



Dr Monika Murcha

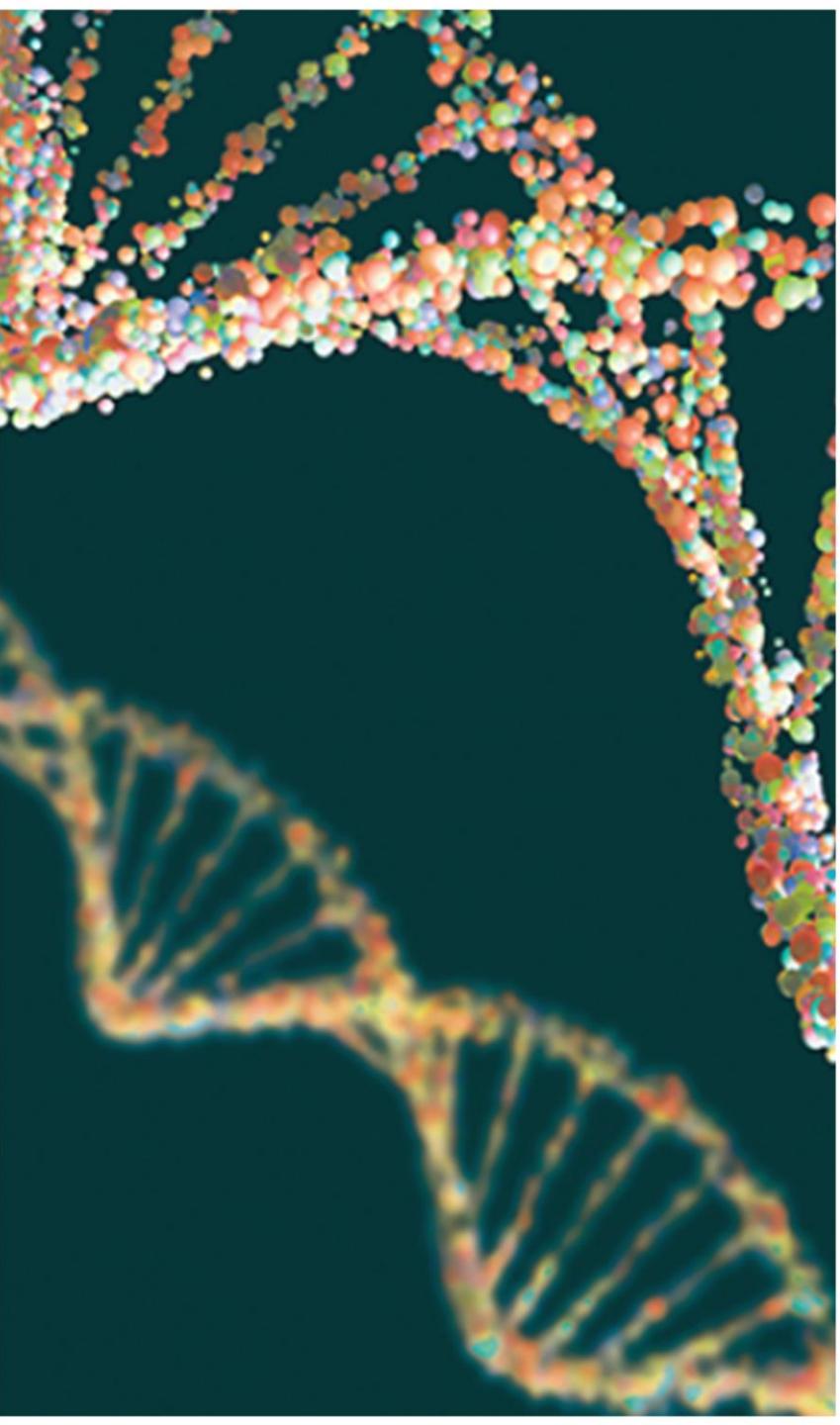
Associate Prof

School of Molecular Sciences

SCIE4001
genotype to
phenotype

Lecture Outline:

- Lecture 1: genotype to phenotype
 - Functional genomics
 - Forward genetic approaches
 - Reverse genetic approaches
 - Phenotypic analysis
- Lecture 2: examples of functional genomics



A bit about me...

BSc at UWA majoring in Biochemistry and Molecular Biology in 1995

Honours 1999, Biochemistry at UWA (Prof. J Whelan)

Started my PhD 2000

"Identification and characterisation of plant mitochondrial import components"

Post-doc ICHR

Post-doc ARC Coe PEB

2014 ARC Australian Post-doctoral Fellow 4 yrs

2014-started my own research group –affiliated with the Centre of Excellence Plant Energy Biology

2014 ARC Future fellowship

2015 ARC Discovery Project

DAAD grant Jurgen Soll (Munich)

STINT grant (Stockholm University)

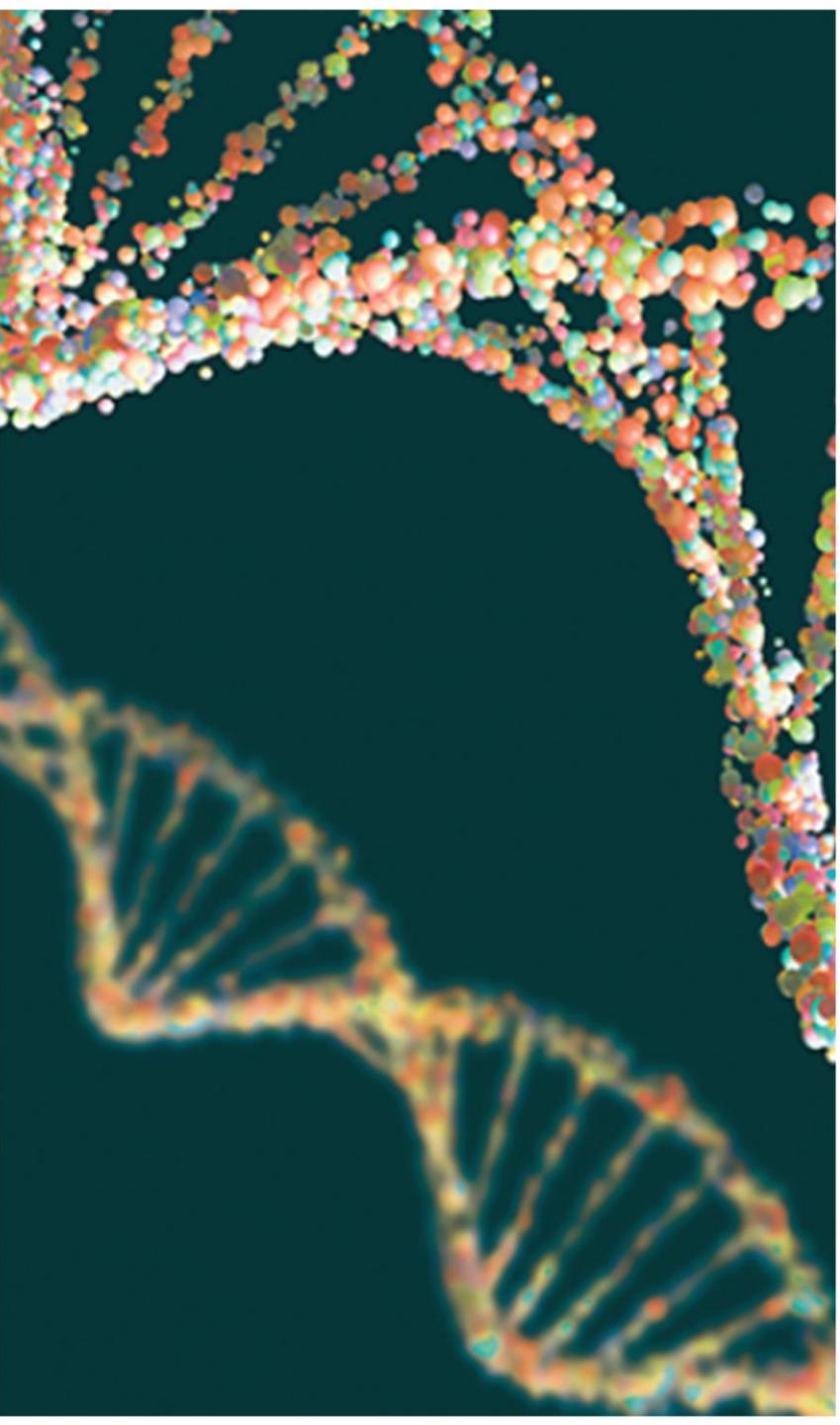
Murchalab.com

Come chat to me about Masters/Honours research projects, vacation projects etc



Learning outcomes: Functional genomics:

- Describe various targeted genetic approaches to determine the function of a protein.
- Describe how plant phenotypes can be used to unravel a protein's function.
- Understand the current research themes and approaches used in reverse genetics and forward genetic screens in the model *Arabidopsis thaliana*
- Critically evaluate the discussed techniques available for researchers today.



Reverse genetics:
Discovering the function of a gene by analysing the phenotypic consequences of a gene



APS > Education > Advanced > Topics in Plant Pathology > An Introduction to Reverse Genetic Tools for Investigating Gene Function

An Introduction to Reverse Genetic Tools for Investigating Gene Function

Tierney, M.B. and Lamour, K.H. 2005. An Introduction to Reverse Genetic Tools for Investigating Gene Function. *The Plant Health Instructor*. DOI: 10.1094/PHI-A-2005-1025-01.

Melinda B. Tierney¹ and Kurt H. Lamour²

¹University of Tennessee and Oak Ridge National Laboratory Genome Science and Technology Graduate Program; Knoxville, TN

²The University of Tennessee, Department of Entomology and Plant Pathology; Knoxville, TN

Forward genetics/classical genetics
Determining the genetic basis responsible for a phenotype

THE ART AND DESIGN OF GENETIC SCREENS: ARABIDOPSIS THALIANA

Damian R. Page and Ueli Grossniklaus

Molecular genetic studies rely on well-characterized organisms that can be easily manipulated. *Arabidopsis thaliana* — the model system of choice for plant biologists — allows efficient analysis of plant function, combining classical genetics with molecular biology. Although the complete sequence of the *Arabidopsis* genome allows the rapid discovery of the molecular basis of a characterized mutant, functional characterization of the *Arabidopsis* genome depends on well-designed forward genetic screens, which remain a powerful strategy to identify genes that are involved in many aspects of the plant life cycle.

The plant kingdom constitutes most of the biomass of our planet and is crucial in the ecosystem. Owing to the huge diversity of plants, and their ability to adapt to a wide variety of environments, it was thought for a long time that conclusions drawn from a plant model system could not easily be applied to other plant species. Indeed, studying plant model organisms is a relatively new approach compared with studies on animals. Until the 1980s, experiments on plants mainly

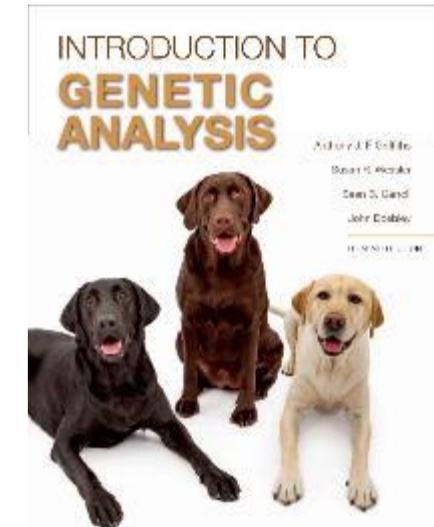
be used as a plant model system in 1943. Several features make *Arabidopsis* amenable to classical experimental genetics: a small size, a rapid generation time (5–6 weeks under optimum growth conditions), the ability to grow well under controlled conditions (either in soil or in defined media), high fecundity (up to 10,000 seeds per plant), and the ease with which a mutant line can be maintained (by self-fertilization) and out-crossed.

If you want some background reading,
I have uploaded several reviews and papers for you .

There is no dedicated textbook, but if you do not have a strong genetic background please refer to:



Benjamin A. Pierce, *Genetics: A Conceptual Approach* (2016, 6th edition)



Also recommended: A.J.F. Griffiths, S.R. Wessler, S.B. Carroll & J. Doebley, *Introduction to Genetic Analysis* (c 2015, 11th edition)

For Quantitative Genetics: F.W. Nicholas, *An Introduction to Veterinary Genetics* (2010, 3rd edition)

All in High Demand in the CMO, Biological Sciences Library.

What is functional genomics.....Functional genomics uses genomic data to study gene and protein expression and function on a global scale (genome-wide or system-wide), focusing on gene transcription, translation and protein-protein interactions, and often involving high-throughput methods.

What is the aim of functional genomics..... to understand the complex relationship between genotype and phenotype



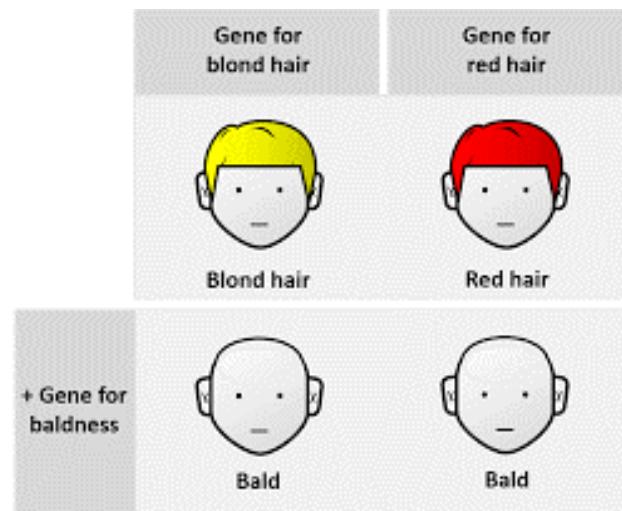
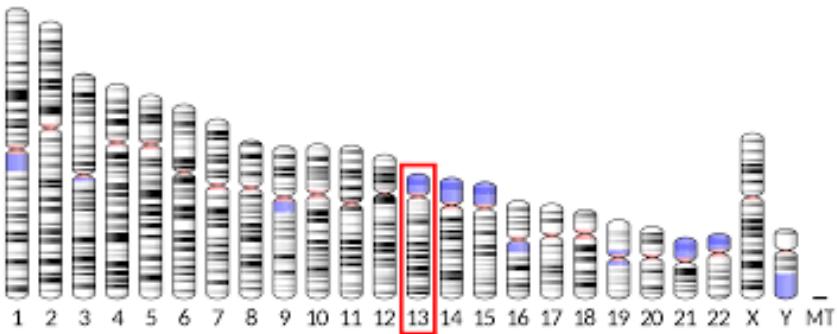
Figure 1. Functional genomics integrates information from various molecular methodologies to gain an understanding of how DNA sequence is translated into complex information in a cell (DNA → RNA → Proteins → biological process).

What is functional genomics?

Functional genomics is the study of how genes (and intergenic regions) contribute to different biological processes.

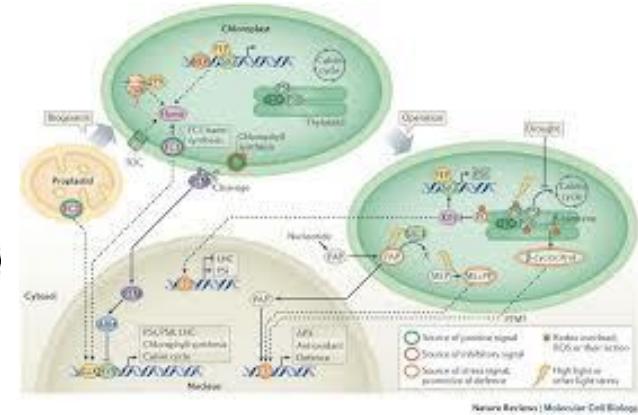
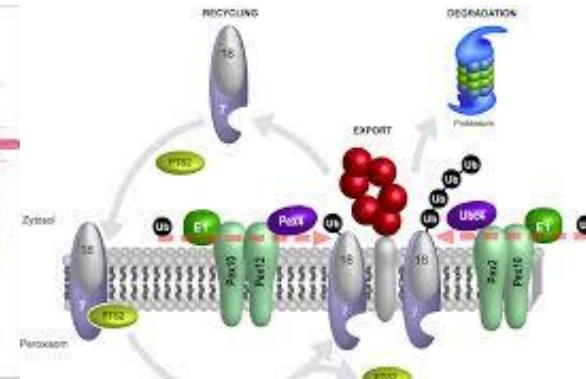
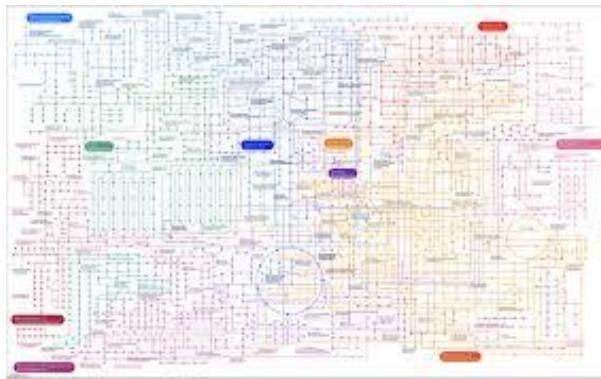
Using a variety of genomic methods to understand a gene/proteins function

GENE PHENOTYPE



Why do we need functional genomics?

What are the roles of specific genes/proteins and how are they regulated.



Bioinformatic approaches
Genomes
Microarray data sets
Phylogenetics
Protein structure

Create variants and analyse the phenotypic Consequences

Predict pathways functions, further biochemical characterisation

disclaimer?

Functional genomics will not replace the time-honored use of genetics, biochemistry, cell biology and structural studies in gaining a detailed understanding of biological mechanisms.

The extent to which any functional genomics approach actually defines the function of a particular protein (or set of proteins) will vary depending on the methodology and gene involved.

(Hieter & Boguski 97)

Why do we need functional genomics?

Organism	# genes	% of genes with inferred function	Completion date of genome
<i>E. coli</i>	4288	60	1997
yeast	6,600	40	1996
<i>C. elegans</i>	19,000	40	1998
<i>Drosophila</i>	12-14K	25	1999
<i>Arabidopsis</i>	25,000	40	2000
mouse	~30,000?	10-20	2002
human	~30,000?	10-20	2000

27 000 genes, only ~9% have been functionally characterised experimentally

Functional genomics in the model plant *Arabidopsis thaliana*

- Reverse genetic approaches using *Arabidopsis*
- Forward genetic approaches using *Arabidopsis*



Model organism for plant biology although it is not of agronomic importance it offers import advantages with regards to genetic and molecular biological research

1. Small genome size, many crop species large genomes due to polyploidization maize 19X wheat 128X
2. Many tools, techniques and resources are available
3. Easily manipulated, short life cycle, broad knowledge base
4. Translational research-basic understanding can be directed to crop species

Reverse genetics:

Discovering the function of a gene by analysing the phenotypic consequences of a gene

How do we do that: we need to modify that gene

1. DELETE IT!!!!!!!, knock-out, Gene silencing-RNA interference

1. MUTATE IT!!!!!!!Directed deletions and point mutations- change regions in the promoter, point mutations to codons in the ORF or create null mutations

2. OVEREXPRESS IT!!!!!!!!!!!!!!, results in the overexpression of a protein

All these methods can be used to create variants which modulates a genes expression or the protein function. From there we can then look for an informative phenotype.

Forward genetics: classical genetics

This is the opposite approach, where you already have a phenotype or you know what phenotype you want, then you find the genetic basis responsible for that phenotype.

1. Generation random mutants to obtain a desired phenotype.
 - Mutagens are used to create point mutations
 - Map the mutation to a particular gene.
1. Classical-crossbreeding methods

Mutagen-application of mutagen to generate random point mutations.

Reverse genetics:

Discovering the function of a gene by analysing the phenotypic consequences of a gene

How do we do that: we need to modify that gene

1. DELETE IT!!!!!!!, knock-out, Gene silencing- RNA interference

**T-DNA insertional
knock-out lines**

**SALK-TDNA
GABI-kat
SAIL
FLAG**



Howard Hughes
Medical Institute

Published as: *Methods Mol Biol.* 2015 ; 1284: 323–342.

A User's Guide to the Arabidopsis T-DNA Insertional Mutant Collections

Ronan C. O'Malley^{1,2}, Cesar C. Barragan¹, and Joseph R. Ecker^{1,2,3}

¹Genomic Analysis Laboratory, Salk Institute for Biological Studies, 10010 N. Torrey Pines Rd, La Jolla CA, 92037

²Plant Biology Laboratory, Salk Institute for Biological Studies, 10010 N. Torrey Pines Rd, La Jolla CA, 92037

³Howard Hughes Medical Institute

Once upon a time ...



ARABIDOPSIS AS A GENETIC TOOL

G. P. Rédei

Department of Agronomy, University of Missouri, Columbia, Missouri 65201

Annual Review of Genetics, 1975



George Rédei (second from right) with (from L-R): Ed Coe, UMC; Charles Levings, NC State; Kathy Newton, UMC; Chris Leaver, University of Edinburgh; David Lonsdale, Plant Breeding Institute, Cambridge. (Source: IPG Archives)

Advantages of using *Arabidopsis thaliana* as a genetic tool:

- The diploid chromosome number is five pairs
- The life cycle maybe completed within one month

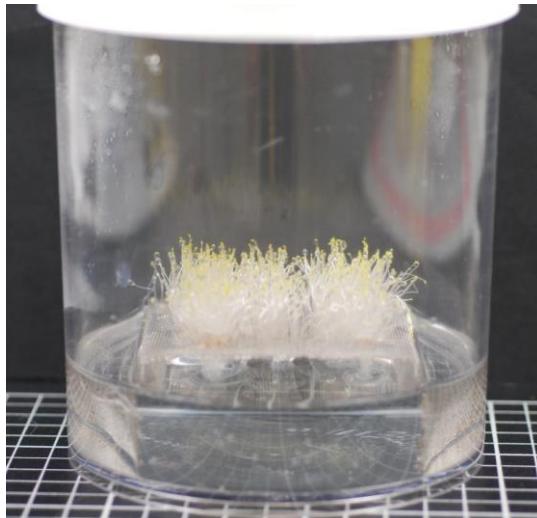
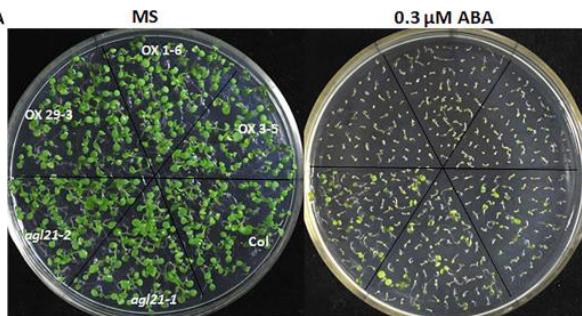
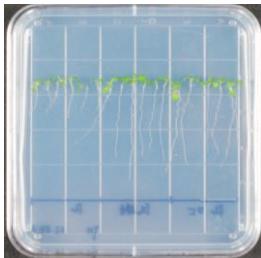
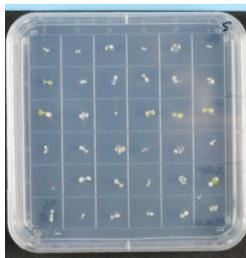
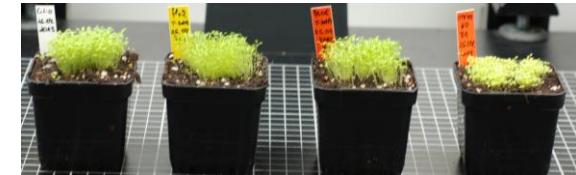
or it may be extended, depending on photoperiodic exposure

- Outcrossing (cross-pollination) is minimal (2% in nature)
- One plant can produce more then 50,000 seeds
- Plant can be grown on soil but also as cell cultures



Arabidopsis as a genetic tool...

Plants can be grown on soil but also as cell cultures

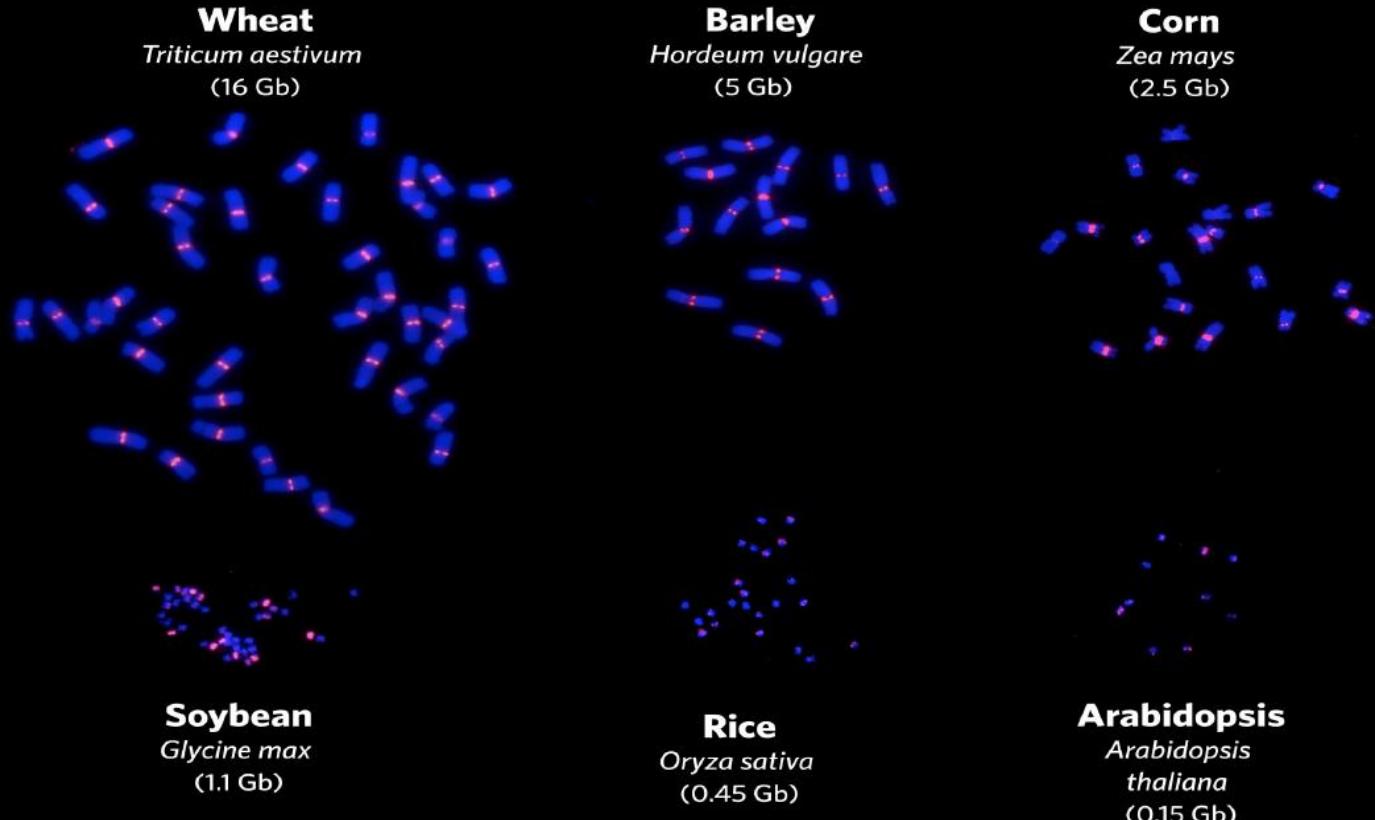


Arabidopsis cell culture

Arabidopsis hydroponically grown

Why wheat is not a model organism

Wheat (*Triticum aestivum*) has a large genome



Dal-Hoe Koo, Wheat Genetics Resource Center, Kansas State University

Gb = 1,000,000,000 DNA base pairs

10 μm

The sequence of the first plant genome was completed and published at the end of 2000

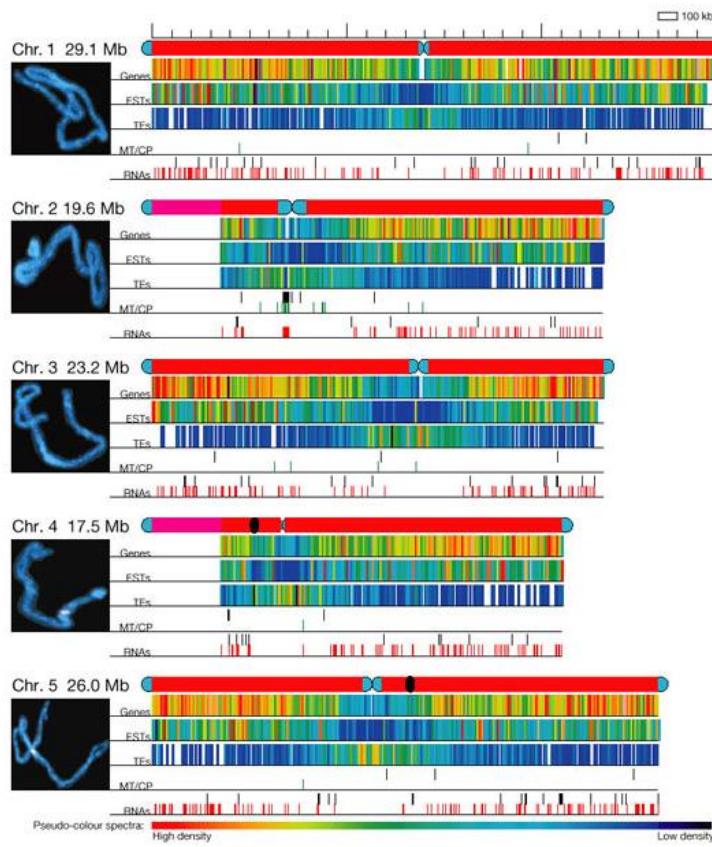
From the following article:

[Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*](#)

and The Arabidopsis Genome Initiative

Nature **408**, 796-815(14 December 2000)

doi:10.1038/35048692



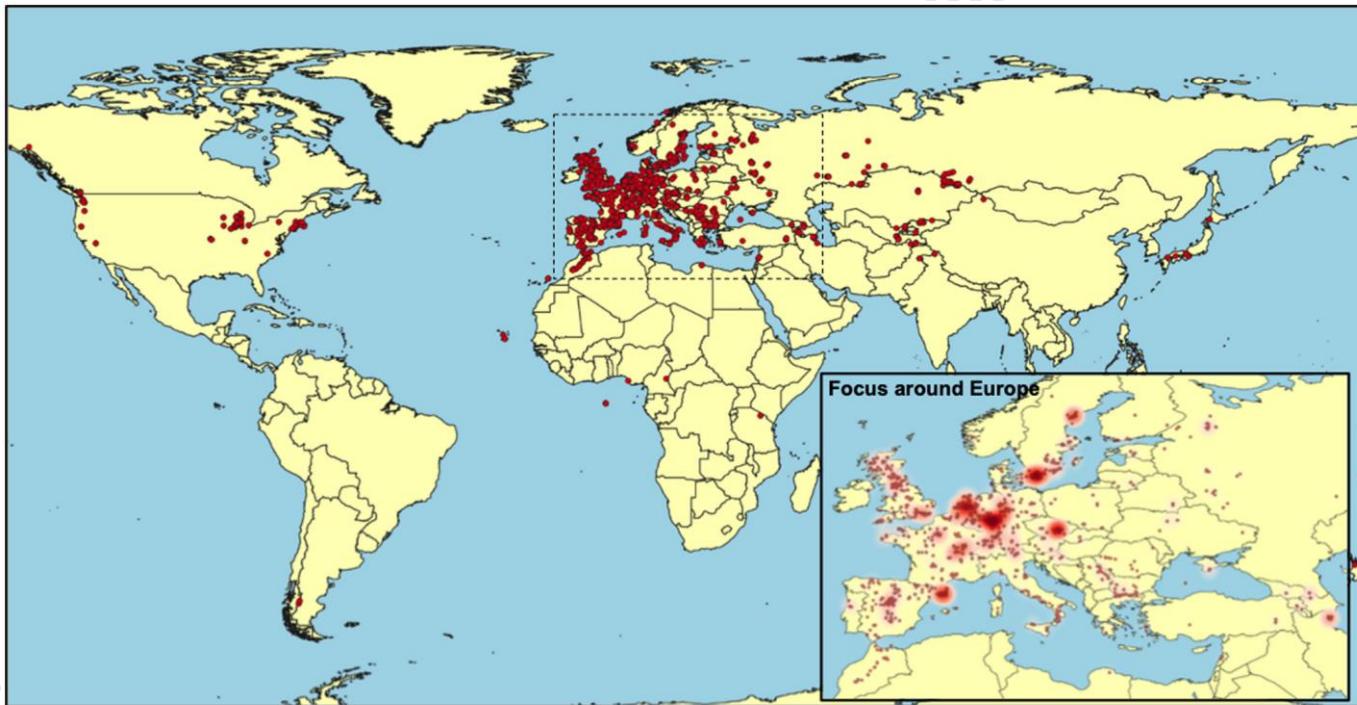
27,000 genes are present in the *Arabidopsis* genome (they encode 35,000 proteins)

Genes are annotated along the chromosome arms and the loci identifier look like AT5g15960



The sequence of the first plant genome was completed and published at the end of 2000

Current status of the multinational *Arabidopsis* community



Genome-Wide Insertional Mutagenesis of *Arabidopsis thaliana*

José M. Alonso,^{1*} Anna N. Stepanova,^{1*} Thomas J. Leisse,¹
Christopher J. Kim,¹ Huaming Chen,¹ Paul Shinn,¹
Denise K. Stevenson,¹ Justin Zimmerman,¹ Pascual Barajas,¹
Rosa Cheuk,¹ Carmelita Gadrinab,¹ Collen Heller,¹ Albert Jeske,¹
Eric Koesema,¹ Cristina C. Meyers,¹ Holly Parker,¹
Lance Prednis,¹ Yasser Ansari,¹ Nathan Choy,¹ Hashim Deen,¹
Michael Geralt,¹ Nisha Hazari,¹ Emily Hom,¹ Meagan Karnes,¹
Celene Mulholland,¹ Ral Ndubaku,¹ Ian Schmidt,¹ Plinio Guzman,¹
Laura Aguilar-Henonin,¹ Markus Schmid,^{1†} Detlef Weigel,^{1†}
David E. Carter,² Trudy Marchand,² Eddy Risseeuw,²
Debra Brogden,² Albana Zeko,² William L. Crosby,²
Charles C. Berry,³ Joseph R. Ecker^{1‡}

Over 225,000 independent *Agrobacterium* transferred DNA (T-DNA) insertion events in the genome of the reference plant *Arabidopsis thaliana* have been created that represent near saturation of the gene space. The precise locations were determined for more than 88,000 T-DNA insertions, which resulted in the identification of mutations in more than 21,700 of the ~29,454 predicted *Arabidopsis* genes. Genome-wide analysis of the distribution of integration events revealed the existence of a large integration site bias at both the chromosome and gene levels. Insertion mutations were identified in genes that are regulated in response to the plant hormone ethylene.

One of the most significant findings revealed through analysis of genomes of multicellular organisms is the large number of genes for which no function is known or can be predicted (*1*). An essential tool for the functional analysis of these completely sequenced genomes is the ability to create loss-of-function mutations for all of the genes. Thus far, the creation of gene-indexed loss-of-function mutations on a whole-genome scale has been reported only for the unicellular budding yeast *Saccharomyces cerevisiae* (*2–4*). Although targeted gene replacement via homologous recombination is

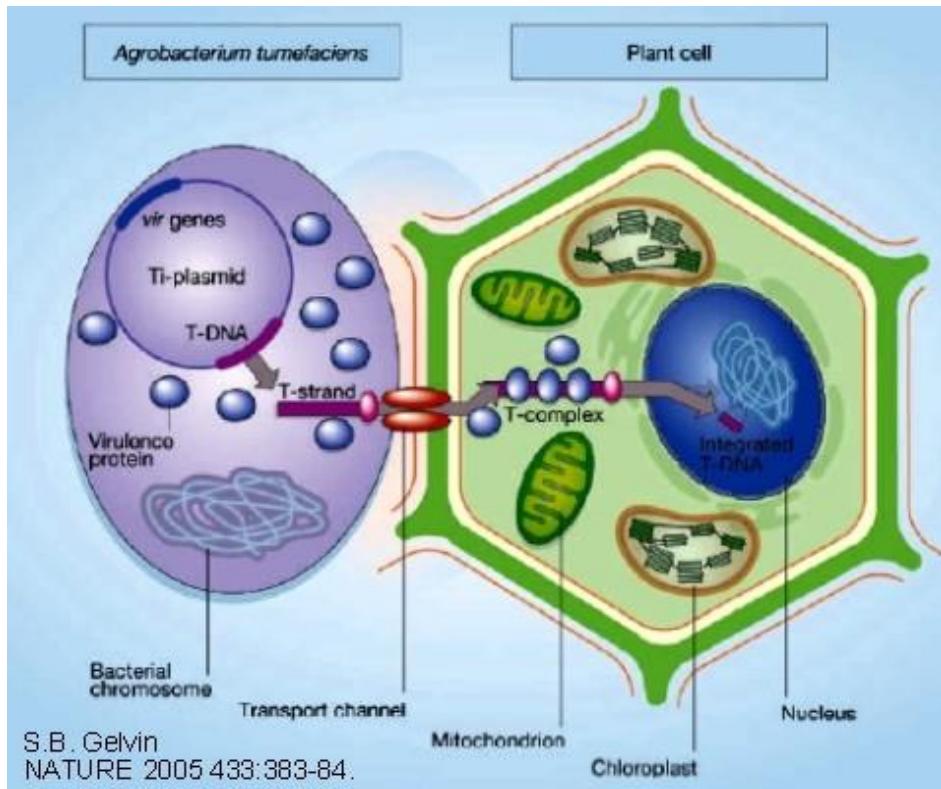
extremely facile in yeast, its efficiency in most multicellular eukaryotes does not yet allow for the creation of a set of genome-wide gene disruptions (*5, 6*). Gene silencing has recently been used to study the role of ~86% of the predicted genes of the *Ceae-rhabditis elegans* genome in several developmental processes (*7, 8*). The RNA interference (RNAi) method has, however, several drawbacks, including the lack of stable heritability of a phenotype, variable levels of residual gene activity (*9–11*), and the inability to simultaneously silence several unrelated genes (*12*).

Generation of T-DNA insertional Knock-out lines:

- 225 000 independent T-DNA insertions
Created.
- Represent almost a complete library of T-DNA insertions for the whole *Arabidopsis* genome (29K genes)
- Sequencing/PCR of T-DNA identifies location of the T-DNA insertion
- Invaluable resource for plant researchers that has allowed the functional characterisation of genes.

Generation of T-DNA insertional Knock-out lines:

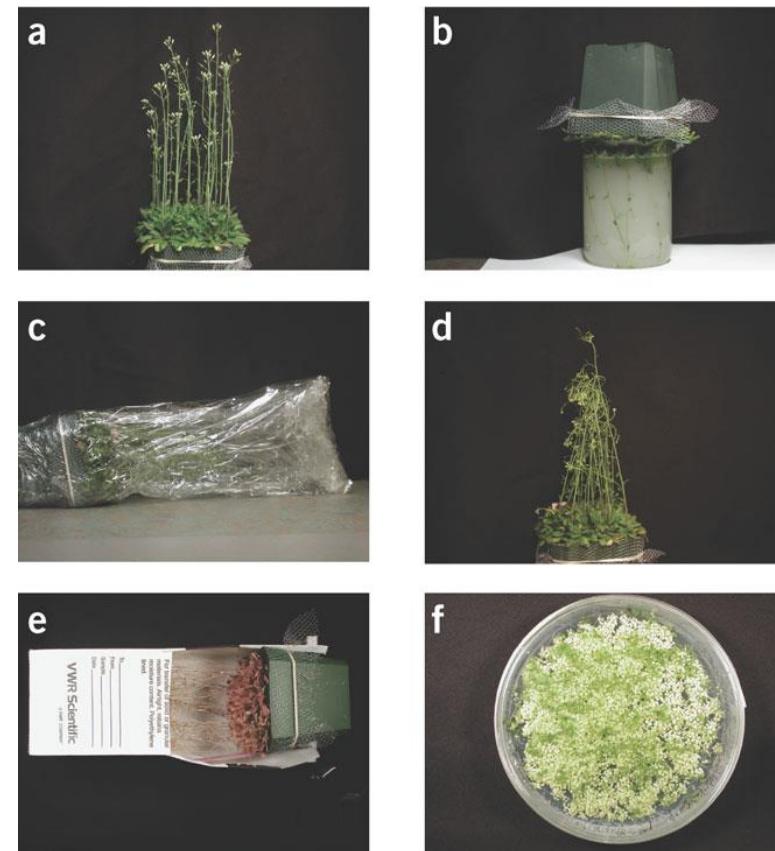
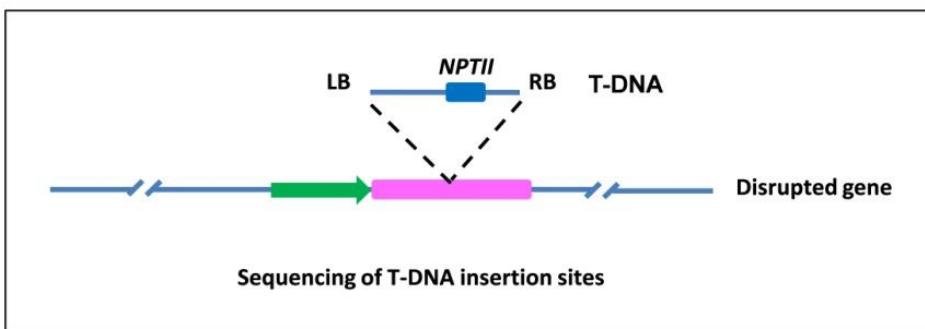
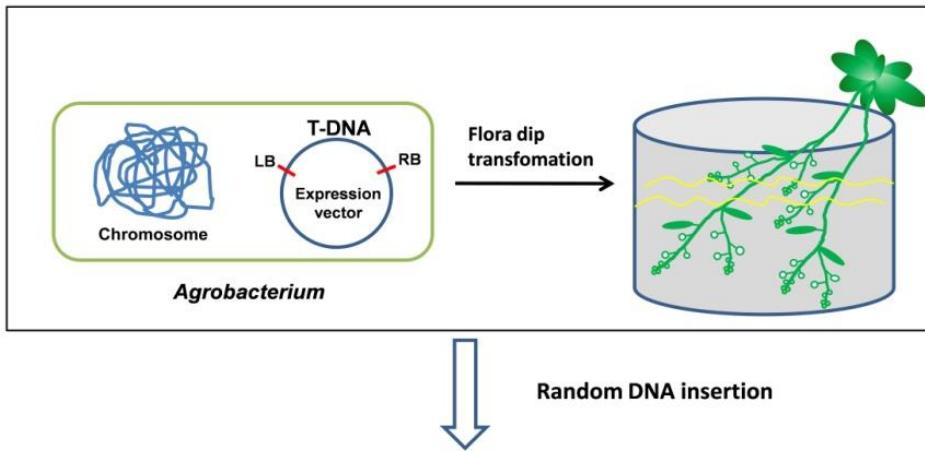
Agrobacterium mediated transformation of plasmid containing a homologous gene short strand sequence. Common soil pathogen that transfers DNA into host plant Transferred DNA (T-DNA). Single or multiple T-DNA can be inserted into host genome.



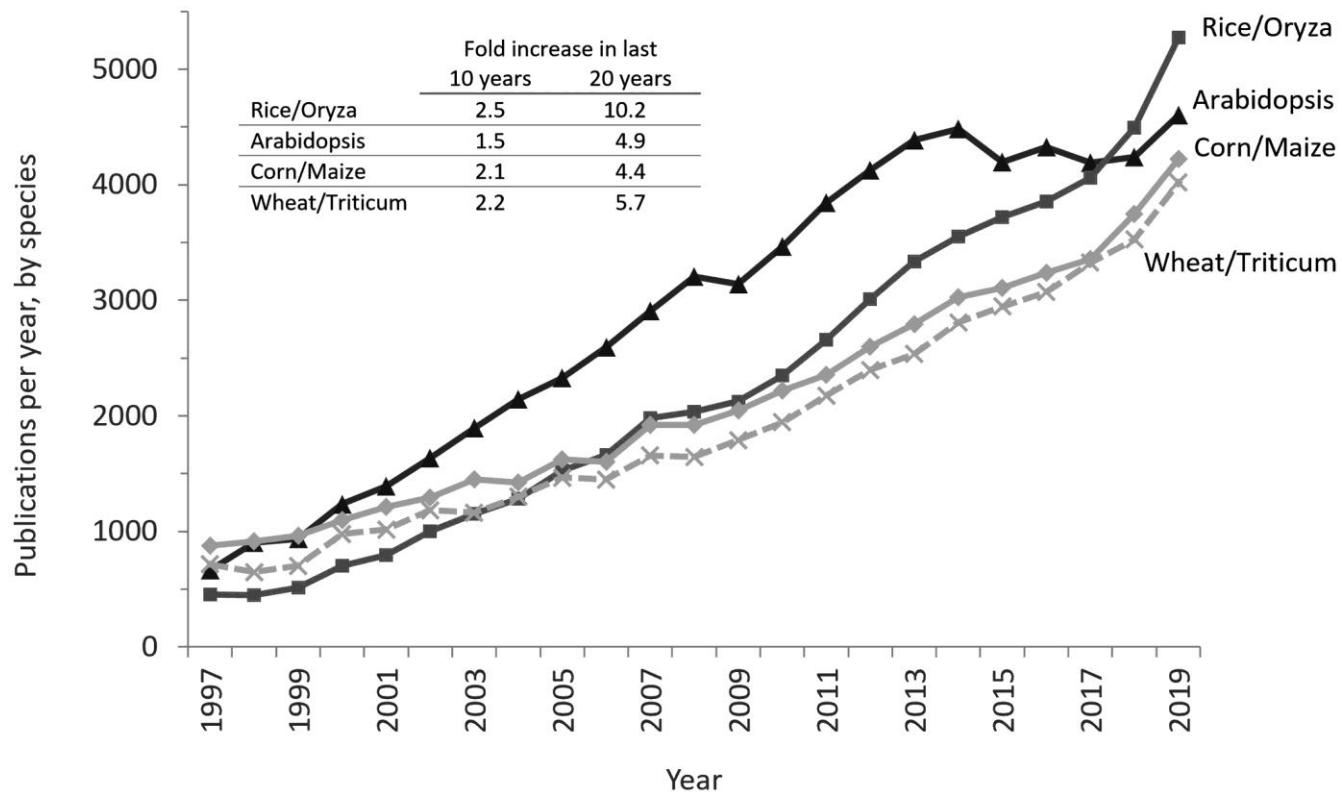
- T-DNA containing plasmid
- Plasmid transformed into agrobacterium
 - Random insertion within plant genome
 - Selection markers (antibiotic resistance) within the T-DNA 25 base pairs direct repeat border sequence are inserted within genome
 - Allows for an introduction of DNA
 - Allows for the inactivation of endogenous genes

Generation of T-DNA insertional Knock-out lines:

Agrobacterium mediated transformation is a simple process of infecting the immature flowers with the soil pathogen.



Arabidopsis research drives plant science



How to you find mutant lines ?



T-DNA Express: Arabidopsis Gene Mapping Tool

T-DNA Express: Arabidopsis Gene Mapping Tool (Dec. 19, 2014)

Powered by gebd 4.18.3

Arabidopsis thaliana [TAIR V10]
chr1 - 40001

out in

1. Search : [?]

Type: Query:

Chr: Posn:

Display:

2. iSect Tool :
 Please Select one

3. Data Source : a. [Data Source, Detail and Summary](#).
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 c. [Data Release Policy](#).
 d. [FAQs](#).

4. Blast :

Program: E-value: Hits:

Cut and paste your sequence into here.

1. Help:
[GE Browser Help](#)
[iSect Tool Help](#)
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[NetCompare Tool](#)
[Bi2Seq Tool](#)

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How to you screen mutant lines ?

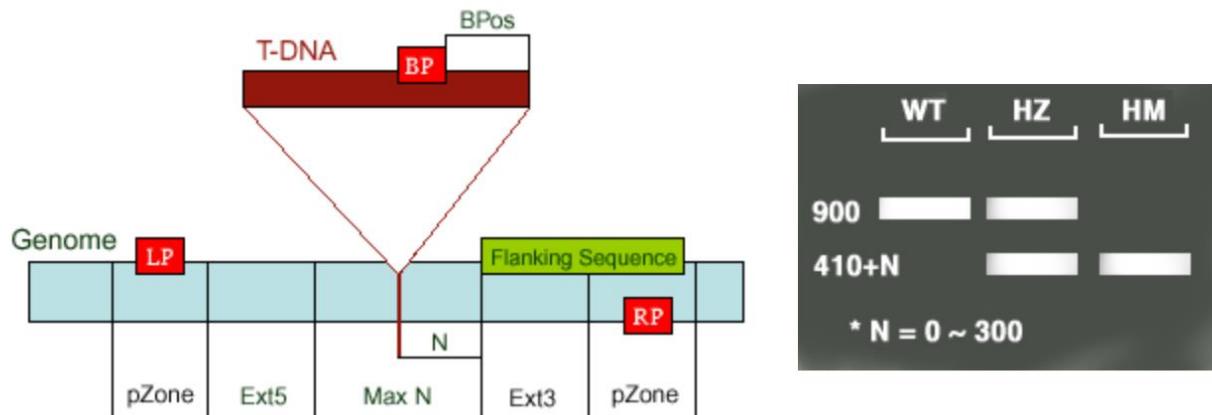
Obtain and screen T-DNA insertion lines, cheap and quick resource, many lines already confirmed.

-optimal to have at least 2 lines, independent insertions within the same gene.

-genomic DNA prep and PCR required to confirm the line

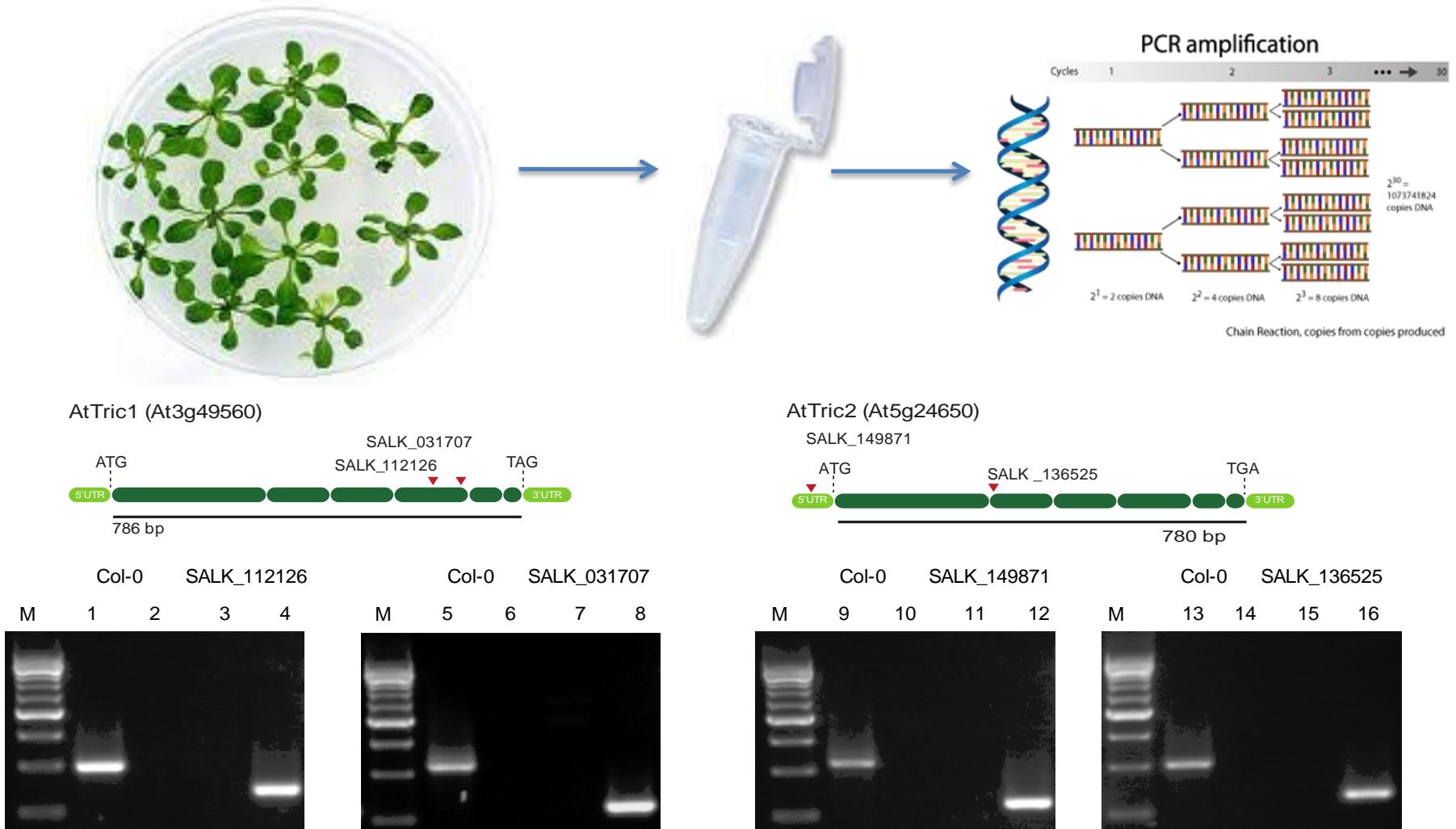
-location of the insert is important, may not produce a KO if insert is in the 5'UTR or 3'UTR. Introns and exons, T-DNA insert may be spliced out.

1. Protocol for SALK T-DNA primer design



How do you screen mutant lines ?

Genotyping: identification of Homozygous mutants



How do you screen mutant lines ?

Genotyping: the identification of a Homozygous mutant

Heterozygous (HET)



Homozygous WT



HET

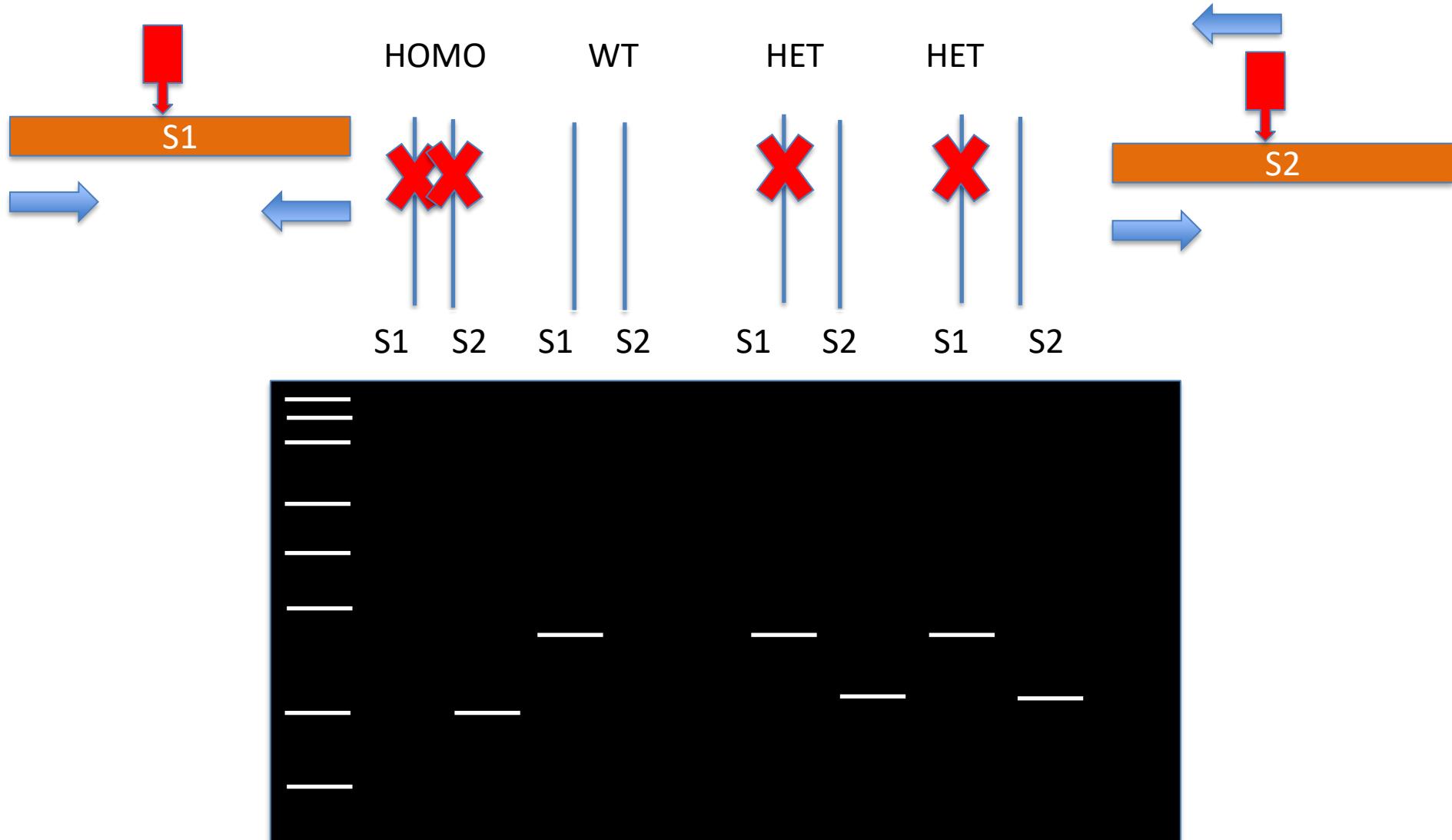


HET



How do you screen mutant lines ?

PCR based genotyping: the identification of a Homozygous mutant



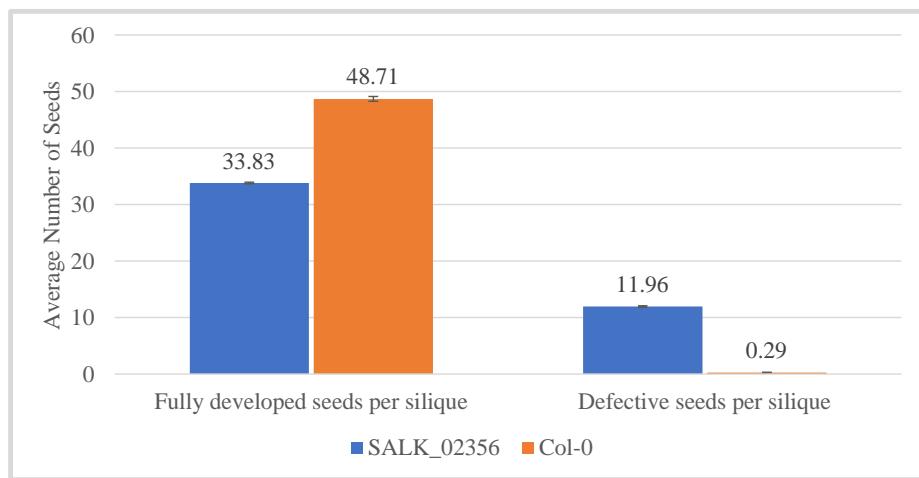
How do you screen mutant lines ?

Genotyping: What happens if you cannot find a homozygous knock-out line?

ii)



i)



Homozygous WT



HET

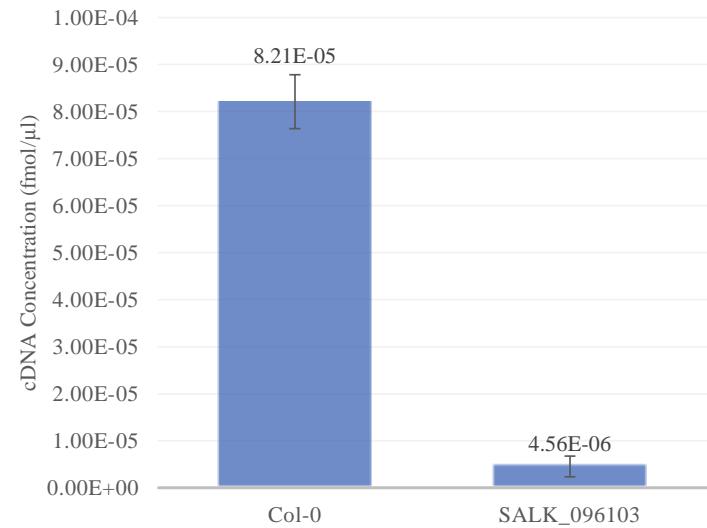
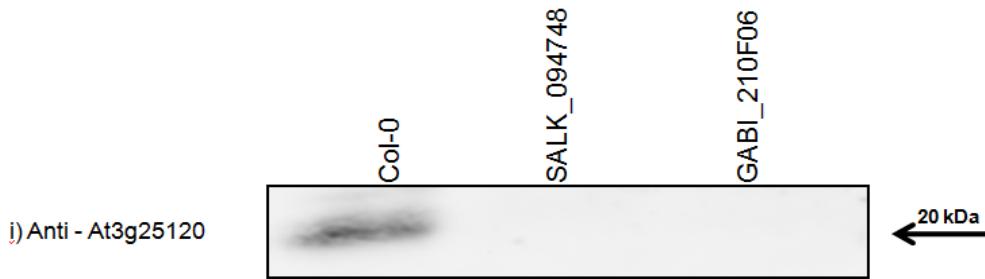


HET



How do you confirm mutant lines ?

Genotyping: confirmation of mutants



Need to confirm transcript level, design primers after the insert.

Transcript level may not correlate to protein level, therefore it is optimal to create antibodies to confirm, total knock-out of protein.

This is the definitive proof..... Looking at protein abundance

How do you confirm mutant lines ?

How effective is T-DNA insertional mutagenesis in *Arabidopsis*?

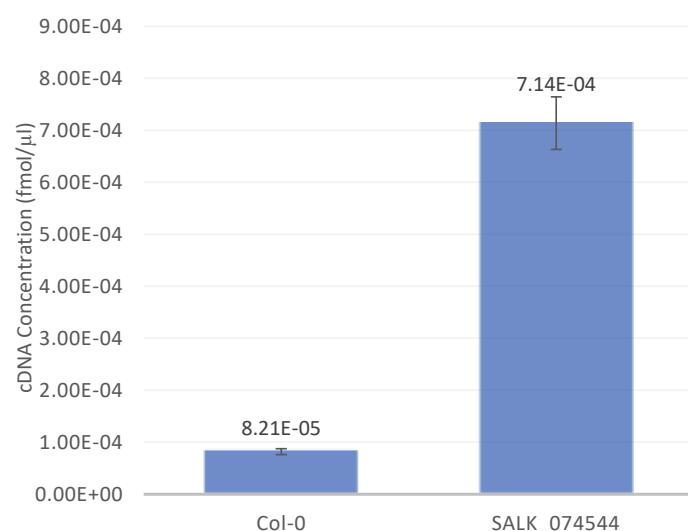
Table 1: Effect of published T-DNA insertion on transcript level of mutated genes in *Arabidopsis*

Insertion site	Number of insertion mutants	No effect on transcript level	Increased transcript level
Exon	609	7 (1.1%)	0 (0%)
Intron	263	2 (0.7%)	0 (0%)
Before start codon	155	17 (11%)	5 (3%)
After stop codon	23	4 (17%)	2 (8%)
Major Deletion	34	0 (0%)	0 (0%)
Total	1084	25 (2.3%)	7 (0.6%)

^aFor each category of insertions, only insertions that showed no or increased effect on expression are listed. The rest are knockout/knockdowns which are not distinguished in exon/intron insertion mutants because a lot of reports used RT-PCR. RT-PCR can show a reduced level of transcript upstream from an insertion site and increased transcript downstream from the insertion site or vice versa, making it difficult to assign the mutant as knockdown or knockout. For "Major Deletions", See Table 5.

Table 2: Effect of T-DNA insertion on protein expression of inserted genes in *Arabidopsis*

Insertion site	Exon	Intron	Before start codon	Total
No protein expression	70(88%)	32(82%)	7(41%)	109(80%)
Reduced protein expression	7(8%)	5(13%)	9(53%)	21(16%)
No effect on protein expression	1(1%)	1(3%)	1(6%)	3(2%)
Truncated protein expression	2(2%)	1(3%)	0(0%)	3(2%)
Total	80	39	17	136



How do you confirm mutant lines ?

A T-DNA insert may not produce a knock-out effect

May only produce a knock-down, -

-examples of no difference in protein abundance –insert in an intron region, insert spliced

Out

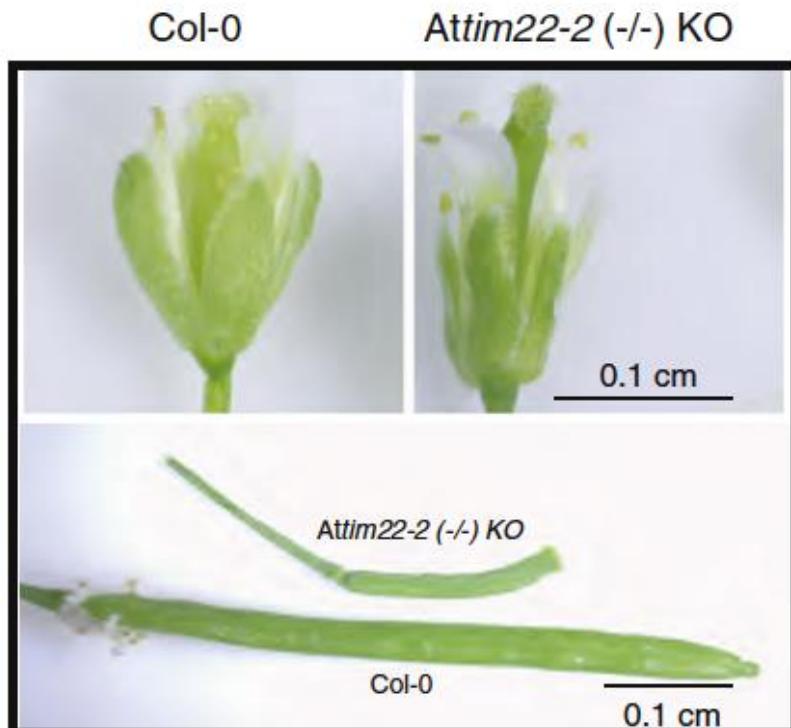
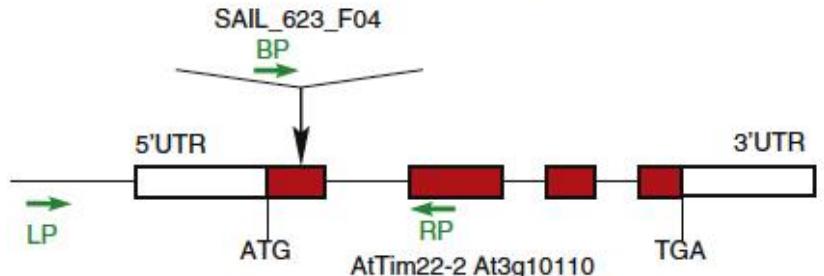
Production a truncated protein- insert towards the end of the gene-results in an early termination

Production of an overexpressor-insert within the promoter region affects transcription/

Translation resulting in the over expression of the protein.

Genotyping: Homozygous mutants that are sterile

M.W. Murcha et al / Biochimica et Biophysica Acta 1840 (2014) 1233–1245



The gene is essential for
pollen/embryo/seed
development
Fertilization

Homozygous mutants can grow
but cannot make progeny

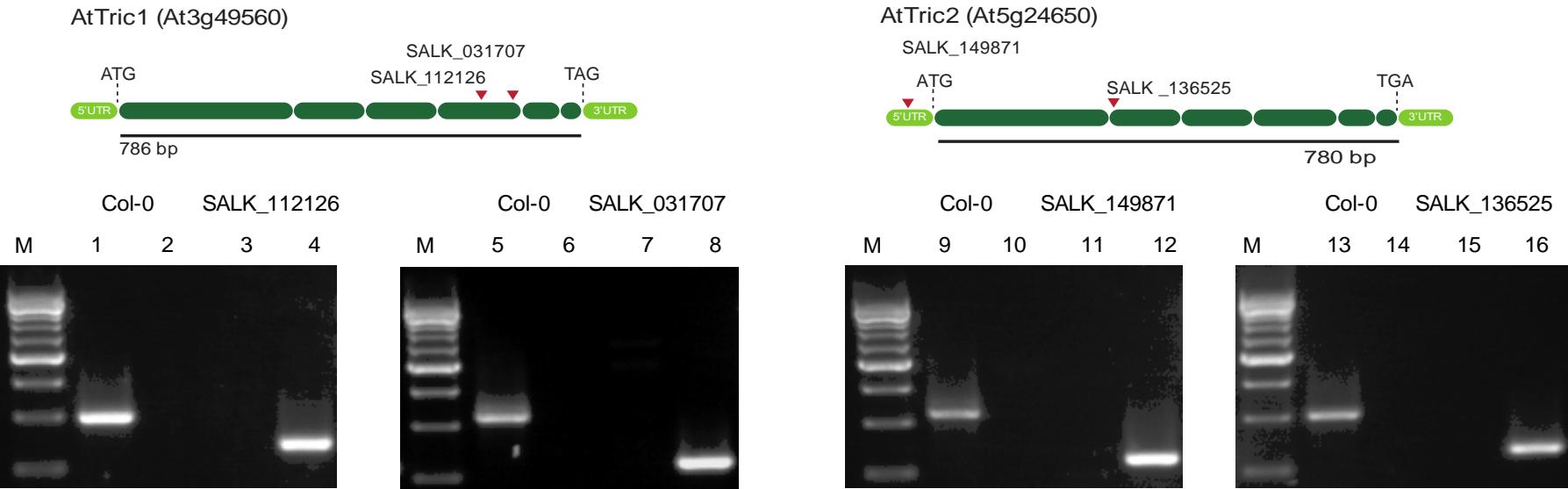
of AtTim22-2 results in plant sterility. The T-DNA insertional knock-out line for AtTim22-2 (At3g10110) (SAIL_623_F03) was screened for homozygosity. Attim22-2 plants exhibit smaller flowers and poorly developed siliques with little to no seed. Scale bar, 0.1 cm, (-/-) = homozygous knock-out.

Multiple genes encode for the same or similar proteins = MULTIGENE FAMILIES

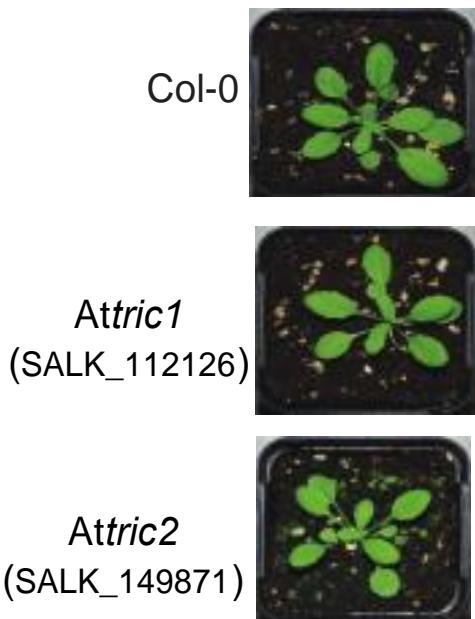
Many genes can have redundant functions. Therefore deletion of a gene will have no consequences to the phenotype. The “other” genes can do the same job.

Tom = Translocases of the Outer Membrane, Tim = Translocase of the Inner Membrane, NdufA11 = NADH dehydrogenase [ubiquinone] 1 subunit 11 (Human), B14.7 = NADH dehydrogenase [ubiquinone] 1 subunit 14.7 (Bovine), HM = Homozygous, Het = Heterozygous, bp = base pair, Ref = Reference, At = *Arabidopsis thaliana*.

Name	Viral in yeast	Gene name (AGI)	Line details	Insert location	Genotype	Altered Phenotype	Details	Ref
Tom20	Yes	AtTom20-2 (At1g27390)	SALK_067986 SALK_134973	+1255 bp intron +126 bp intron	HM HM	- -	Non-essential gene	[24] [24]
		AtTom20-3 (At3g27080)	GK-554C03 SAIL_88_A08	+971 bp intron +733 bp exon	HM HM	- -	Non-essential gene	[24] [24]
		AtTom20-4 (At5g40930)	SALK_147093 SALK_004057	+579 bp exon +422 bp intron	HM HM	- -	Non-essential gene	[24] [24]
		AtTom40-1 (At3g20000)	SALK_128170	+1796 bp exon	Het	Defective embryo	essential gene	unpublished data
		AtTom40-2 (At5g40930)	SALK_004057	+422 bp intron	HM	-	Non-essential gene	unpublished data
OM64	NA	OM64 At5g09420	SALK_068772 SALK_089921		HM HM	- -	Non-essential gene	[24] [24]
Sam37/Metaxin	No	Sam37/Metaxin At2g19080	SALK_107629 SALK_039892		HM	Delayed growth	Non-essential gene	[24] [24]
Tim23	Yes	AtTim23-1 (At1g17530)	SALK_030470 SALK_107963	+699 bp 3'utr +656 bp 5'utr	HM HM	- -	Attim23-2: Attim23-3 not viable Essential gene	
		AtTim23-2 (At1g72750)	SALK_143656 GK-689C11	-135 bp 5'utr +43 bp exon	HM HM	Delayed growth	Over-expressor Knock-out Attim23-1: Attim23-3 not viable Essential gene	[13] [13]
		AtTim23-3 (At3g04800)	SAIL_1151_B01 SALK_129386	+548 bp exon +415 bp exon	HM HM	- -	Attim23-1: Attim23-2 not viable Essential gene	
		AtTim17-1 (At1g20350)	SALK_091528 SALK_092885	-400 bp 5'utr +360 bp exon	HM HM	Faster germination Faster germination	Non-essential gene	unpublished data
		AtTim17-2 (At2g37410)	GK-561ED3	+160 bp exon	Het	Defective embryo	Essential gene	
Tim17	Yes	AtTim17-3 (At5g11690)	SALK_048425 SALK_125567	+543 bp 3'utr +471 bp 3'utr	HM HM	- -	Non-essential gene	
		AtTim22-1 (At1g18320)	GK-848H04	+71 bp exon	HM	-	Non-essential gene not expressed	
		AtTim22-2 (At3g10110)	SAIL_623_F03	+60 bp exon	HM	Sterile plant	Essential gene	
Tim50	Yes	AtTim50 (At1g55900)	SALK_000523 SALK_059376	+2211 bp intron +1177 bp intron	HM HM	Delayed growth -	Early termination Knock-down Essential gene	[112]
Tim44	Yes	AtTim44-1 (At2g20510)	SALK_015319	+34 bp exon	HM	-	Attim44-1: Attim44-2 not viable Essential gene	
		AtTim44-2 (At2g36070)	SALK_146901	-277 bp 5'utr	HM	-	Attim44-1: Attim44-2 not viable Essential gene	
Tim21	No	AtTim21 (At4g00026)	sd3-2	intron	HM	seedling lethal	Essential gene	[111]
NdufsA11/B14.7	NA	AtB14.7 (At2g42210)	SAIL_434_E06	+619 bp intron	Het	Defective pollen	Essential gene	



28 d



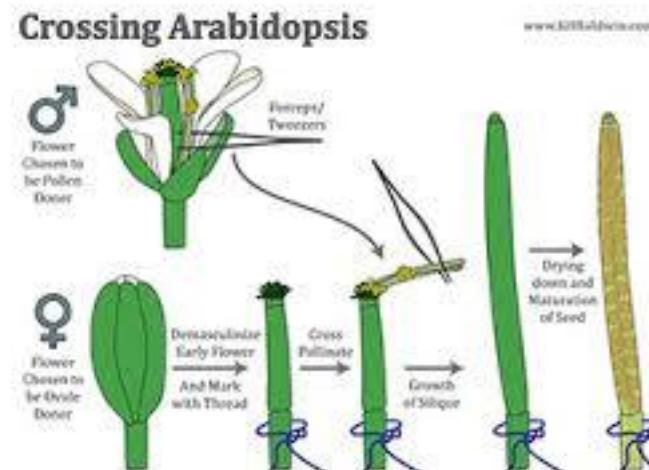
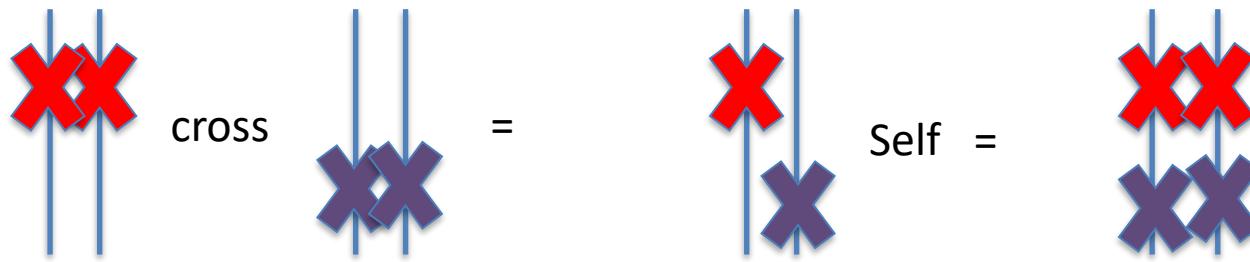
Multiple genes encode for the same or similar proteins?

- Example AtTric1/2 single deletions have no consequence to phenotype
- Need to make a double mutant ??
HOW??

Generation of double Homozygous mutants

- Single lines had no obvious defect/effect on plant development.
- Need to make a double mutant plant
- May need to make triple/ quadruple (1-2 yrs)

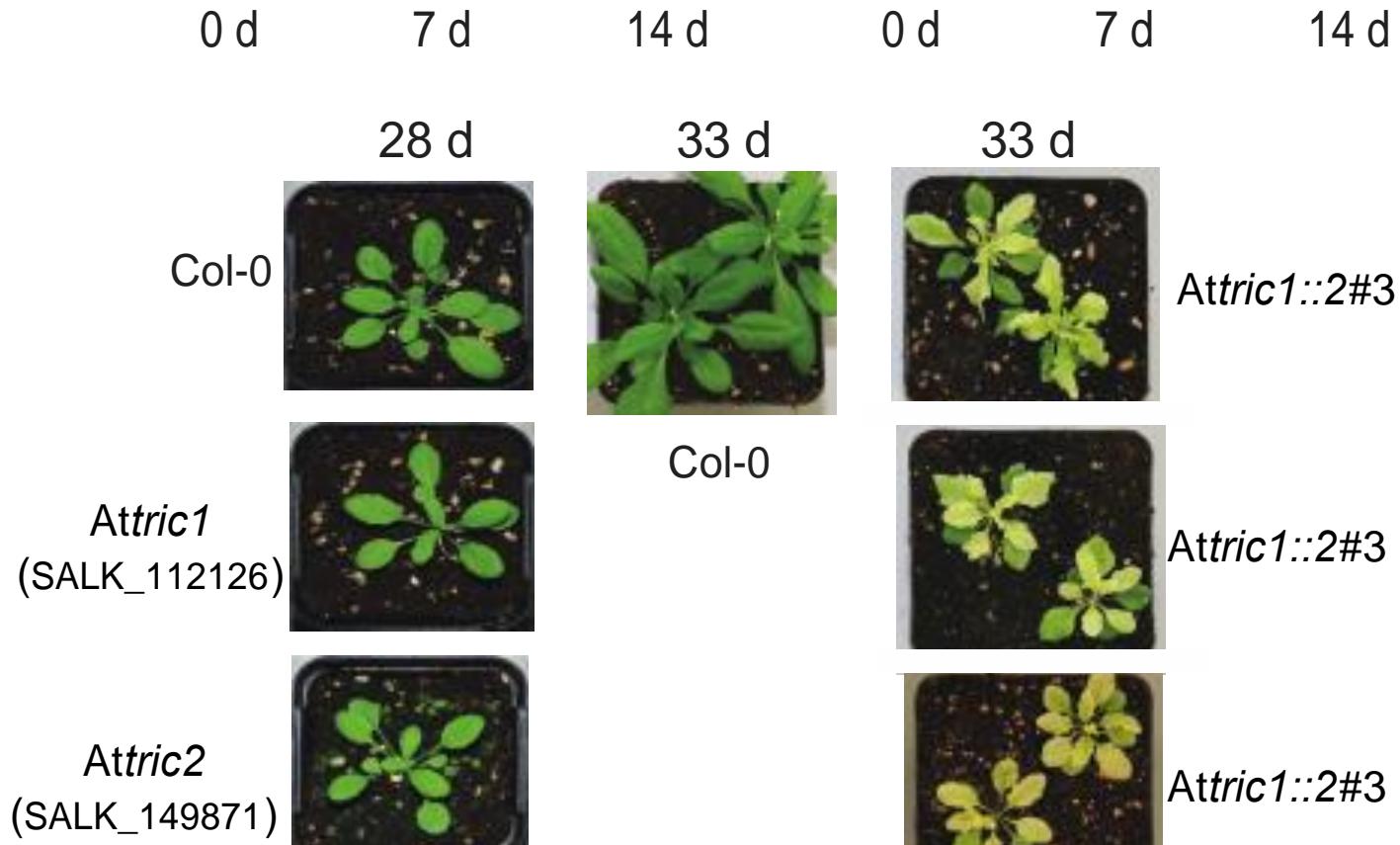
HM A HM B = Het A Het B = HM A and HM B 1/16



Genotyping: generation of double Homozygous mutants

Generation of double Homozygous mutants

- Single lines had no obvious defect/effect on plant development.
- May need to make triple/ quadruple (1-2 yrs)



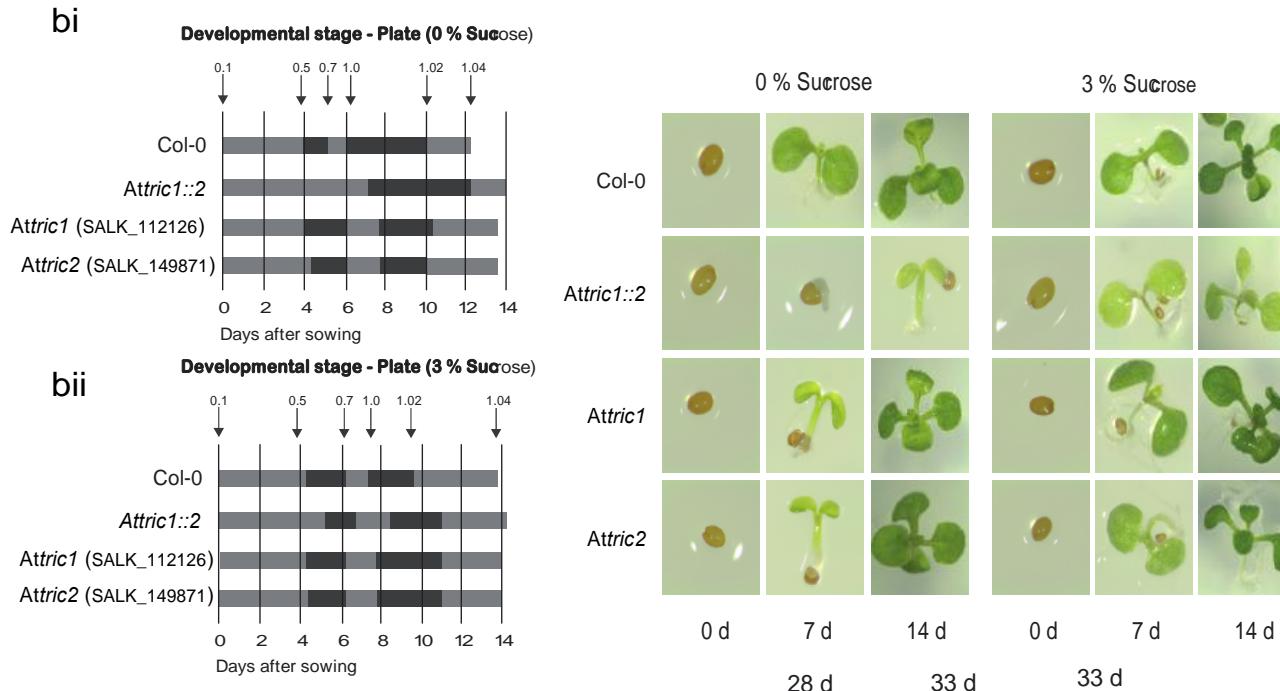
Phenotypic analysis of mutants (KO's, knock-downs, overexpressors etc)

What are the observable physical consequences of modifying that gene?

1. Normal conditions on MS media and Soil.
1. Phenotypic analysis on compromised conditions eg stress, high light drought etc.

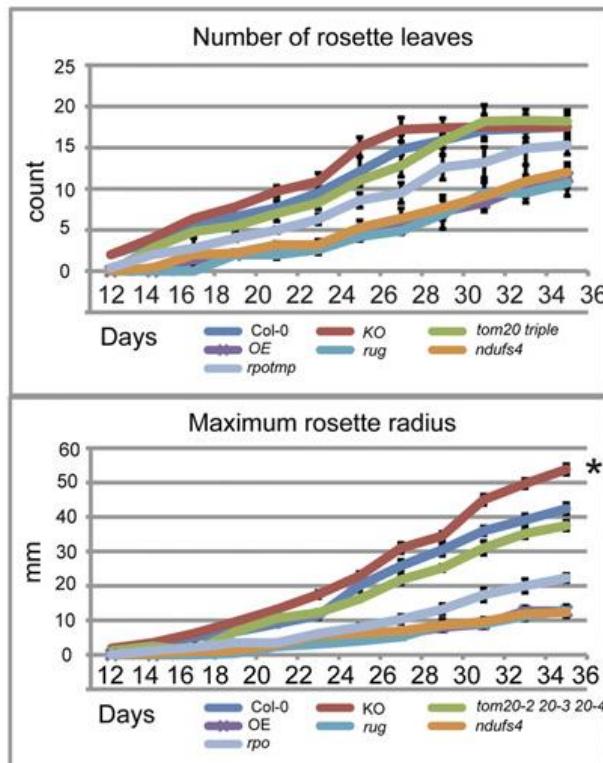
How do we measure phenotypes?

Stage growth progression- how many days does it take to reach a certain developmental stage

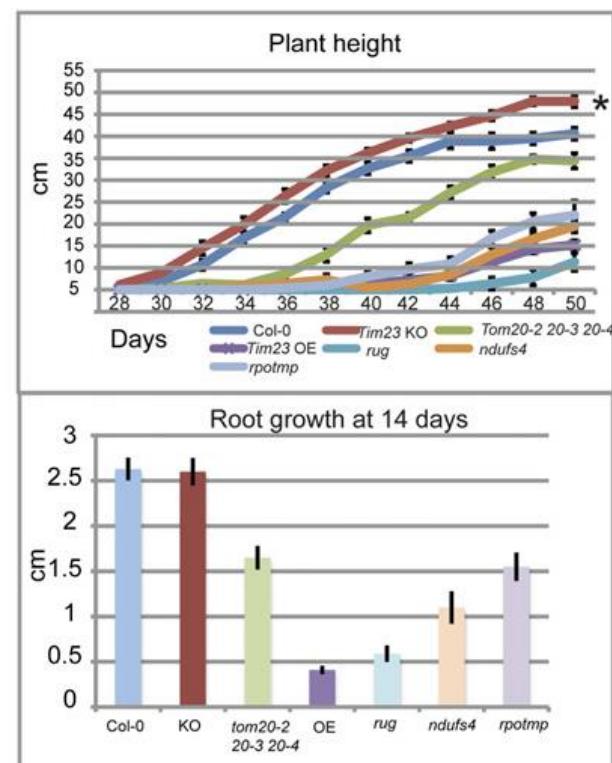


Phenotypic analysis of mutants (KO's, knock-downs, overexpressors etc)

Growth parameters: height, length, root length, leaf radius



representative growth parameters

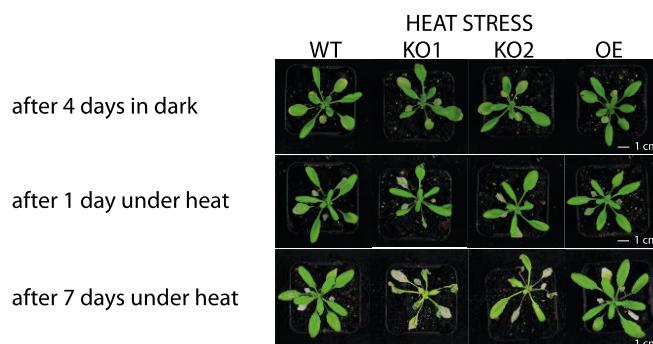
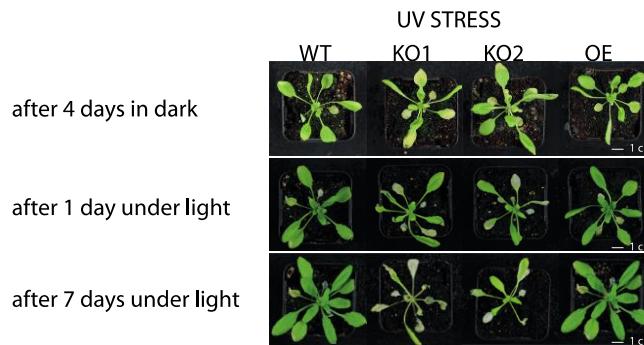
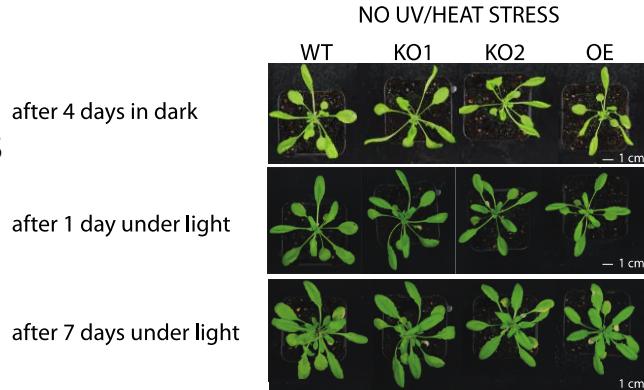


representative growth parameters

Phenotypic analysis of mutants

Phenotypic analysis on compromised conditions

1. High temp 42 deg , return to normal
2. High light
3. Treat UV light
4. High salinity
5. Stop watering
6. Infect pathogen (biotic)



Phenotypic analysis of mutants

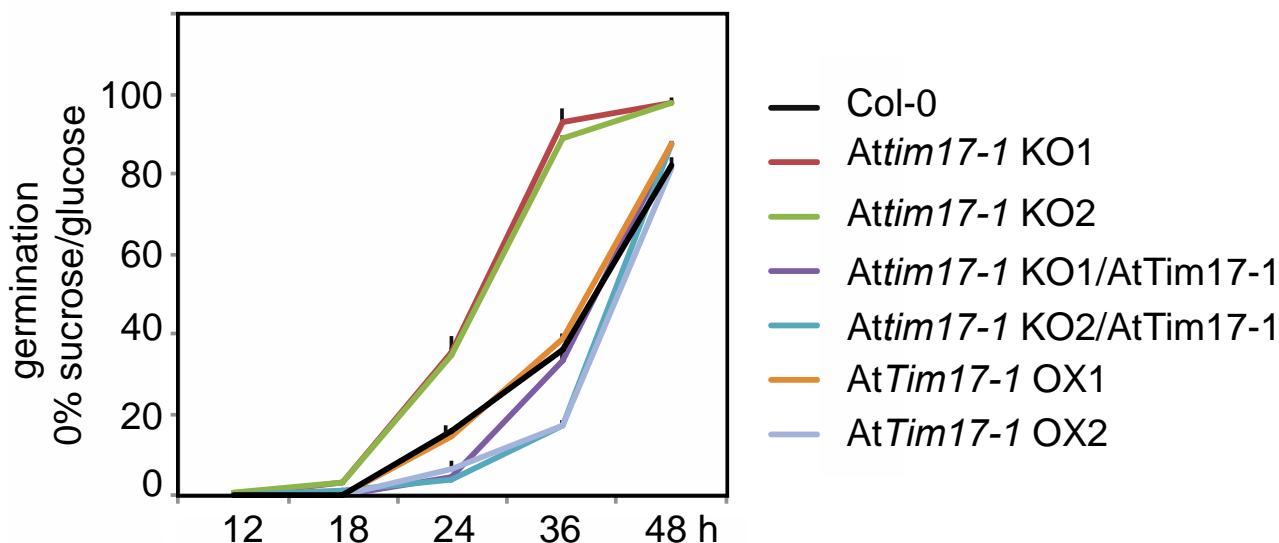
developmental parameters

Eg. Germination, senescence

If your gene is expressed in a developmental specific manner



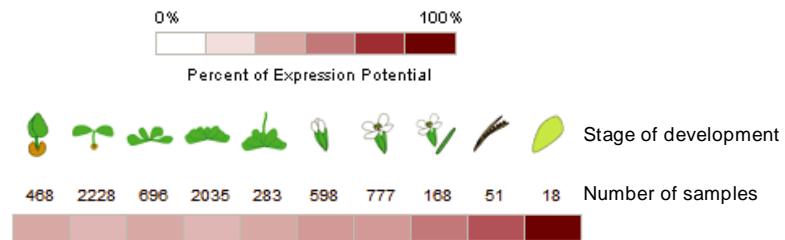
You should only observe the phenotype at a particular stage



Phenotypic analysis of mutants

developmental parameters

A



B

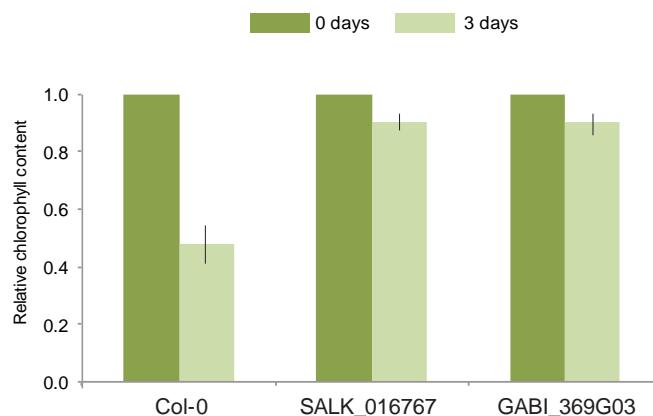
The effects of this gene KO can only be observed at senescence

Look in online expression data to see where your gene is expressed



Leaves after 3 days of dark-induced senescence treatment

C



Molecular and Biochemical analysis of mutants

No obvious developmental growth defect

Can have a biochemical or Molecular defect

Eg. Defective in a biochemical pathway

Know what your looking for..

Whole transcriptome, proteome,
metabolome analysis.

Generation of mutants

Overexpressor lines

Introduce another copy of the gene into the genome. Can be under the native promoter (endogenous expression) or under a strong constitutive promoter.

You may Tag your new protein- FLAG tag pull downs, GFP tag localisation

RNAi knock-downs

The Nobel Prize in Physiology or Medicine in 2006 was awarded to Craig Mello and Andrew Fire for the development of essentially a new field, RNA Interference or RNAi In *c. elegans*.

Using RNA molecules to inhibit gene expression, siRNA molecules bind to mRNA.

In plant research it is a very powerful technique as many species are polyploidal, eg wheat

CRISPR/cas9- directed mutations/deletions/insertions

Specifically target regions within the genome, make truncations, make specific mutations, make knock-outs where none are available.

Complementation of Knock-out mutants

The only way to confirm that your gene is responsible for the phenotype observed is to complement it.

Return a functional copy of the gene back into the genome..... but will it be under the native promoter, will it be in the same position within the genome, will expression levels be restored as in wildtype.

Generation of complementation lines and overexpressor lines.

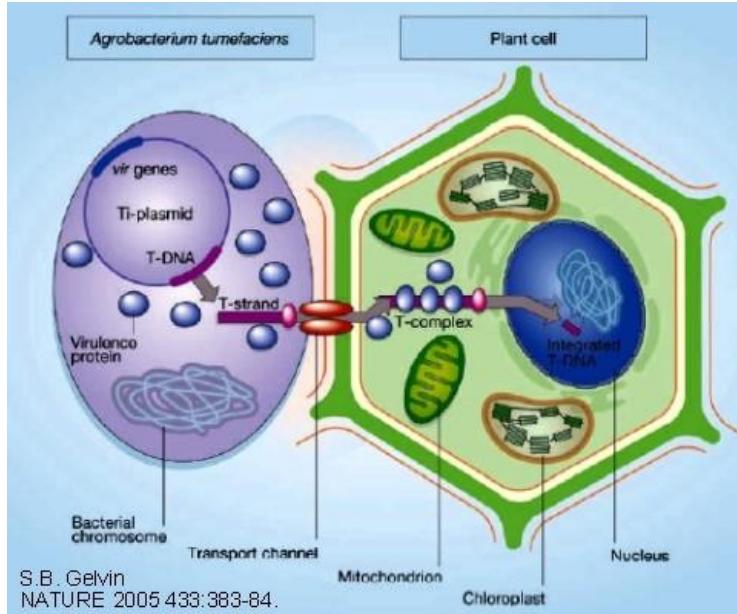
Restore the gene of interest and restore the phenotype observed. Can create lines with both a strongly expressed promoter (CaMV) or an endogenous promoter (native promoter). CaMV will most likely produce an overexpression lines, though it can get silenced over generations

Obtain varying levels of transcript and protein expression due to multiple individual

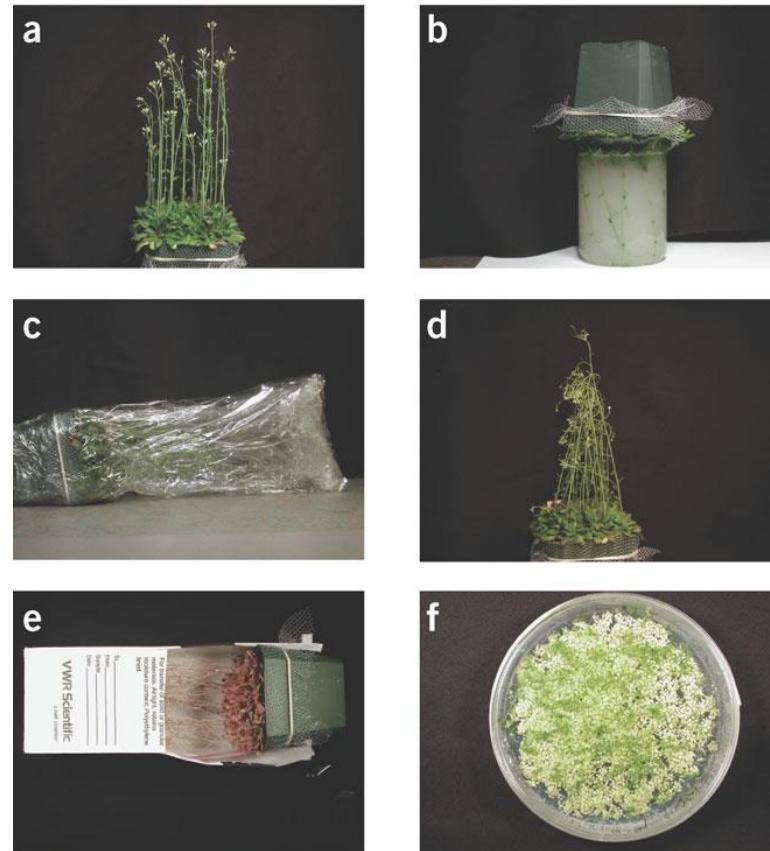
T-DNA lines with varied location and number of T-DNA inserts.

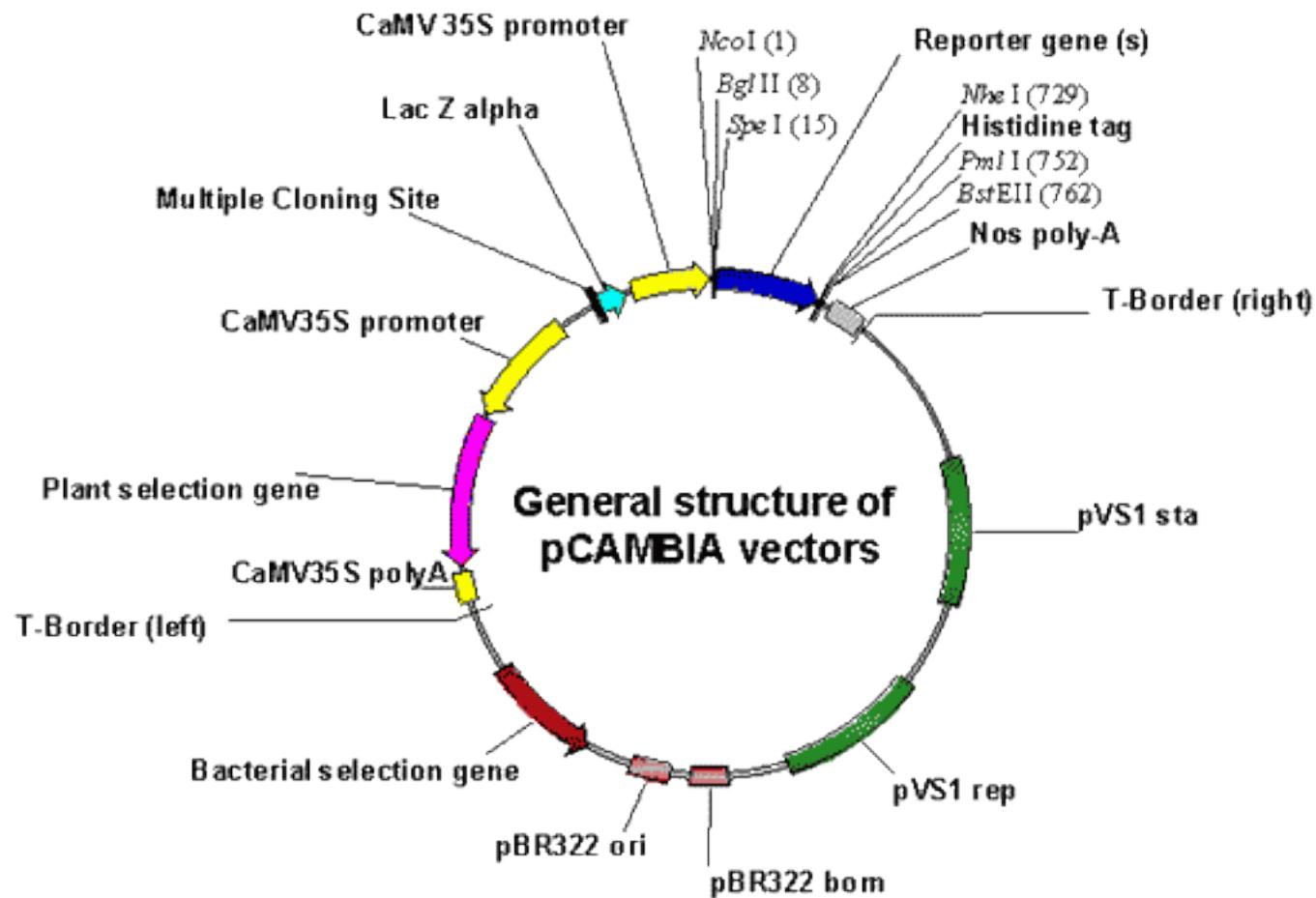
Allow you to make tagged variants for biochemical characterisation- Pull down approaches.

Agrobacterium mediated transformation of plasmid containing a homologous gene short strand sequence. Common soil pathogen that transfers DNA into host plant Transferred DNA (T-DNA). Single or multiple T-DNA can be inserted into host genome.



Nature Protocols 1, 641 - 646 (2006) Published online: 29 June 2006
doi:10.1038/nprot.2006.97

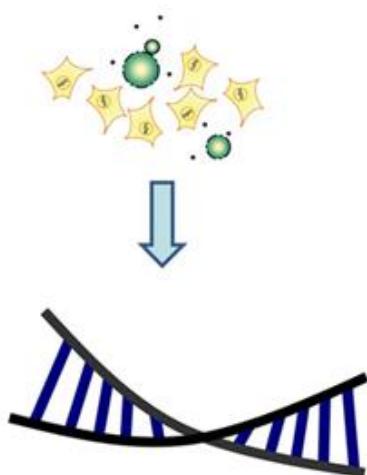




Forward genetics: classical genetics
Determining the genetic basis responsible for a phenotype

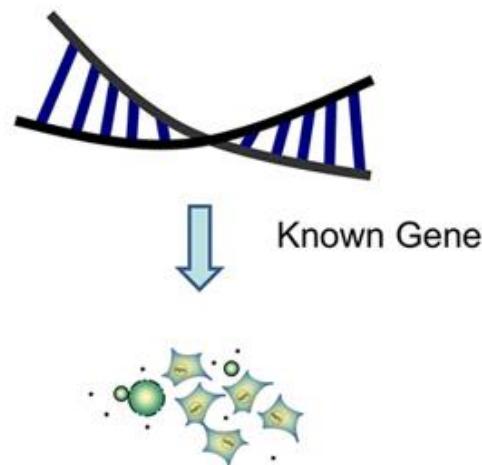
Reverse genetics:
Discovering the function of a gene by analysing the phenotypic consequences of a gene

Forward Genetic Screens



Discover
Gene
underlying
Phenotype

Reverse Genetic Screens



Phenotype
Resulting
from
Alteration

Forward genetics: classical genetics

Determining the genetic basis
responsible for a phenotype



1. Mutagen treat seeds-EMS
2. Screen for desired phenotype
3. Identify the gene responsible by traditional mapping techniques and deep sequencing

EMS mutagenesis

Ethyl Methanesulphonate

Introduces random point mutants, single amino acids base change, favours a C to T change.

Can produce a single amino acid change or a stop codon (only 5%).

Required at least 125 000 seeds

Multiple mutations can occur per line depending on the concentration and length of treatment. ~700

Forward genetics: classical genetics

Determining the genetic basis
responsible for a phenotype



1. Mutagen treat seeds- use mainly EMS

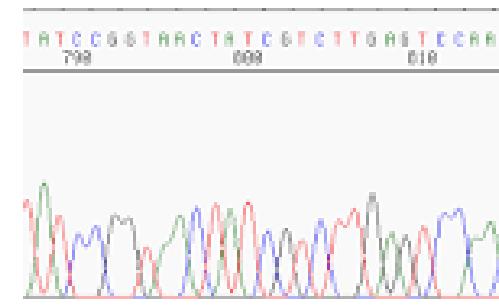
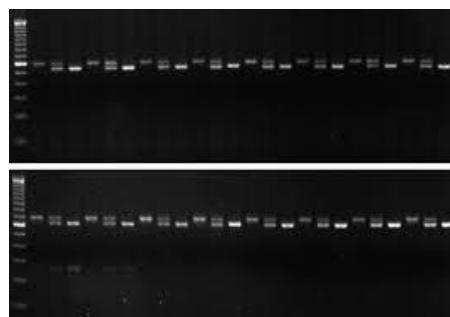
(radiation, natural variation, recombination)

1. Screen for desired phenotype

Simple, easy to identify screen- visual to allow screening of 10's-100's mutants at one time



1. Identify the gene responsible by traditional mapping techniques and deep sequencing



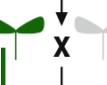
Mapping

Positional mapping

1. Use mutagenised Columbia seed stock



Mutant (*mutmut*)

2.  Phenotype

CC

LL

Genotype

3. 

F1

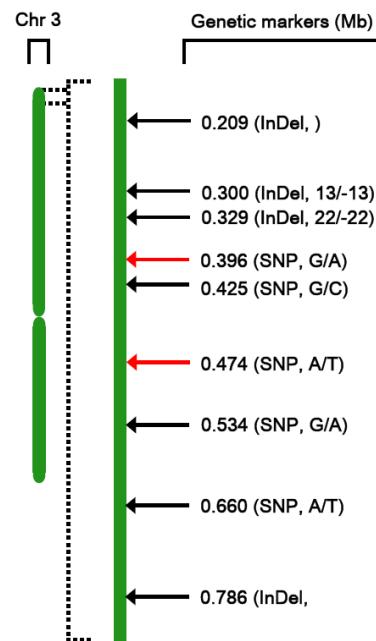
CL

4. 

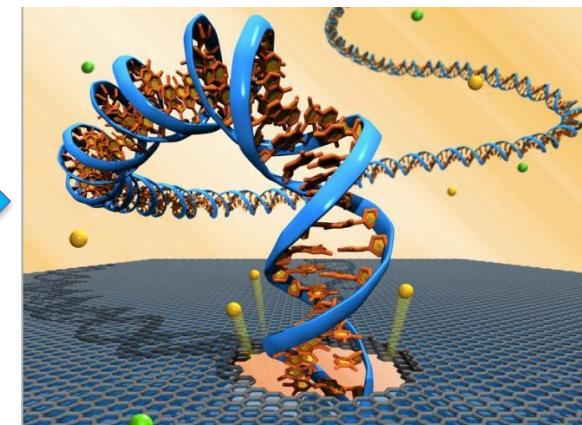
F2 generation



1-3 Mb chromosomal
region



Next Generation Sequencing



Forward genetics:

Positional mapping.

- single nucleotide polymorphism (SNP) mapping is employed to narrow down a genomic region
- cross is set up between mutant and alternative strain of the same species that contains polymorphic nucleotides
 - eg. Col-0 and Ler
- recombination during meiosis, the polymorphisms from the mutant and mapping strains will be distributed 50/50 ratio,
- except for the mutation, it will be linked SNPs that are genetically linked to the causal mutation.

Positional mapping can take years to do PCR, sequencing, crossing segregating etc.

Traditionally this was the bottleneck in EMS mutant screens.

Thanks to NGS we can sequence genomes. So we use a combination of mapping and sequencing.

Still need to confirm the mutation is responsible???????

How would you do this

Complementation!!!

Genetic screen

EMS treatment of
seeds



M1 plants
2000

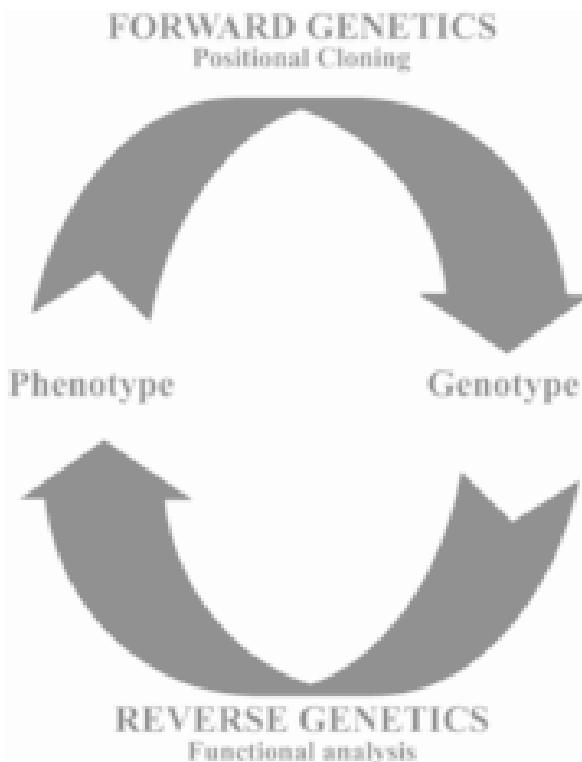


Screen of M2
phenotype



Forward genetics:

- Time consuming
- Expert geneticists needed
- Need easy to screen phenotype- pale plant, small plant, large plant etc
- unbiased discovery
- Still need to work out the mechanisms



Reverse genetics:

- Faster (still take month to make mutants)
- T-DNA cheap and available
- Proven techniques and methodology
- Multiple genes
- Cant find a phenotype
- Is the observed phenotype a direct consequence, or due to downstream effects.
- Researcher bias
- Still need to work out mechanisms