and calculate the RNA concentration of your samples: OD_{260} x 40 x dilution factor 100 = RNA-concentration in µg/ml

Note:

RNA: 1 OD_{260} = 40 µg/ml

double-stranded DNA: 1 OD_{260} = 50 µg/ml

Oligonucleotide: 1 OD_{260} = 33 µg/ml

4. Check the integrity of your RNA on a gel: Transfer 1 µg of RNA to a new tube, add 8 µl of water and 1 µl of sample buffer. Load onto a 0.8 % agarose gel.

5. To pour the agarose gel, add agarose to electrophoresis buffer and heat in microwave (attention: wear safety goggles!). Let solution cool down to approx. 60°C, add Ethidium bromide solution, mix and pour into gel tray.

DNAse digestion of RNA samples

In order to remove any DNA from the extracts, we first incubate the RNA with DNAse.

Solutions and Materials

solutions used in the course
10x DNase buffer[1]
RNase-free water[1]
DNase I (1 U/µl)[1]
50 mM EDTA[1]

[1]: Will be prepared/provided by the supervisor.

Instructions

1. Take a 1.5 ml tube and add:

Total RNA	1 µg
DNase-Buffer	2 µl
RNAse-free H_2O	ad 19 µl

2. Add 1 µl of DNase I per tube

3. Incubate for at least 30 min at 37 ^{o}C.

4. Add 2 µl of EDTA (50 mM) and incubate for 10 min at 65 ^{o}C in order to inactivate the DNase.

Check for remaining DNA contamination

1. 2 µl DNase treated RNA is used for standard PCR with UBQ10 primers.

PCR mix	
RNA	2 µl
10x Taq buffer	2 µl
10 µM forward primer	1 µl
10 µM reverse primer	1 µl
dNTPs	0.5 µl
Taq polymerase	1 µl
H_2O	ad 20 µl

2. PCR products are checked on an agarose gel afterwards (use genomic DNA as control).

Question: In which sample do you expect to see a band from the gel and why?

Reverse Transcription of all mRNAs

We will quantify transcript levels using RT-PCR (reverse transcription-PCR). In a first step, the enzyme reverse transcriptase copies all mRNA molecules into cDNA. The primer for this reaction is an oligo-dt primer which anneals to the polyA tail of the mRNAs. In a second step, we will amplify specific cDNAs using gene-specific primer pairs in a normal PCR reaction.

Solutions and Materials

solutions used in the course	
5x First strand buffer[1]	20 mM MgCl2 250 mM KCl 50 mM DTT 200 mM Tris/HCl, pH 8.3
50x dNTP-solution1	10 mM dATP, dCTP, dGTP, dTTP
RevertAid H Minus-MuLV Reverse Transcriptase (200 U/µl)[1]	
10 µM Oligo-dT primer[1]	

[1]: Will be prepared/provided by the supervisor.

Instructions

1. Take a PCR tube. Add the following components to the PCR tube placed on ice:

Total RNA	1 µg (20 µl of DNase digestion)
Oligo-dt primer (10 µM)	2 µl
H_2O (RNase-free!)	ad 27 µl

2. Denature RNA by incubating tube for 5 min at 65°C in a preheated PCR machine.

3. Transfer tubes to ice immediately to prevent renaturing of RNA.

4. Add the following components (on ice!):

5x first strand buffer	8 µl
dNTP-Mix (10 mM/dNTP)	4 µl
Reverse Transcriptase (RevertAid H Minus M-MuLV)	1 µl

5. Place PCR tubes into a PCR machine that was preheated to 37 °C programmed as follows:

5min @ 37°C

60 min @42°C (Synthesis of a DNA strand complementary to RNA)

5 min @70°C (Inactivate Reverse Transcriptase)

6. Store samples on ice or at –20°C.

Quantification of transcript levels using REAL-TIME PCR

Now the cDNA generated in the reverse transcriptase reaction will be PCR-amplified using gene-specific primers. The amount of PCR-product obtained directly correlates with the amount of mRNA that was present in the RNA sample. However, determining the amount of PCR product produced after 40 cycles is not sufficient because the increase in PCR product abundance increases in a sigmoid (i.e. non-linear) fashion during the PCR (see Fig. 6). Towards the end of the PCR reaction PCR reagents are used up and therefore no further increase in the amount of PCR product is possible. This end point is reached „earlier" for abundant templates and „later" for rare templates (see Fig. 6), thus after 40 cycles all samples might have reached this end point and differences are no longer detectable. Hence, the abundance of PCR products derived from different RNA samples needs to be compared at an earlier cycle step when reagents are not yet used up and the PCR thus proceeds linearly. Here, REAL-Time PCR comes in handy: The advantage of the REAL-TIME PCR method is that the abundance of PCR product can be measured during the PCR reaction.

To a REAL-TIME PCR-reaction, a dye („SYBR-Green") is added which specifically binds double-stranded DNA and then fluoresces. In the course of a PCR reaction, the amount of PCR product increases which also results in a proportional increase in SYBR-Green fluorescence. This fluorescence is quantified after every PCR cycle using a CCD camera. At the end of the PCR reaction, a curve is obtained for every sample (see Fig. 6). During the first PCR cycles the fluorescence is below the limit of detection, then it increases linearly. Eventually, the increase levels off and fluorescence intensity approaches a maximum. To quantitatively determine and compare transcript levels a relatively early step during the PCR is used. This step is chosen by placing a horizontal line, the so-called „threshold" just above the background fluorescence. The PCR cycle at which the PCR curve crosses the threshold is called the „threshold cycle" (C_T-value). The fold difference in transcript abundance between two samples X and Y (e.g. light and darkness) can be calculated according to the formular 2^n , with n being the difference between the C_T-value of X and that of Y.

To normalize results, the abundance of a transcript that is not regulated by light is used (e.g. ubiquitin).

Question: Since ubiquitin is not light-regulated and used as normalization/control gene, what kind of expression level do you expect?

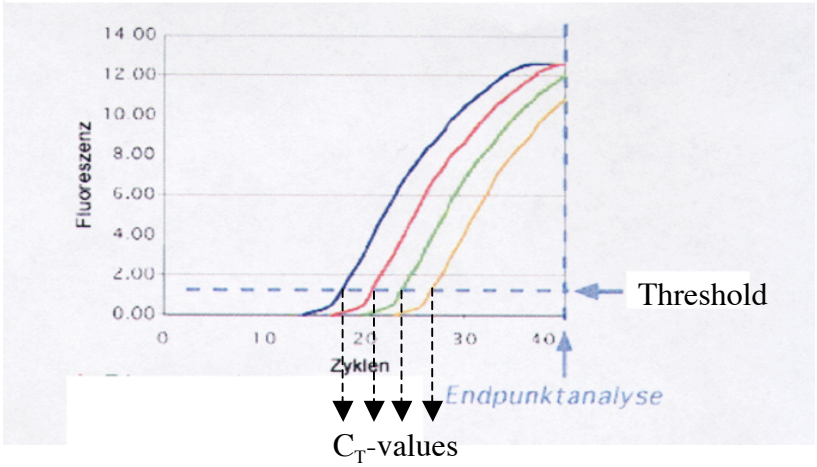


Figure 7 Fluorescence intensity in the course of a PCR reaction (number of cycles)

Solutions and Materials

solutions and materials used in the course
2x Power SYBR-Green PCR Master Mix[1]
Gene-specific primers (UBQ10, gene of interest)[1]
96-well PCR plates with lids[1]
cDNA samples

[1]: Will be prepared/provided by the supervisor.

Instructions

1. Prepare Master Mix 1 and Master Mix 2 (per reaction):

    12.5 µl Power-SYBR-Green

    x µl Primer 1 (100 nM final concentration)

    y µl Primer 2 (100 nM final concentration)

    ad 23 µl dH_2O (new)

2. Aliquot (23 µl) Master-Mix 1 / 2 into 96-well plate.

3. Add 2 µl of cDNA per well.

4. Close the PCR plate with a lid.

5. Switch on ABI7300 and run REAL-TIME PCR program (as follows).

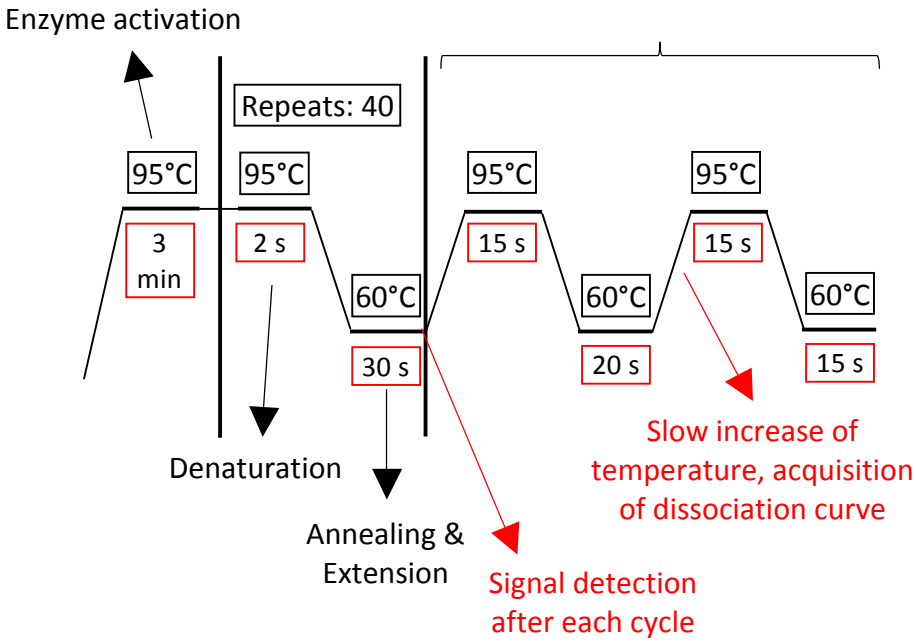


Figure 8 Scheme of the qRT-PCR program

Statistical analysis of data according to the $2^{-\Delta\Delta Ct}$ method

Definitions

Threshold Cycle (Ct): The threshold cycle (Ct) is the cycle during a quantitative real time - PCR run in which the SDS software detects the first significant fluorescence of the reporter dye above background level (which is defined by the baseline).

ΔCt (Normalization): The Ct value of a target gene is subtracted from the Ct value of an endogenous control (reference gene):

ΔCt = Ct (target gene) – Ct (endogenous control).

ΔΔCt (Calibration): The ΔCt value is subtracted from the ΔCt value of a calibrator:

ΔΔCt (test sample) = ΔCt (test sample) – ΔCt (calibrator sample).

The ΔΔCt value is employed to calculate the magnitude of change in the expression of the target gene, using the following formula:

$$2^{-\Delta\Delta Ct}$$

Question: What ΔΔCt value would you get from the calibrator sample?

Example

Here is an example of how you calculate your real time PCR result:

The standard error (SEM) of the average ΔΔCt is given by:

Standard deviation (SD) of ΔΔCt/ (Square root (number of biological replicates))

			Step 1				Step 2	Step 3	Step 4	Step 5	Step 6	
Samples	Biological Replicates	Technical replicates	Ct_{TAR}	Average Ct_{TAR}	Ct_{REF}	average Ct_{REF}	$\_Ct_{TAR-REF}$	Average $\_Ct_{control}$	$\_\_Ct_{\_Ct-Avg.\_Ct}$	$__$Ct Expression	Average $__$Ct Expression	Standard error of Average $__$Ct Expression
Treatment (Sample1)	1	1	33,6	33,27	26,3	26,40	6,87	N/A	4,81	0,0356	0,0405	0,003
		2	33		26,4							
		3	33,2		26,5							
	2	1	33,3	33,6	26,7	26,9	6,70		4,64	0,0400		
		2	33,6		27							
		3	33,9		27							
	3	1	33,6	33,4	26,9	26,9	6,50		4,44	0,0459		
		2	33,2		26,8							
		3	33,4		27							
Control (Calibrator)	1	1	28,9	28,97	26,5	27,23	1,73	2,06	-0,32	1,2503	1,0149	0,125
		2	29		27,6							
		3	29		27,6							
	2	1	29,1	29,17	27	27,07	2,10		0,04	0,9697		
		2	29,1		27							
		3	29,3		27,2							
	3	1	29,9	29,87	27,5	27,53	2,33		0,28	0,8249		
		2	29,9		27,6							
		3	29,8		27,5							

In this case, the target gene of the mutant was 24.7 (1/0.0405) fold down-regulated compared with the wild type.

Task: With the calculated results, make a clear graph of the test samples and calibrator sample on your computer. Analyze the expression of our gene of interest (don´t forget to add the error bars!)

Estimated time schedule

Day 1	Day 2	Day 3	Day 4	Day 5
Experiment 2 RNA extraction DNase treatment RT-PCR	**Experiment 1** Protein isolation Co-IP preparation of gels	**Experiment 1** Electrophoresis Western Blot 1° AB incubation **Experiment 2** Discussion of results	**Experiment 1** 2° AB incubation HA-AB incubation detection (Coomassie stain) Discussion of results Clean up!	**free**