Molekulare Physiologie der Pflanzen und Mikroorganismen

WS2014/15

Script for the lab course (AG Höcker) Interactions between plants and the environment

part 2

A. Transient expression of GFP-fusion proteins using particle bombardment of plant tissue:

Determination of the subcellular localization of a protein

Especially for proteins with a function in signaling it is important to know whether the protein is localized in the nucleus and thus might participate in the control of gene expression. Certain motifs in the sequence of a protein can allow to predict that a protein is transported into the nucleus. Such nuclear localization sequences (NLS) consist of a short stretches of basic amino acids (Lys K and Arg R). One distinguishes SV40-type NLS (5 basic amono acids in a row, e.g. KKRKR) and a bipartite NLS (2-3 basic amino acids– $(X)_{10-12}$ – 4 basic amino acids, with X being any amino acid).

If a protein contains a NLS, chances are high that the protein localizes to the nucleus. An experimental confirmation of this hypothesis is nevertheless necessary. To this end, the protein-coding sequence (cDNA) is fused with a reporter gene, whose gene product can be easily detected. Thus, the purpose of the reporter gene is to visualize the fusion protein in the cell. In plants, one frequently uses the bacterial uidA gene which codes for a β-Glucuronidase (GUS). When 5-Brom-4-Chlor-3-Indolyl-β-D-Glucuronide (X-Gluc) is used as a substrate, GUS activity can be detected histologically because the resultant product is blue and insoluble. Alternative reporter genes are the Green Fluorescent Protein (GFP) and luciferase. An advantage of GFP and luciferase is the possibility of in vivo imaging of living tissue. GUS-staining, in contrast, kills the cells and thus is a lethal stain.

GFP is a protein from jellyfish („Qualle"). Three Scientists (Shimomura, Chalfie, Tsien) were awarded the Nobel Price for Chemistry in 2008 for its detection and its application as a reporter gene. The sequence of the original GFP has subsequently been mutated to obtain spectral GFP variants that flouresce more in the blue or yellow part of the spectrum (CFP, YFP, Fig. 1a, b). This allows the expression of two GFP variants in one cell for applications such as protein co-localization and FRET.

To express GFP-fusion proteins in plants, there are two main possibilities:

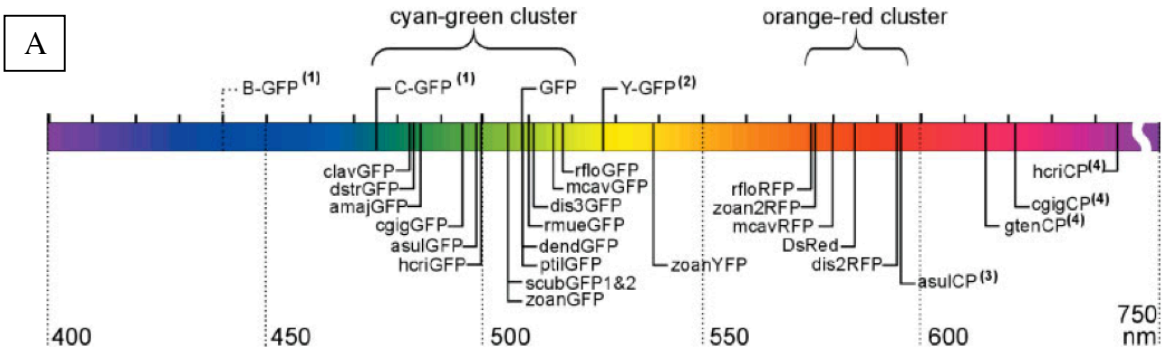
a) generate stably transformed Arabidopsis plants by Agrobacterium-mediated transformation (takes 2-4 months) and analyze GFP-expression in these transgenic plants.

b) transiently express GFP-fusion protein in plant tissue (takes 1 day) and analyze GFP-expression in the transformed cells

Transient expression does not require stable integration of the GFP plasmid into the plant chromosomes. It only requires that the GFP plasmid is introduced into plant cells. Because the plasmid DNA usually is not stably integrated into the plant genome it is degraded over time. The coded protein is therefore synthesized only over a short period of time (approx. 48 h). This type of

transformation is therefore called transient transformation. In animal systems it is also referred to as transfection. Methods for transient expression in plant cells are particle bombardment and transfection of plant protoplasts (= plant cells after removal of the cell wall).

For particle bombardment, plasmid DNA is precipitated onto small gold particles (diameter 1 μm) using high salt concentrations (see Fig. 2). These loaded gold particles are subsequently accelerated by the release of helium pressure so that they are propelled towards the plant tissue. They break through the cell wall of the upper cell layer and somehow reach the nucleus where the DNA is transcribed. The produced mRNA is then translated into protein.



Spectral properties of variants of the GFP family (Matz et al., 2002).

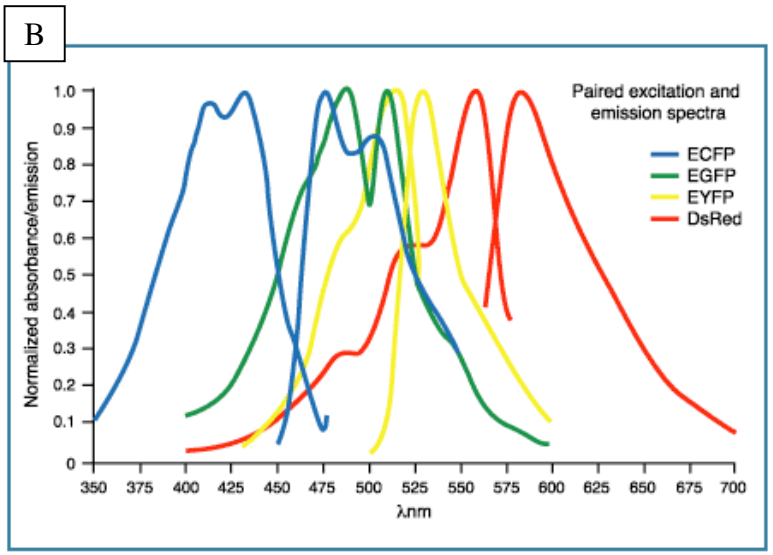


Figure 1: Spectral properties of GFP variants
B: The excitation and emission spectra for ECFP (cyan), EGFP (green), and EYFP (yellow), which are the most commonly used versions of Aequorea GFP, and DsRed (red). In each case, the excitation and emission spectra for a particular protein are paired by color.

Particle bombardment

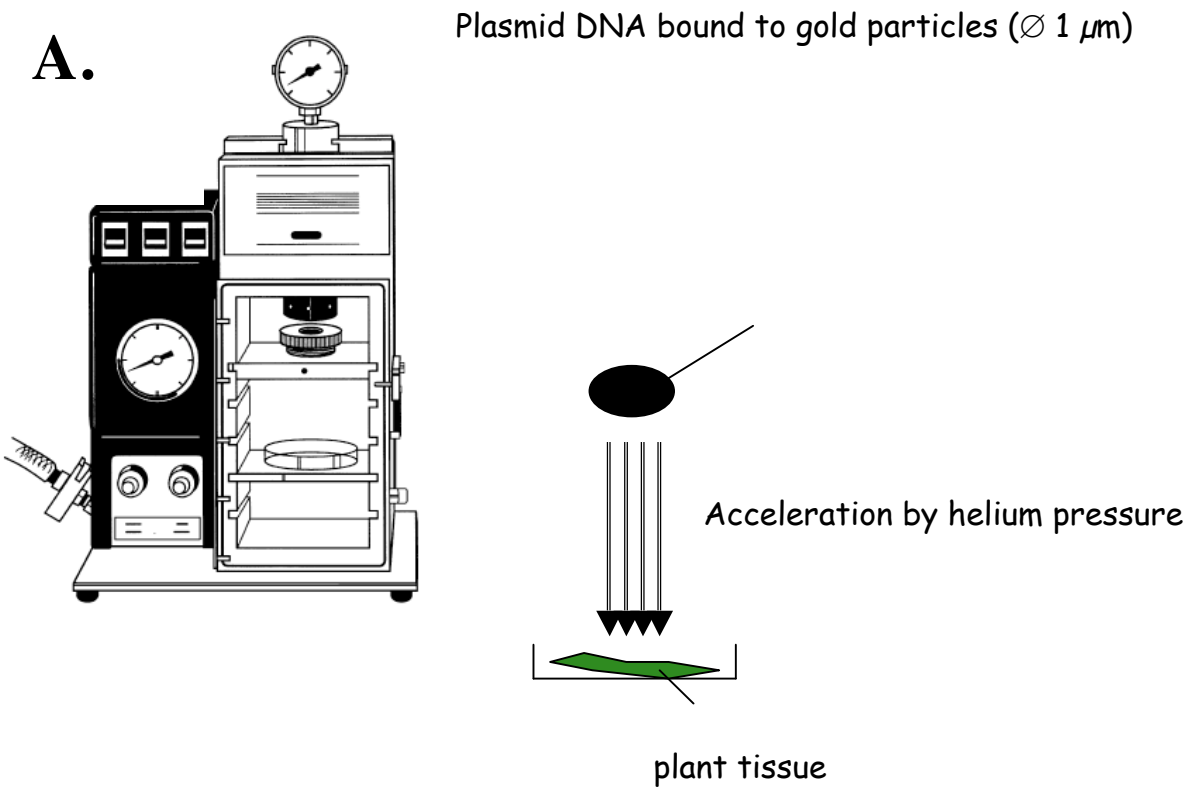


Fig. 2: Biolistic transformation of plant tissue using the Particle Gun

A. Principle of Particle Bombardment

B. Particle Gun

The Particle Gun consistes of (from top to bottom, see Fig. 2):

- an acceleration tube which carries a rupture disk at the bottom end.
- a thin plastic plate („macrocarrier"), onto which the DNA-gold particles (= „microcarriers") are pipetted. In Fig. 1, the gold particles are on the bottom-facing side of the macrocarriers.
- a „stopping screen"
- the plant tissue to be bombarded

In the acceleration tube helium pressure builds up and eventually at a certain pressure causes rupture of the rupture disks. The pressure is released and propels the macrocarriers carrying the DNA-gold particles towards the plant tissue. The macrocarriers are stopped by the stopping screen while the DNA-gold particles reach the plant tissue.

Tasks:

- Express GFP fusion proteins in onion and leek epidermal cells and localize the GFP fusion proteins by fluorescence microscopy.

Biolistic transformation of onion epidermal cells

Solutions and materials:

1. petri dishes (diameter 9 cm) with wet paper (12 petri dishes for the whole course)
2. 2.5 M $CaCl_2$ (50 ml for the whole course, autoclave)
3. 0.1 M spermidine (will be provided)
4. 100 % ethanol (p.a.) (2 x 50 ml for the whole course)
5. 70 % ethanol (p.a.) (2 x 50 ml for the whole course)
6. Goldparticle solution (will be provided)

   Weigh 30 mg gold particles (diameter 1.0 μm) into a 1.5 ml Eppendorf tube and add 1 ml of 70% ethanol, vortex and let sit for 15 min. Centrifuge for 15 sec, discard supernatant and wash twice with 1 ml of sterile water (sonicate 3 sec). At last add 1 ml of sterile water, sonicate briefly and make 50 μl aliquots while vortexing. These can be stored at -20°C.

Materials:

1. 2 onions / leek
2. rupture disks (900 psi), macrocarriers, stopping screens

Protocol:

1. Remove outer layers from the onion and cut onion in quaters and place onion tissue with the epidermal layer facing up onto wet paper in petri dishes. Remove outer layers from the leek, cut pieces from the lower (white) part of the leek and place with the inner side facing up onto wet filter paper.
2. Add 400 ng of each plasmid to an Eppendorf tube and add the following solutions:
    - 5 µl gold particles
    - 10 µl 2.5 M $CaCl_2$
    - 4 µl 0.1 M spermidine
3. incubate at room temperature for 10 -15 min, flick the tube gently several times during this step.
4. Gently pellet gold by pulse-centrifugation (10 sec, 10.000 rpm). Carefully remove and discard supernatant.
5. Wash pellet by adding 100 µl 70% ethanol. Briefly vortex and gently pellet gold by pulse-centrifugation (10 sec, 10.000 rpm), remove and discard supernatant.
6. Wash a second time by adding 50 µl 100% ethanol (p.a.), briefly vortex and spin as above. Discard supernatant.
7. Add 12 µl of 100% ethanol. Resuspend by pipetting up and down thoroughly, make sure that the mixture is homogenous! Store on ice until use.
8. Pipet gold particles on macrocarrier and wait until the ethanol evaporated.
9. Perform particle bombardment.
10. Incubate onion tissue for ca. 24 h at room temperature. Then examine by microscopy.

B. Identifying components of the light signalling cascade using the yeast two-hybrid system

Proteins for signal transduction usually act as a complex with other proteins and are therefore equipped with protein-protein interaction domains. As soon as a protein involved in a signalling cascade is found, there is a great interest in isolating interacting proteins. The yeast two-hybrid system may be utilised in these cases. This approach was, for example, used to identify proteins which can bind phytochrome. A transcription factor of the basic helix-loop-helix protein family (PIF3) was isolated by this method. PIF3 can exclusively bind phytochrome in its active form (P_{fr}), but not in its inactive form (P_r) (Ni et al., 1998; Ni et al., 1999). Therefore, the probability of PIF3 being an essential component in the phytochrome signal transduction pathway is very high.

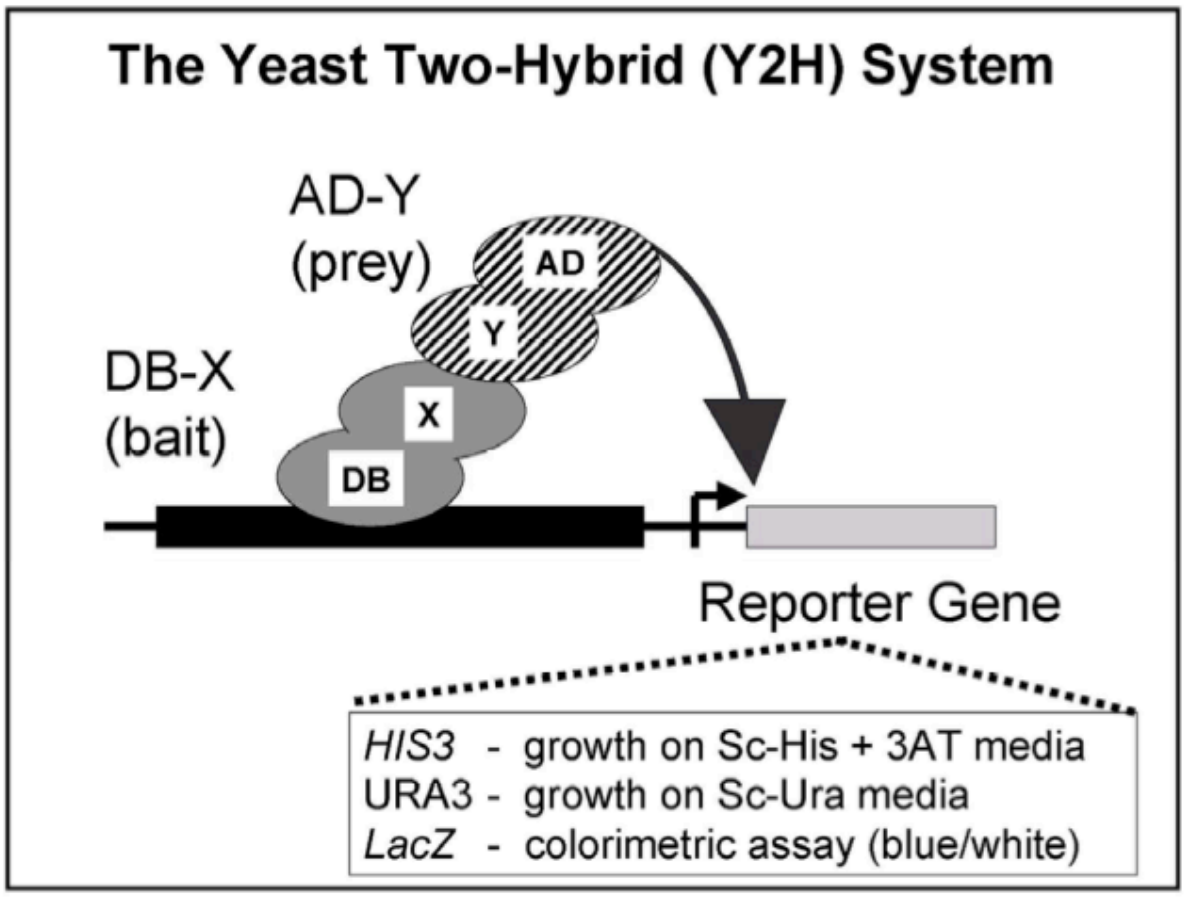


Fig. 5: The two-hybrid system for analysis of protein-protein interaction. The provided bait plasmid encodes a fusion of protein X with the Gal4-DNA-binding domain. The prey protein contains a fusion of protein Y with the GAL4 transcriptional activation domain.

Basis for the two-hybrid system is the fact that transcription factors possess a domain structure. A DNA-binding domain can, for instance, be combined as a fusion protein with transcription activation domains of a variety of other transcription activators without interfering with their function. The principle of the two-hybrid system is displayed in Fig. 5. Figs. 6 and 7 show commercially available vectors for expression of the different fusion proteins.

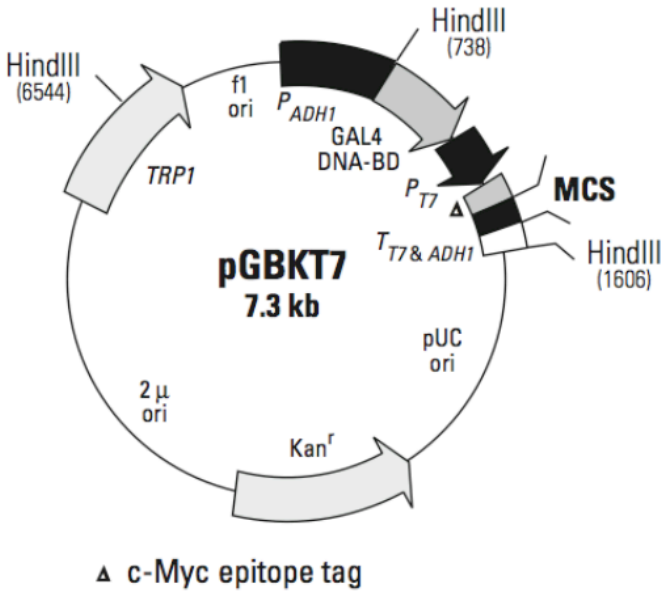
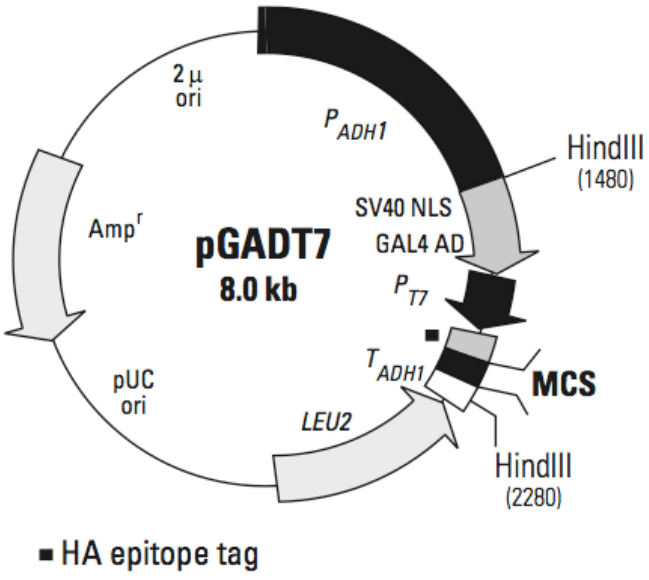


Fig. 6: The bait vector pGBT9 from the two-hybrid system by Clontech. The sequence of the bait protein is cloned into the multiple cloning site (MCS) behind the gene sequence for the DNA-binding domain (BD) of the GAL4 transcription factor.

Fig. 7: The cDNA-GAL4AD fusion vector pGADT7 from the two-hybrid system by Clontech. The cDNAs, whose corresponding proteins are to be tested for binding to the bait protein, are cloned behind the gene sequence of the activation domain (AD) of the GAL4 transcription factor in the multiple cloning site (MCS).



If one aims at identifying phyA-binding proteins, the first step is to fuse the phyA protein to the DNA-binding domain of the transcription factor GAL4 and then to express this fusion protein in the cells of the yeast tester strain. This yeast tester strain cannot grow on minimal medium without addition of the amino acid histidine, because it possesses a mutation in the *HIS* gene, which encodes an essential enzyme in the histidine biosynthesis pathway of yeast. Furthermore, the yeast tester strain contains a genomic reporter gene, which consists of a promoter with a GAL4-*cis* element and the HIS3-coding sequence.

For screening of phyA-binding proteins, these yeast cells are transformed with a cDNA library derived from Arabidopsis seedlings. The single cDNAs are inserted on a plasmid behind (in 3' direction) the sequence for the activation domain of the GAL4 transcription factor from yeast (see fig. 7). When both sequences are in phase, fusion proteins consisting of random plant protein fragments and the GAL4 activation domain can form. The selector gene - *HIS3* - is controlled by a GAL4-dependent promoter. It can only be activated if the GAL4 binding domain, which is fused to the so-called bait protein (phyA in this case), physically links the activation domain to the DNA binding domain through the putative interaction partner, the so-called prey protein. This will lead to the formation of a functioning GAL4 protein complex. Such an interaction between bait protein and prey protein will lead to growth of yeast cells on histidine-free minimal medium. To make it possible to identify those from the large amount of cDNAs that can produce phytochrome-binding proteins, a great number of yeast transformants is plated on histidine-free medium. Those yeast cells that can grow, and thus form colonies, express a phytochrome-binding protein.

To test and confirm this interaction, the yeast tester strain contains a second reporter gene. This reporter gene consists of the β-galactosidase gene, which is also placed behind a GAL4-dependent promoter. The intensity of the interaction can be measured by analysing β-galactosidase activity.

References:

Inouye C, Remondelli P, Karin M & Elledge S (1994): Isolation of a cDNA encoding a metal response element binding protein using a novel expression cloning procedure: the one hybrid system. DNA Cell Biol *13*: 731-742.

Brent R & Finley RL jr (1997): Understanding gene and allele function with two-hybrid methods. Annu Rev Genet *31*: 663-704.

MATCHMAKER One-Hybrid System, User Manual. Clontech

GAL4 Two-Hybrid Phagemid Vector Kits, User Manual. Stratagene.

Schiestl RH & Gietz RD (1989): High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr Genet *16*: 339-346.

Plasmids:

Designation	Origin	Description
pGAD424 pGADT7	Clontech	*E. coli*/yeast shuttle vector: GAL4 activation domain, **LEU2**, 2µ origin of replication
pGBT9 GBKT7	Clontech	*E. coli*/yeast shuttle vector: GAL4-(1-147)-DNA-binding domain, **TRP1**, 2 µ origin of replication

Yeast strains:

Designation	Origin	Description
AH109	Clontech	his 3-200, ade 2-101, leu 2-3, lys 2-801, trp1-901, ura 3-52, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$-HIS3, URA3::MEL1$_{UAS}$-lacZ

Tasks:

- The genetic screening process of the two-hybrid system is to be comprehended in the context of this practical course. You will receive different bait plasmids (phyA, phyB) and the prey plasmid PIF3. It is your task to test the binding specificity of the PIF3 protein. To do so, you will use two selection methods:
  1) Growth on histidine-free minimal medium and
  2) Production of the enzyme β-galactosidase, which can be quantitatively and qualitatively detected.

Group 1: plate on medium + 20 mM 3-AT

1. GAD-PIF3 + GBD-phy**A**CT
2. GAD-424 + GBD-phy**A**CT
3. GAD-PIF3 + GBT9

Group 2: plate on medium + 20 mM 3-AT

4. GAD-PIF3 + GBD-phy**B**CT
5. GAD-424 + GBD-phy**B**CT
6. GAD-PIF3 + GBT9

Group 3: plate on medium + 20 mM 3-AT

7 GAD- phy**B**CT + GBD-phy**B**CT

8. GAD-424 + GBD-phy**B**CT

9 GAD- phy**B**CT + GBT9

Group 4: plate on medium without 3-AT

10 COP1-GAD + SPA1-GBD

11. GADT7 + SPA1-GBD

12. COP1-GAD + GBKT7

Group 5: plate on medium without 3-AT

13 COP1-GAD + COP1-GBD

14. GADT7 + COP1-GBD

15. COP1-GAD + GBKT7

Abbreviations:

GAD	= Activation domain of transcription factor GAL4
GBD	= DNA-binding domain of transcription factor GAL4
GAD424, GADT7	= Vectors for expression of GAD fusion proteins
GBT9, GBKT7	= Vectors for expression of GBD fusion proteins

Solutions and media:

1. 40 % glucose (200 ml for the whole course, autoclave)

   400 g/l glucose

2. YAPD medium (liquid) (750 ml for the whole course, autoclave)

   20 g/l Difco-peptone
   10 g/l yeast extract
   100 mg/l adenine hemisulfate

   adjust pH to 5.8

   after autoclaving, add 50 ml/l 40 % glucose (sterile)

   Adding adenine hemisulfate to the YAPD medium is not essential, since adenine is present in both yeast and peptone extract. However, it will enhance growth of yeast cells with a mutation in the *ADE* gene.

3. Synthetic "drop-out" medium (solid)
   -Leu, -Trp

   -Leu, -Trp, -His

   -Leu, -Trp, -His, + 20 mM 3-AT

   6.7 g/l nitrogen base without amino acids
   40 mg/l adenine hemisulfate
   x g/l drop-out supplement (-Leu, -Trp; or -Leu, -Trp, -His, -Ade)

   adjust pH to 5.8
   put 18 g/l agar into bottles
   autoclave
   add 50 ml/l 40 % glucose (sterile)
   add 3-AT ad 20 mM where necessary, mix well
   pour plates

4. 3-amino-1,2,4-triazol (3-AT) stock solution (15 ml for the whole course, filter-sterilize)
   1 M 3-AT

5. 100% DMSO (20 ml for the whole course)

6. 1 M lithium acetate · 2 H_2O (20 ml for the whole course, autoclave)

7. 50% (w/v) polyethyleneglycol (PEG, MW 3350) (20 ml for the whole course)
   Slowly add water to 10 g PEG under constant stirring (final volume 20 ml) and filter-sterilise or autoclave.

8. 10x TE buffer (50 ml for the whole course, autoclave)

0.1	M	Tris/HCl
10	mM	EDTA

Adjust pH to 7.5

Freshly prepare the following solutions from these two stock solutions under sterile conditions prior to transformation:

9. 1x TE

10. 1x TE/0.1 M lithium acetate (2x 10 ml for the whole course)

11. PEG/lithium acetate solution (2x 10 ml for the whole course)

40	%	PEG
1	x	TE
0.1	M	lithium acetate

Since the PEG solution has a much higher density than water, the solution has to be mixed well until fully intermixed.

12. sterile water (2x 100 ml for the whole course, autoclave)

13. salmon sperm DNA (10 mg/ml) (will be provided)
Since transformation efficiency is directly proportional to the average length of the carrier-DNA used, all steps during the generation of the DNA have to be performed with great care. The optimum fragment length, with a maximum at approx. 7 kb, falls into a range from 2 to 15 kb. Therefore, pulverised salmon sperm DNA must not be used. Additionally, the solution must not be autoclaved.

- Add 100 mg of salmon sperm DNA to 10 ml 1x TE buffer (pH 8.0). Fragment bigger clumps by repeated absorption with a pipette and dissolve on a stir plate at 4 °C overnight.
- Sonicate the solution 3x for 30 seconds (Branson Sonifier, level 7). During that procedure mix the solution well and cool on ice.
- Extract the solution twice with buffer-saturated phenol/chloroform and precipitate the DNA with 1/10 vol. 3 M sodium acetate and 2 vol. ethanol overnight.
- Wash the pellet twice with 70% ethanol, resuspend in 10 ml 1x TE buffer, divide into 100 µl aliquots and denature in boiling water for 10 minutes before storing in the freezer (-20 °C).

14. Z buffer (2x 100 ml for the whole course, autoclave)

10.7	g/l	$Na_2HPO_4 \cdot 2\ H_2O$ (=60 mM)
5.5	g/l	$NaH_2PO_4 \cdot H_2O$ (=40 mM)
0.75	g/l	KCl
0.246	g/l	$MgSO_4 \cdot 7\ H_2O$ (=1 mM)

15. Z buffer with β-mercaptoethanol (2x 20 ml)
54 µl β-mercaptoethanol/20 ml Z buffer
Prepare freshly immediately before use.

16. X-Gal-stock solution for Filter-LacZ-Assay (1 ml for whole course)
dissolve 20 mg X-Gal/ml in dimethylformamide (DMF). Store at -20°C in darkness.

17. Z-Puffer/X-Gal-solution for Filter-LacZ-Assay (20 ml for whole course)

20 ml	Z-buffer
54 µl	ß-mercaptoethanol
335 µl	X-Gal stock solution

1. Generation of transformation-competent yeast cells

1. Inoculate 50 ml / 100 ml / 200 ml YAPD medium with a small single colony from the yeast tester strain AH109 and incubate at 30 °C and 200 rpm overnight. On the next morning, OD_{600} should be around 0.6.
2. Prepare the 1x TE, 1xTE/0.1 M LiAc and the PEG/LiAc solution (sterile).
3. The cells are spun down by centrifugation at 4000 rpm at room temperature for 5 min, washed with 20 ml sterile water, centrifuged again and resuspended in 1.5 ml 1x TE buffer/0.1 M lithium acetate.

2. Yeast transformation

1. For each transformation sample, prepare a 1.5 ml Eppendorf tube with 1 µg of each plasmid DNA - bait and prey (sterile at room temperature). Additionally, prepare a control sample without plasmid DNA to rule out reversion of the selection marker in the cells.
2. Denature salmon sperm DNA by boiling for 2 minutes. Cool on ice immediately to prevent renaturing.
3. Add 5 µl (50 µg) salmon sperm DNA to each tube (on ice!).
4. To each sample add 50 µl competent cell suspension and mix. Then add 300 µl PEG/lithium acetate solution and mix again (vortex for 2 seconds).
5. Incubate the samples on a shaker at 30 °C for 30 minutes.
6. Add 35 µl DMSO to each sample. Mix carefully by inverting the Eppendorf tube.
7. Incubate the samples at 42 °C in a water bath for 15 minutes.
8. Pellet the yeast cells by centrifugation in a table-top centrifuge at 10,000 rpm for 1 minute and carefully resuspend in 100 µl sterile 1x TE.

9. Plate 50 µl of each transformation sample on SD-Leu-Trp plates and 50 µl on SD-Leu-Trp-His (+3-AT) plates and incubate at 30 °C for three days.

3. Qualitative determination of LacZ-activity (Filter-Assay)

1. Label one petri dish and two round filter papers for each transformation.
2. Prepare Z-buffer/X-Gal-solution (chemical hood).
4. Place a round filter paper into a petri dish and wet well with Z-buffer/X-Gal-solution (approx. 1 ml)
5. Place filter paper onto plate with yeast colonies (from SD-Leu-Trp plates) with the help of a pair of forceps. Lightly rub filter paper and remove it again. Immediately submerge filter paper in liquid nitrogen (ca. 5 sec, wear protective glasses!), then let it thaw at room temperature and place - with the colony side facing upwards – onto the moist round filters. Make sure that the upper filter paper is soaked evenly with buffer.
4. Close petri dish with parafilm and incubate it at 30°C until blue color formation is visible. Open petri dishes under fume hood and let it dry. Take a picture as a record.

Analysis:

- Which proteins interact with each other? Compare the results of the selection by growth with those from measuring LacZ activity.
- What does this interaction mean for the function during light signal transduction?

C. Molecular markers and positional cloning of a gene

Assume you have isolated a mutant with an exciting phenotype. You would like to know which gene is mutated in your mutant. Unfortunately, you used the chemical EMS for mutagenesis, thus your gene probably carries only a missense mutation. How are you going to find this mutation? It´s like finding a needle in a hay stack!

You are going to use positional cloning, i.e. you are going to determine on which chromosome and where exactly on this chromosome your mutation is. To do so, you will need molecular markers.

What are molecular markers?

A phenotypic marker is a trait that allows phenotypic discrimination between two genotypes. Examples for phenotypic markers are color, trichome formation, glossy cuticle etc. Molecular markers allows molecular discrimination between two genotypes and are for example used for genetic fingerprinting among relatives (paternity test). These markers detect sequence polymorphisms between two genotypes, thus they detect changes in DNA sequence caused for example by missense mutation, deletion or insertion.

Just like every human has a different genome sequence, there are many so-called ecotypes (or accessions) of Arabidopsis that were collected all over the world. These ecotypes differ in many phenotypic and molecular traits. Therefore, there are thousands of molecular markers that can distinguish between two ecotypes, e.g. between Columbia (Col) and Landsberg erecta (Ler), the most commonly used ecotypes.

Typical molecular markers are PCR-based markers such as SSLPs (*simple sequence length polymorphic* markers, also called microsatellite markers) or CAPS-markers (*cleaved amplified polymorphic sequences*). Because each marker represents a particular DNA sequence, the exact position of the marker on a chromosome is known. Figure 3 shows the positions of molecular markers on the five chromosomes of Arabidopsis. Table 1 shows further information on the markers used in the practical course.

Positional cloning, also called map-based cloning

During positional cloning, you are going to map the position of the mutation which you are trying to identify. This means that you are going to determine on which chromosome and where exactly on this chromosome your mutation is.

To do so, you need to generate a mapping population (Figs. 4, 5): You will cross your mutant (let´s assume it is in the Ler ecotype and the mutation is called m) with a wild type plant (M) of a different ecotype (here: Col). The obtained F1 plants are all uniform and heterozygous at each locus (Mm). You will self the F1 to obtain the F2 generation where segregation occurs (MM, Mm, mm at

1:2:1). Moreover, recombination occurs during meiosis: cross over events disrupt the linkage („Kopplung“) in Col and Ler chromosomes. **The closer the distance between two loci on a chromosome, the lower the probability that a cross over event occurs between these two loci.** One says the loci are closely linked because they are usually co-inherited.

The F2 population is your mapping population. Only a quater of the F2 plants will be homozygous mutant (mm) and thus exhibit the mutant phenotype (assuming your mutation is recessive). You select these mutant plants and isolate genomic DNA from them. You know now that all selected plants carry your mutation (mm) and thus Ler DNA at and very close to your mutation m. However, you do not know where in the genome this mutation is. You will find its position as follows: you will test many molecular markers on these selected mm plants. For each chromosome, you will use at least five markers that cover more or less the whole chromosome. The marker analysis will tell you whether a plant carries Col DNA at the position of the marker, Ler DNA or both (i.e. heterozygous). Let´s assume you use a marker that is on a different chromosome than your mutation m. You would find that your mm F2 plants carry Col and Ler DNA at equal frequency. Let´s assume you use a marker that lies very close to your mutation m. You will find that most mm F2 plants carry only Ler DNA (because the marker DNA is co-inherited with m, i.e. no cross-over event has occurred).

Tasks

- You will isolate genomic DNA from Col and Ler Arabidopsis plants. You will use several molecular markers (SSLP and CAPS, see Figures 3 and 4) on Col and Ler genomic DNA to detect the polymorphisms between Col and Ler.
- You will map the *ga1* (*GA-deficient*) mutation using the virtual mapping population shown in Fig. 4. The *ga1* mutation causes dwarfism (see Fig. x). Homozygous *ga1 ga1* (Ler) plants were crossed with wild type Col plants. Harvested F1 seeds were propagated by selfing to obtain the F2 mapping population shown in Fig. x). Which plants are you going to use for mapping the *ga1* mutation? With the help of virtual results you will determine the rough position of the *ga1* mutation.

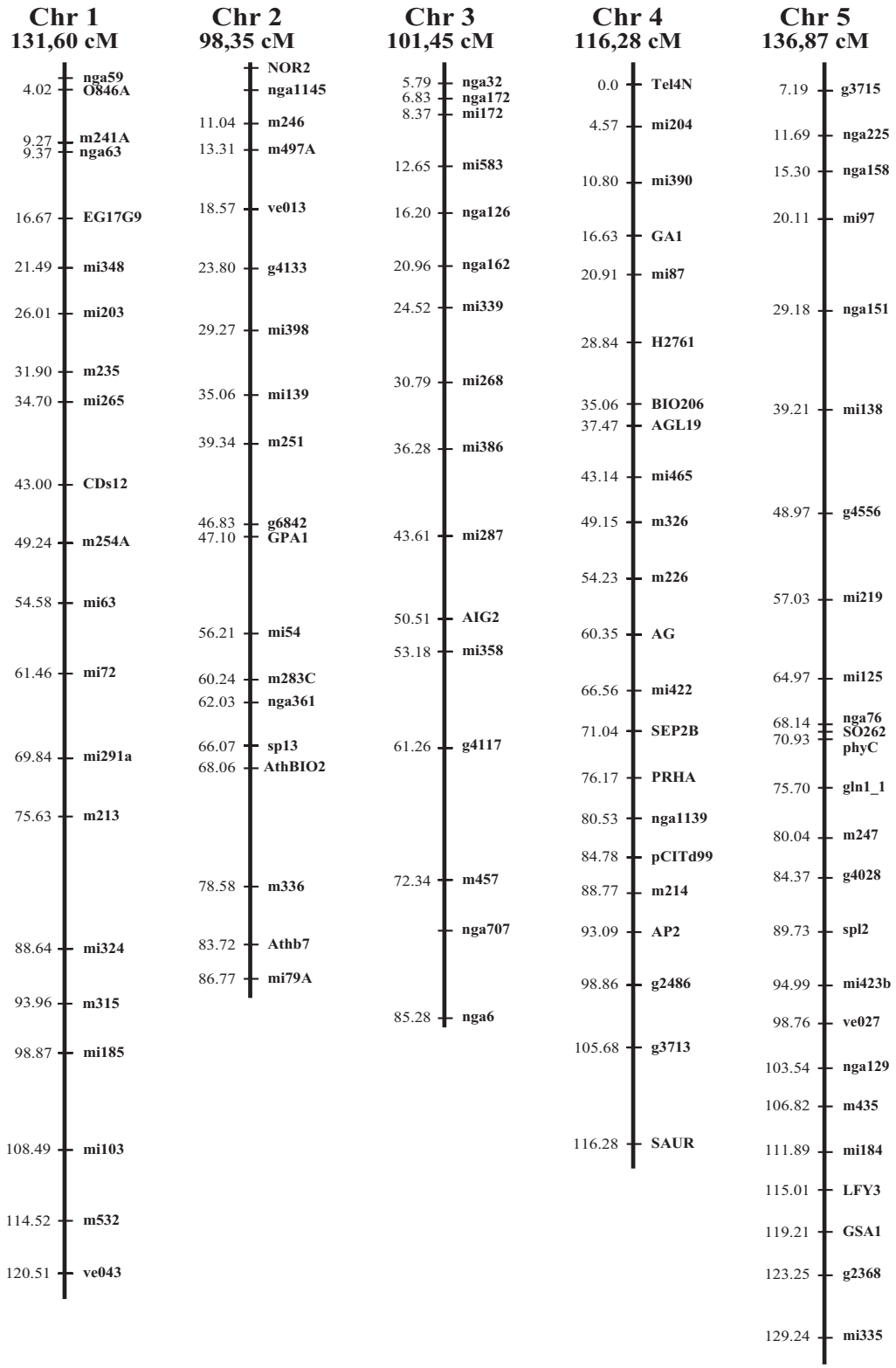


Fig. 3: Genetische Karte der fünf Chomosomen von Arabidopsis mit Lage der molekularen Marker. Ziffern links neben den molekularen Markern geben deren Position in cM (centiMorgan) an. 1 cM ist 1% Rekombinationshäufigkeit. Marker mit Kästchen stellen im Praktikum zu verwendende Marker dar.

Table1: Information on molecular markers used in the practical course.

Marker name	**Chromo-some**	**location**	**marker type**	**Forward primer**	**Reverse primer**	**Restriction-enzyme**	**Fragment size (bp)**	
							Col	Ler
nga 63	1	9.4 cM	SSLP	nga 63A-F	nga 63A-R	-------	111	89
G2395	1	28.08 cM	CAPS	G2395-F	G2395-R	XbaI	183, 153	336
nga280	1	81.4 cM	SSLP	nga280-F	nga280-R	--------	105	85
PhyB	2	34.5 cM	CAPS	PhyB-F	PhyB-R	XhoI	1100	700, 400
nga168	2	73.77 cM	SSLP	nga168-F	nga168-R	--------	151	135
F6E13A	2	90 cM	SSLP	F6E13A-F	F6E13A-R	--------	285	264
nga172	3	6.91	SSLP	nga172-F	nga172-R	---------	162	136
nga162	3	20.56	SSLP	nga162-F	nga162-R	---------	107	89
PUR5	3	93	CAPS	PUR5-F	PUR5-R	RsaI	264,260,132,37	524,132,37
CIW6	4	48.7 cM	SSLP	CIW6-F	CIW6-R	--------	162	148
AG	4	63.16	CAPS	AG-F	AG-R	XbaI	1420	1120, 290
T27E11	4	80 cM	SSLP	T27E11-F	T27E11-R	--------	184	172
nga151	5	29.62 cM	SSLP	nga151-F	nga151-R	--------	150	120
CIW9	5	88	SSLP	CIW9-F	CIW9-R	--------	165	140
MSJ1/SNP1/2	5	125	CAPS	...-F	...-R	MseI	80, 66	146

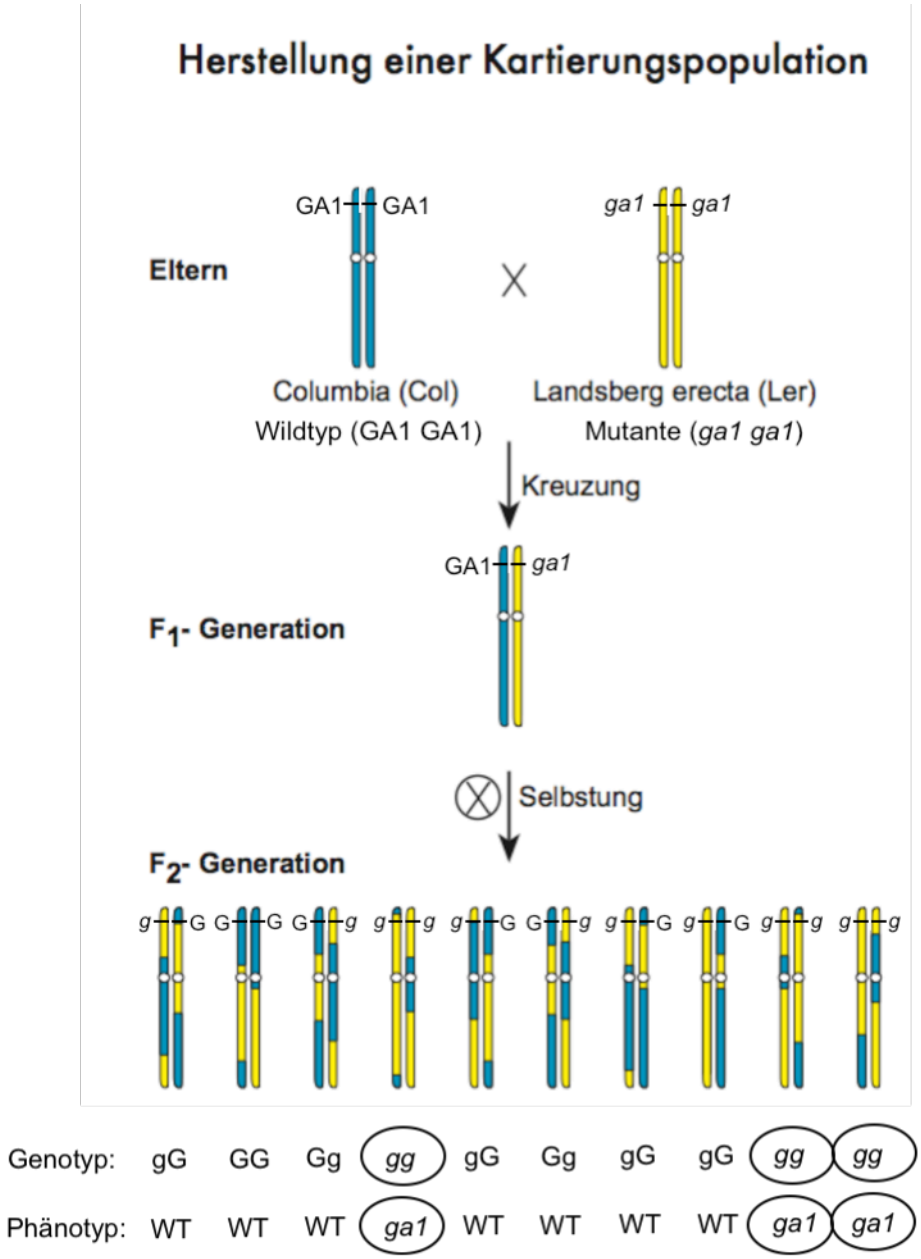
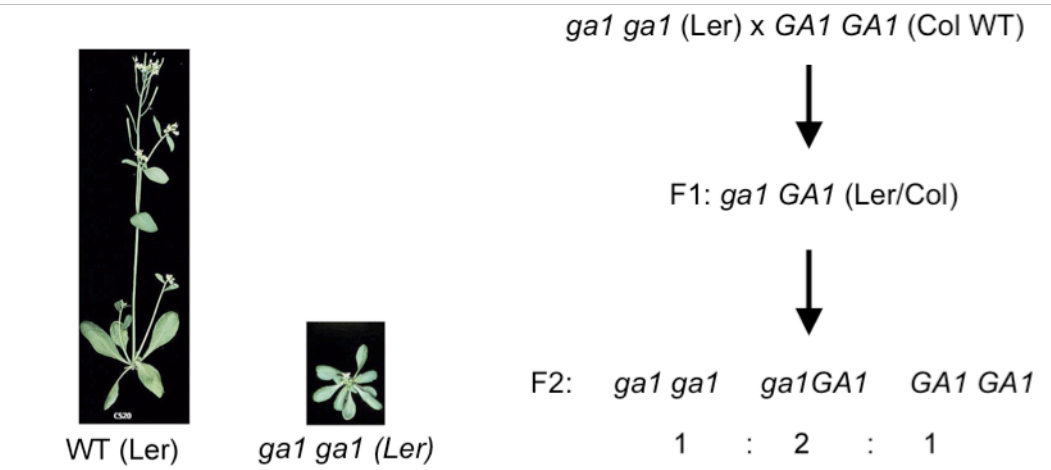
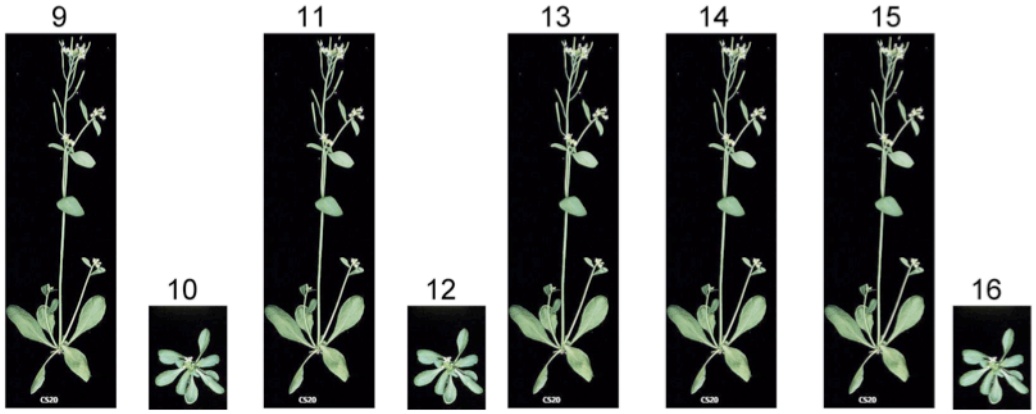


Fig. 4: Cross-over events during meiosis lead to recombination of Col and Ler alleles in the F2 generation. *ga1* mutant F2 plants carry Ler DNA (yellow) at the location of the (unknown!) *GA1* Locus. Thus, when a molecular marker very close to the *GA1* locus is used, then *ga1* mutant F2 plants should all carry only Ler DNA at the marker locus.


ga1 ga1 (Ler) x *GA1 GA1* (Col WT)
F1: *ga1 GA1* (Ler/Col)
F2: *ga1 ga1* *ga1GA1* *GA1 GA1*
1 : 2 : 1
WT (Ler)
ga1 ga1 (Ler)

F2 plants:


1
2
3
4
5
6
7
8


9
10
11
12
13
14
15
16

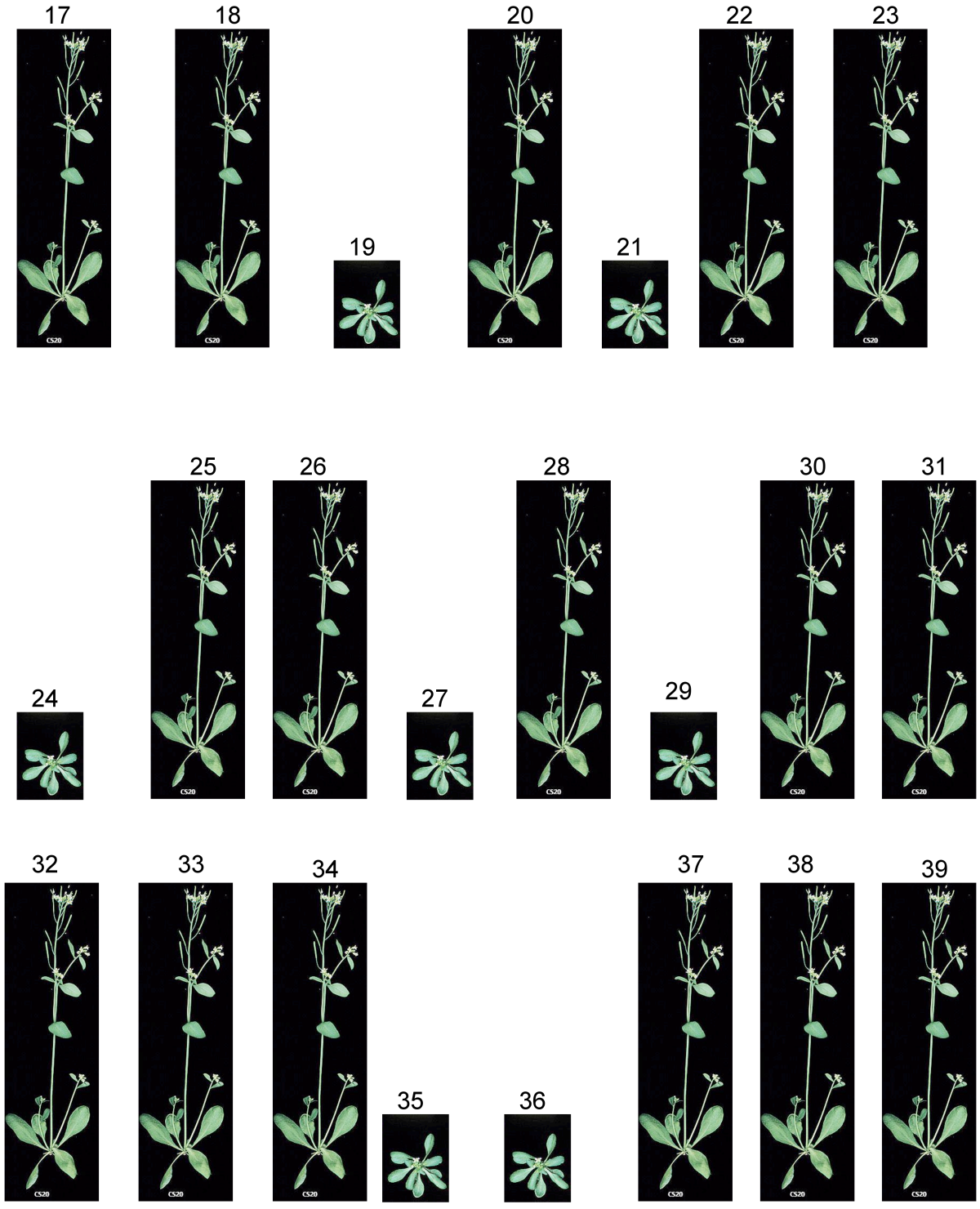


Figure 5. Generating a *ga1* mapping population

1. Isolation of genomic DNA from plants

References:

Edwards K, Johnstone C & Thompson C (1991): A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res *19*: 19-21.

Solutions:

1. Extraction buffer (50 ml)

0.2	M	Tris/HCl, pH 7.5
0.25	M	NaCl
0.025	M	EDTA
0.5	% (w/v)	SDS

2. Isopropanol (= 2-propanol!)

3. 1x TE buffer, pH 8.0

Instructions:

1. Place a piece of a young leaf (ca. 0.5 x 0.5 cm) in a 1.5 ml Eppendorf tube, grind with the help of a micropestle (approx. 5 - 15 seconds), and immediately add 400 µl extraction buffer. Vortex the homogenate for 5 seconds. The homogenate can be kept at room temperature until all samples have been extracted.
2. Centrifuge the extracts at 13,000 rpm for 3 minutes in an Eppendorf centrifuge at room temperature.
3. Collect 300 µl of the supernatant and transfer it to a clean Eppendorf tube. Attention: do not transfer any of the pellet!
4. Add 300 µl isopropanol and vortex, then keep at room temperature for 2 minutes (DNA precipitation).
5. Centrifuge in an Eppendorf centrifuge at 13,000 rpm for 5 minutes (at either room temperature or 4 °C).
6. Remove supernatant completely and place the Eppendorf tube upside down on paper towels to dry. Afterwards keep at room temperature for 10 minutes with the lid open. The pellet will be invisible or up to 1 mm in size, depending on the age and quality of the plant material.
7. Add 100 µl 1x TE buffer to the pellet and dissolve the pellet at 4 °C for at least an hour. Attention: Do not vortex or otherwise vigorously shake! The DNA will dissolve by itself.
8. Use 1 µl of this DNA for the PCR reaction.

2. PCR amplification using primers for the microsatellite- and CAPS-markers

For each marker, four PCR reactions will be performed:

1. Col genomic DNA
2. Ler genomic DNA
3. Col and Ler genomic DNA (1:1)
4. no genomic DNA (negative control)

Solutions:

1. PCR buffer (10x concentrated)

2. dNTP solution (10x concentrated)
   200 µM dATP, dCTP, dGTP, dTTP

3. Primer pairs (Forward and reverse primer)

4. Taq DNA polymerase

Instructions for the PCR reactions will be provided

3. Analysis of the PCR products:

Microsatellite markers:

5 -10 µl of each PCR product are analyzed on an agarose gel.

CAPS-markers:

The PCR products are digested with the appropriate restriction enzyme and subsequently analyzed on an agarose gel.

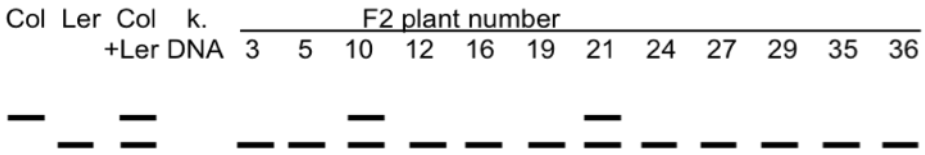
Analysis:

- What polymorphisms do the markers uncover?
- Calculate the recombination frequencies between the *ga1* locus and the markers using the virtual mapping population provided (see page 25ff). Where does the *ga1* locus map?

% recombination frequency between marker and *ga1* = $\frac{\text{number of Col chromosomes}}{\text{total number of chromosomes}}$ x 100

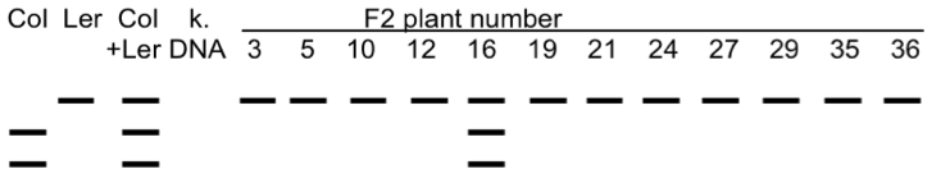
Chromosome 1

nga63 (9.4 cM)



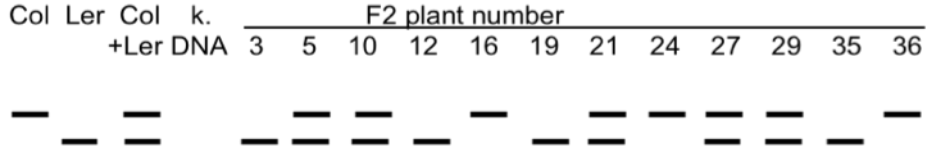
% recombination frequency between marker and *ga1*:

G2395 (28.08 cM)



% recombination frequency between marker and *ga1*:

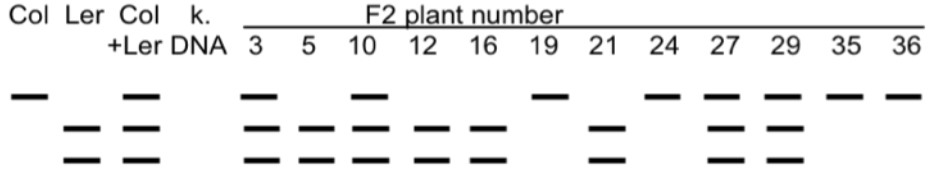
nga280 (81.4 cM)



% recombination frequency between marker and *ga1*:

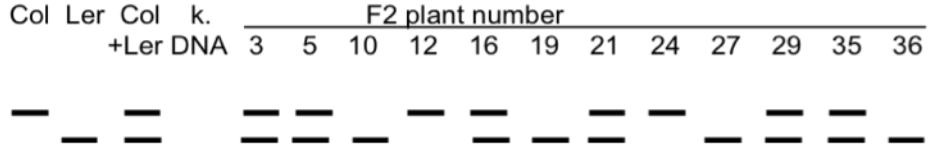
Chromosome 2

PhyB (34.5 cM)



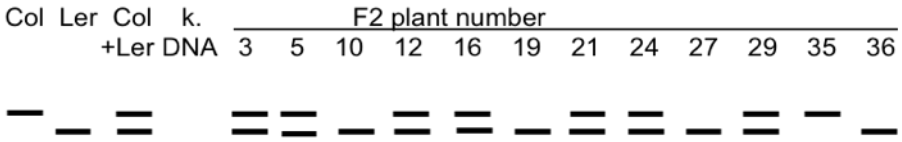
% recombination frequency between marker and *ga1*:

nga168 (73.77 cM)



% recombination frequency between marker and *ga1*:

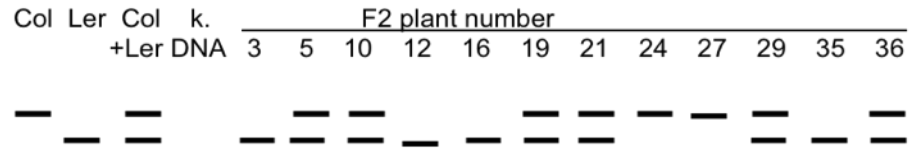
F6E13A (90 cM)



% recombination frequency between marker and *ga1*:

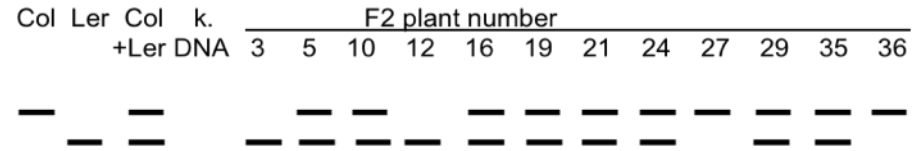
Chromosome 3

nga172 (6.91 cM)



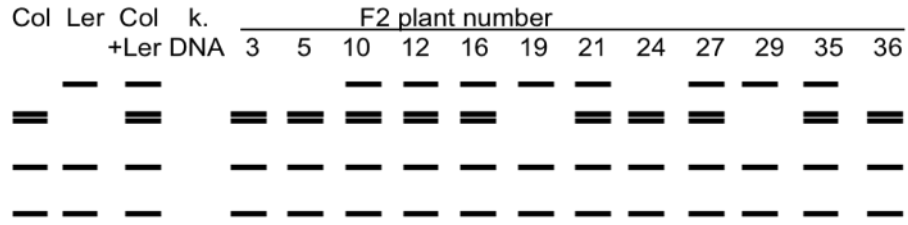
% recombination frequency between marker and *ga1*:

nga162 (20.56 cM)



% recombination frequency between marker and *ga1*:

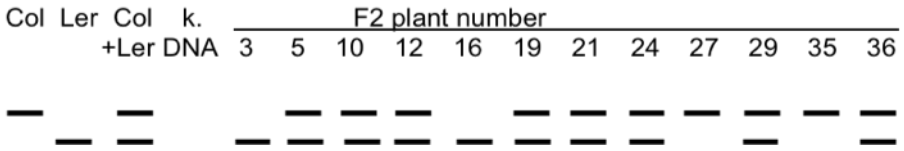
PUR5 (93 cM)



% recombination frequency between marker and *ga1*:

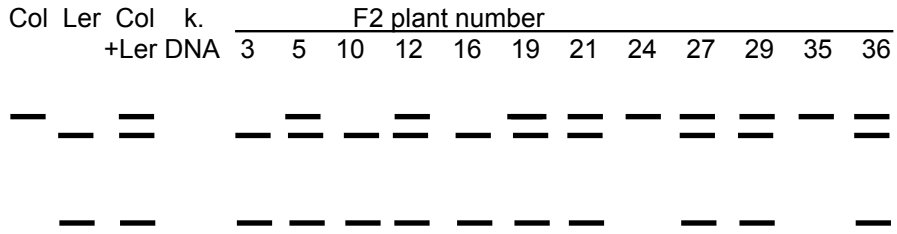
Chromosome 4

CIW6 (48.7 cM)



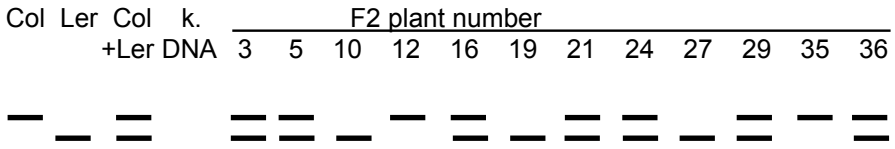
% recombination frequency between marker and *ga1*:

AG (63.16 cM)



% recombination frequency between marker and gai:

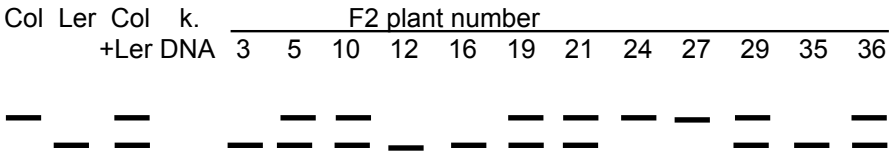
T27E11 (80 cM)



% recombination frequency between marker and gai:

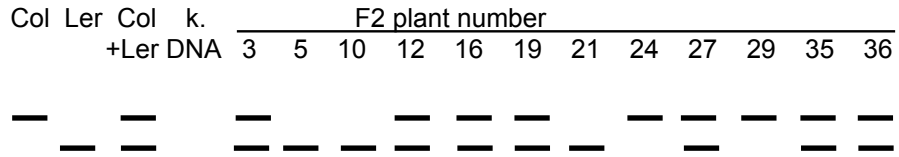
Chromosome 5

nga151 (29.62 cM)



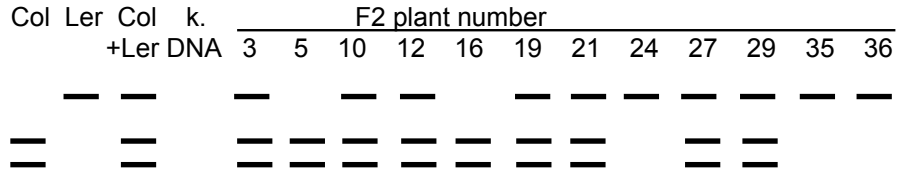
% recombination frequency between marker and gai:

CIW9 (88 cM)



% recombination frequency between marker and gai:

MSJ1/SNP1/2 (125 cM)



% recombination frequency between marker and gai: