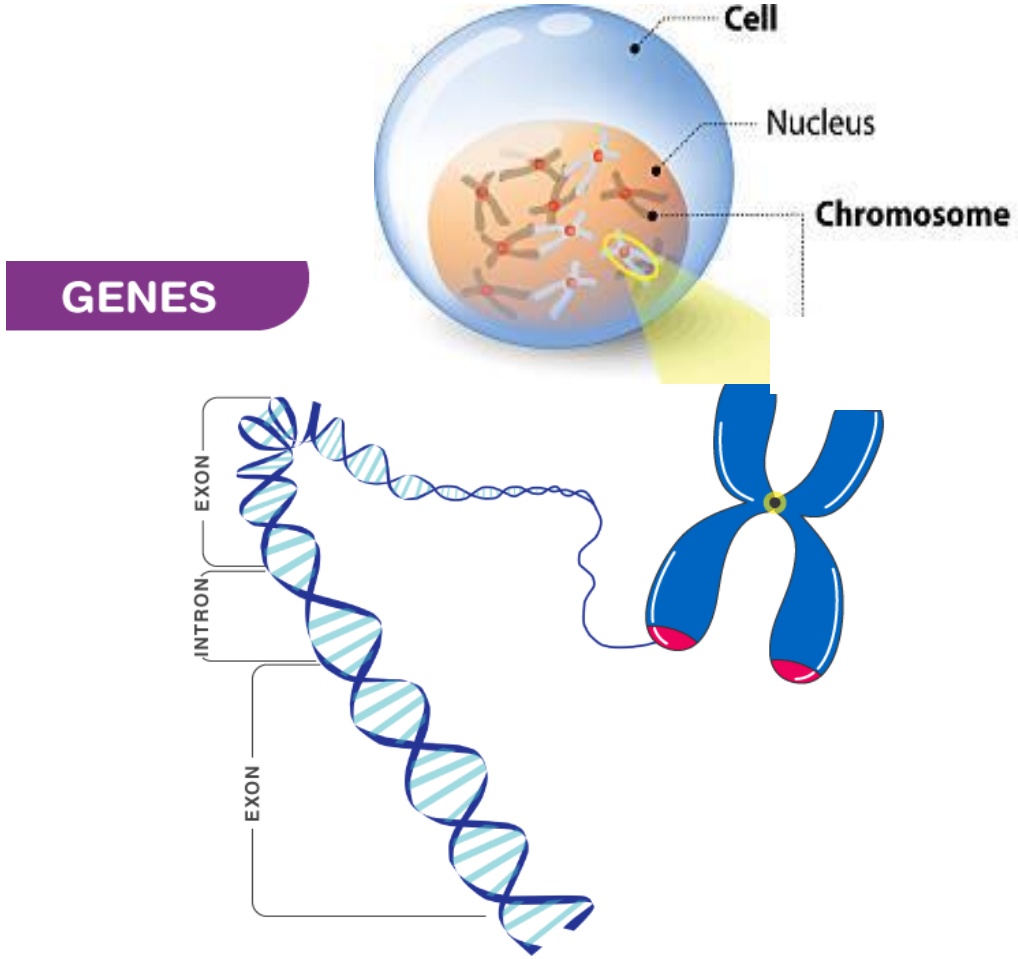
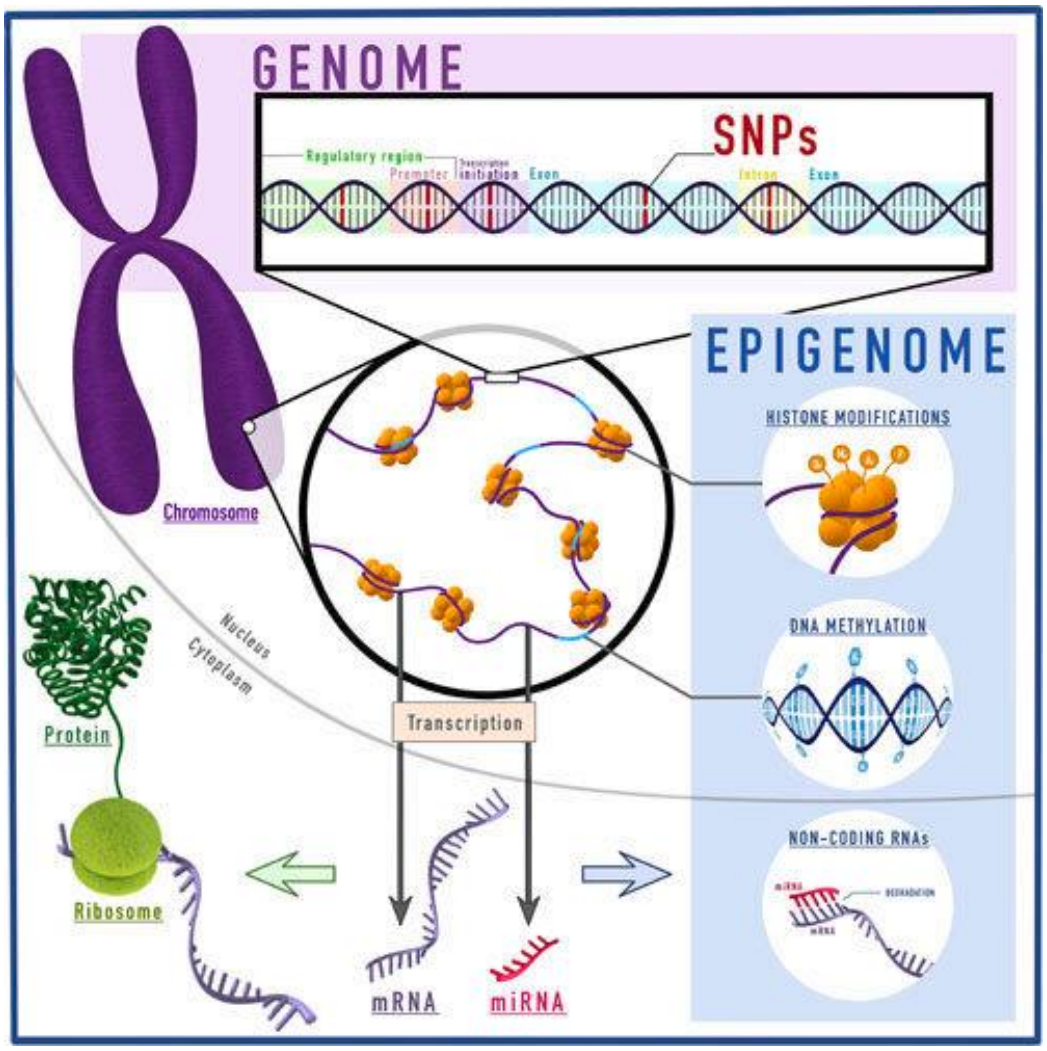
Why understanding gene function is important ?



Genetic variations are there in the crop plants' wild cousins, or in less-improved or heirloom varieties. These relatives can be a **reservoir of genetic diversity -** plant breeders just need to figure out **how to get the right genes into the crop plant**

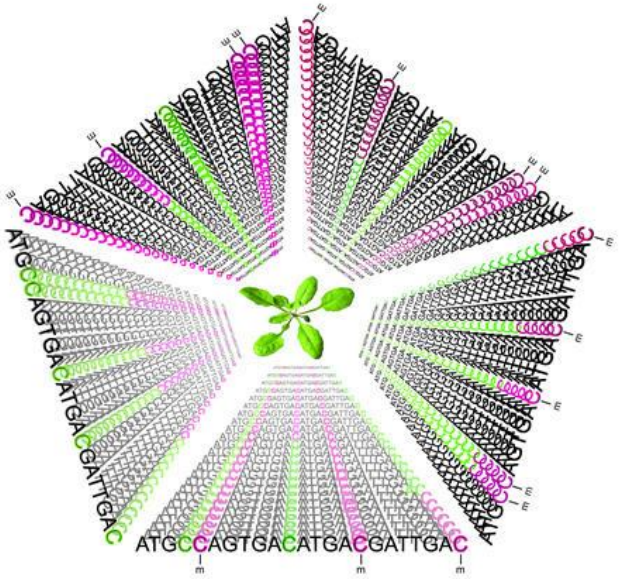
Genome and Epigenome



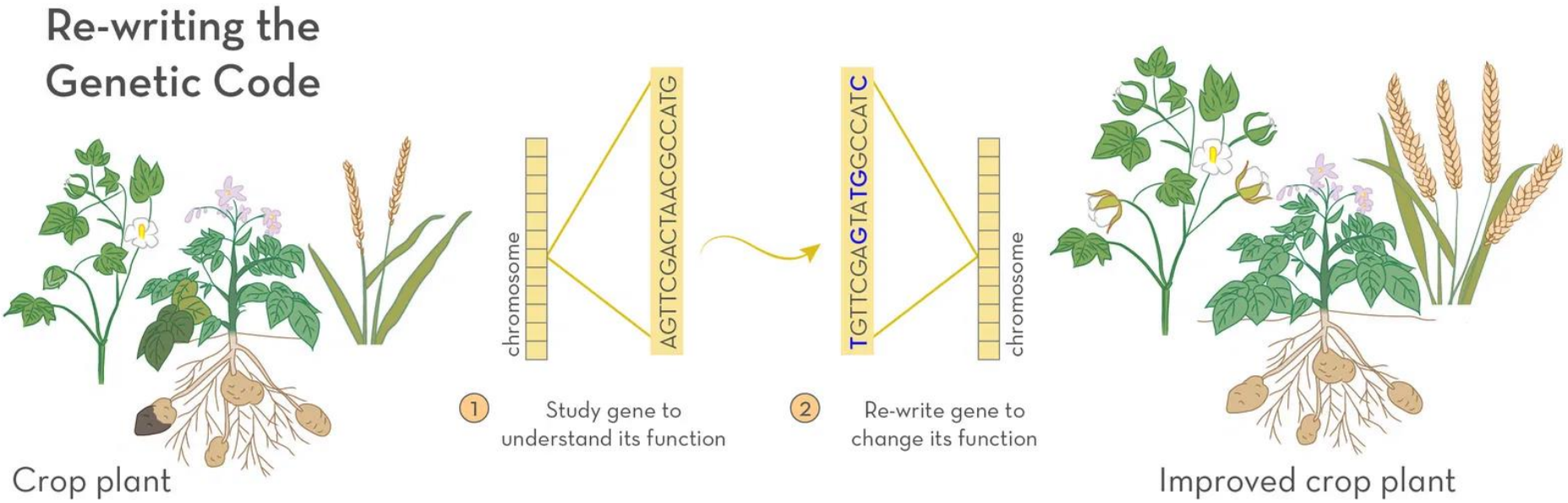
Environment creates further complexity to gene function

Understanding Gene Function –
tools of plant functional genomics

- Gene discovery
- Understanding gene function
- Genetic control of traits

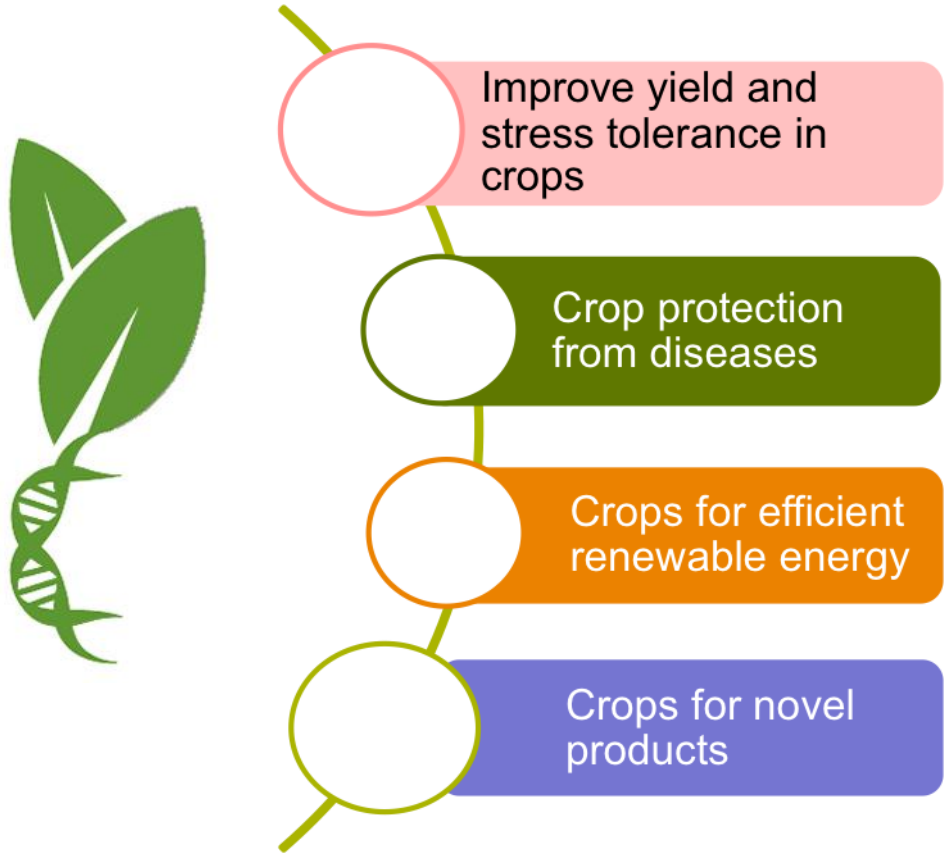


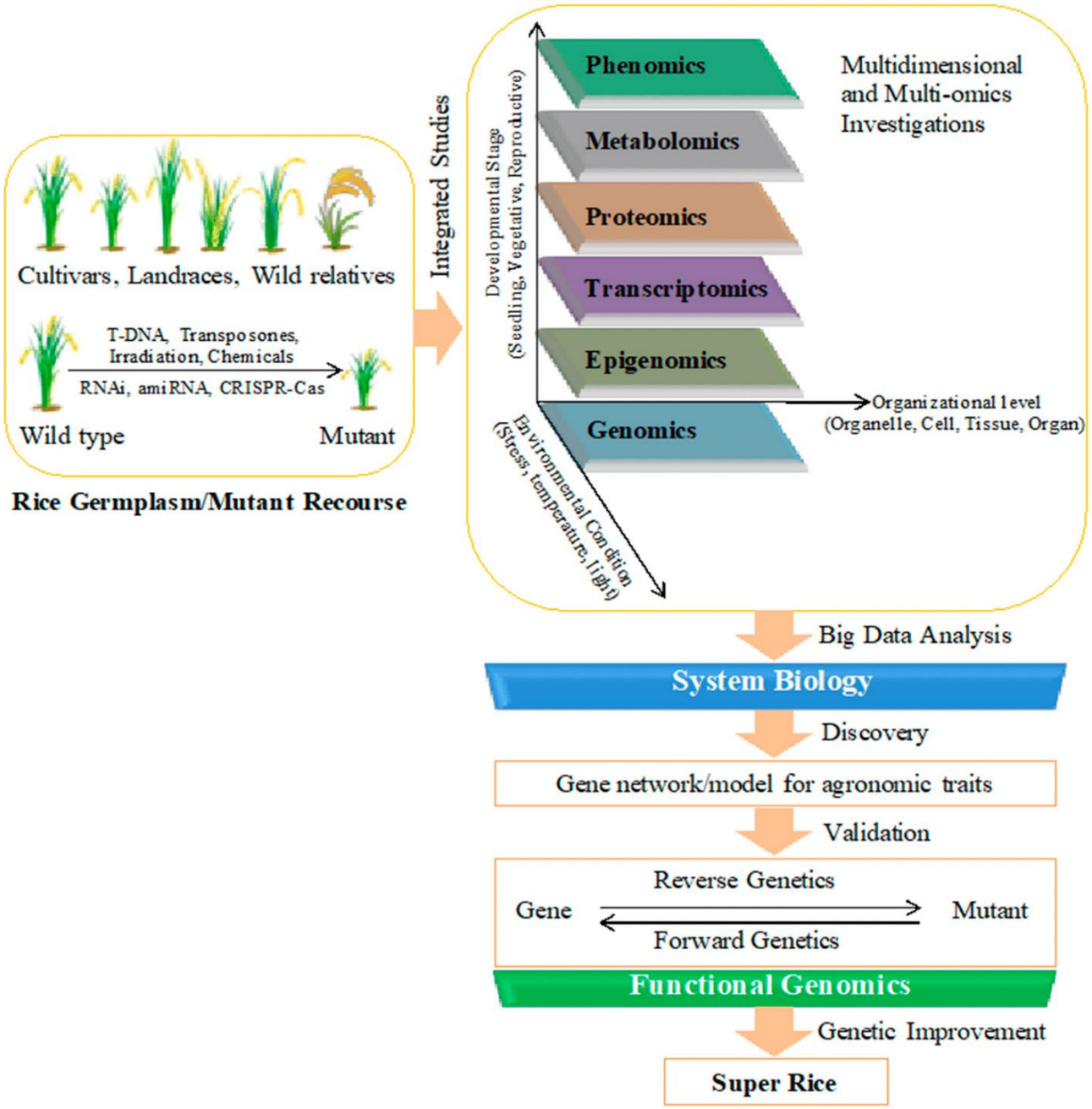
Important to improve crops

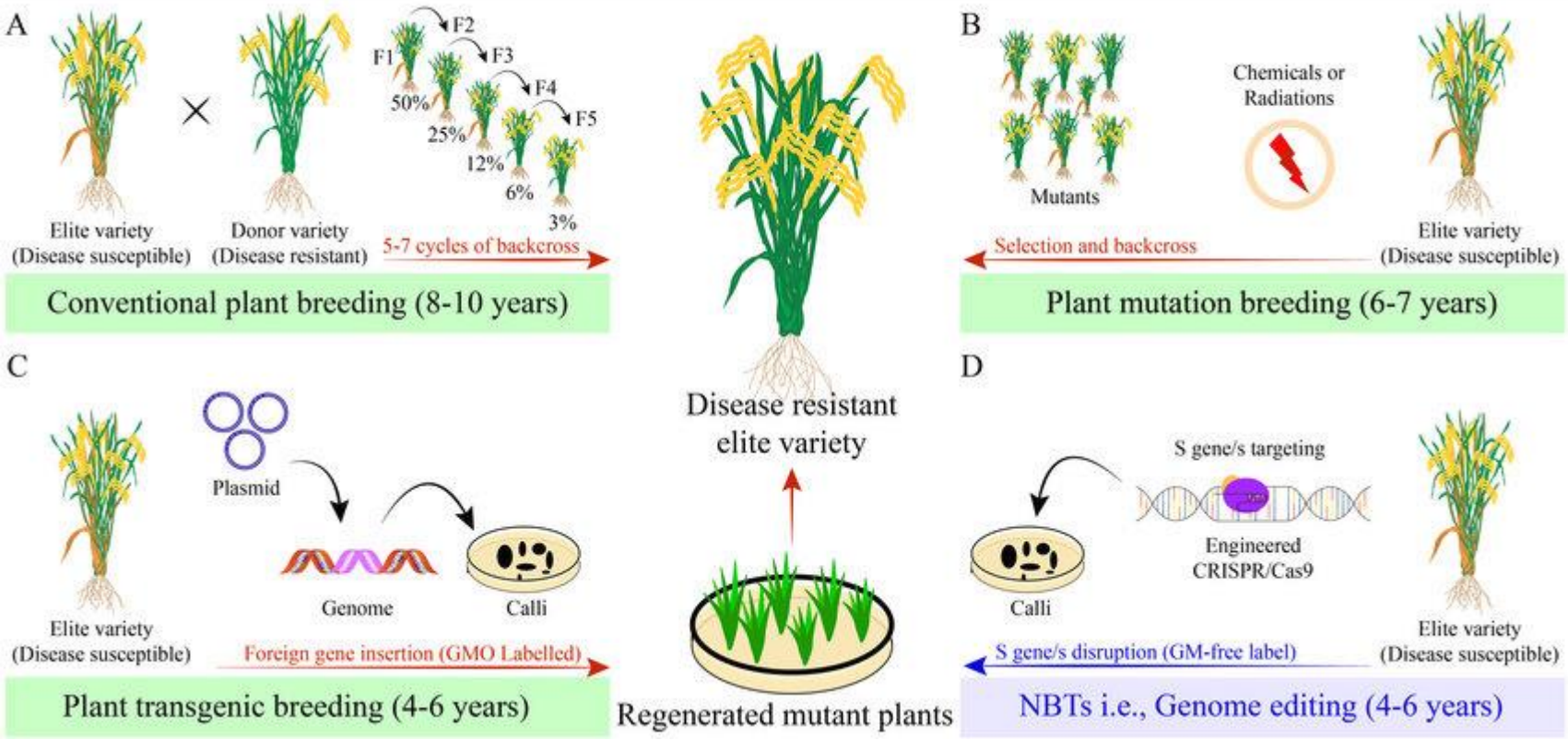


Gene variations that might help them **resist a new challenge** — say a new disease or pest, drought, or a different habitat

Genomics for Crop Improvement




Cultivars, Landraces, Wild relatives
T-DNA, Transposones, Irradiation, Chemicals
RNAi, amiRNA, CRISPR-Cas
Wild type
Mutant
Rice Germplasm/Mutant Recourse
Integrated Studies
Phenomics
Metabolomics
Proteomics
Transcriptomics
Epigenomics
Genomics
Multidimensional and Multi-omics Investigations
Developmental Stage (Seedling, Vegetative, Reproductive)
Organizational level (Organelle, Cell, Tissue, Organ)
Environmental Condition (Stress, temperature, light)
Big Data Analysis
System Biology
Discovery
Gene network/model for agronomic traits
Validation
Reverse Genetics
Gene
Mutant
Forward Genetics
Functional Genomics
Genetic Improvement
Super Rice


A
Elite variety
(Disease susceptible)
Donor variety
(Disease resistant)
F1
F2
F3
F4
F5
50%
25%
12%
6%
3%
5-7 cycles of backcross
Conventional plant breeding (8-10 years)
B
Mutants
Chemicals or
Radiations
Selection and backcross
Elite variety
(Disease susceptible)
Plant mutation breeding (6-7 years)
Disease resistant
elite variety
C
Plasmid
Genome
Calli
Elite variety
(Disease susceptible)
Foreign gene insertion (GMO Labelled)
Plant transgenic breeding (4-6 years)
Regenerated mutant plants
D
S gene/s targeting
Engineered
CRISPR/Cas9
Calli
Elite variety
(Disease susceptible)
S gene/s disruption (GM-free label)
NBTs i.e., Genome editing (4-6 years)

How Crops Are Genetically Modified

Traditional Breeding

Crossing plants and selecting offspring

Almost All Crops

Desired gene(s) inserted with other genetic material. No safety testing requirements.

Mutagenesis

Exposing seeds to chemicals or radiation

Random changes in genome, usually unpredictable. No safety testing requirements.

RNA Interference

Switching off selected genes with RNA

Targeted gene(s) switched off or 'silenced'. Safety testing required.

Transgenics

Inserting selected genes using recombinant DNA methods

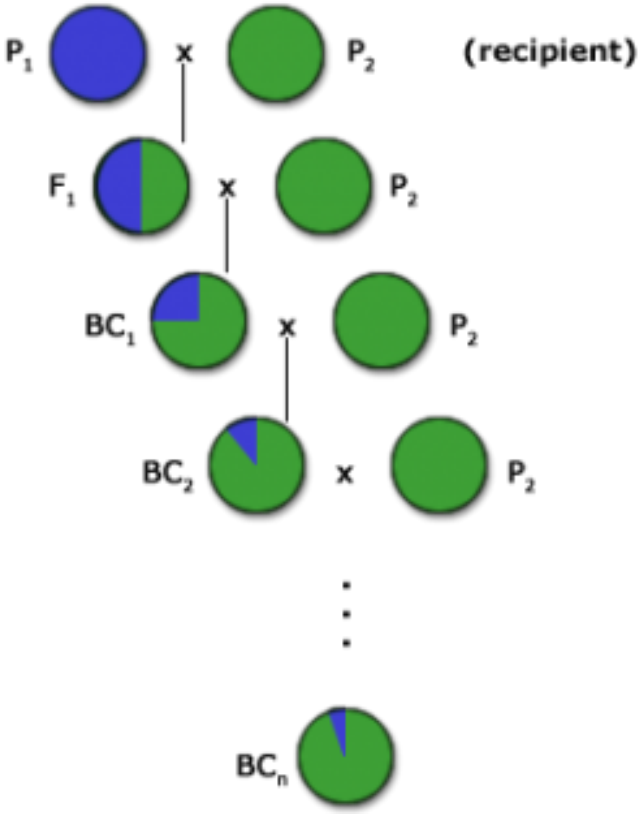
Desired gene(s) inserted only at known locations. Safety testing required.

Number of Genes Affected

10K - >300K ? No way to assess **1- 2** **1 - 4**

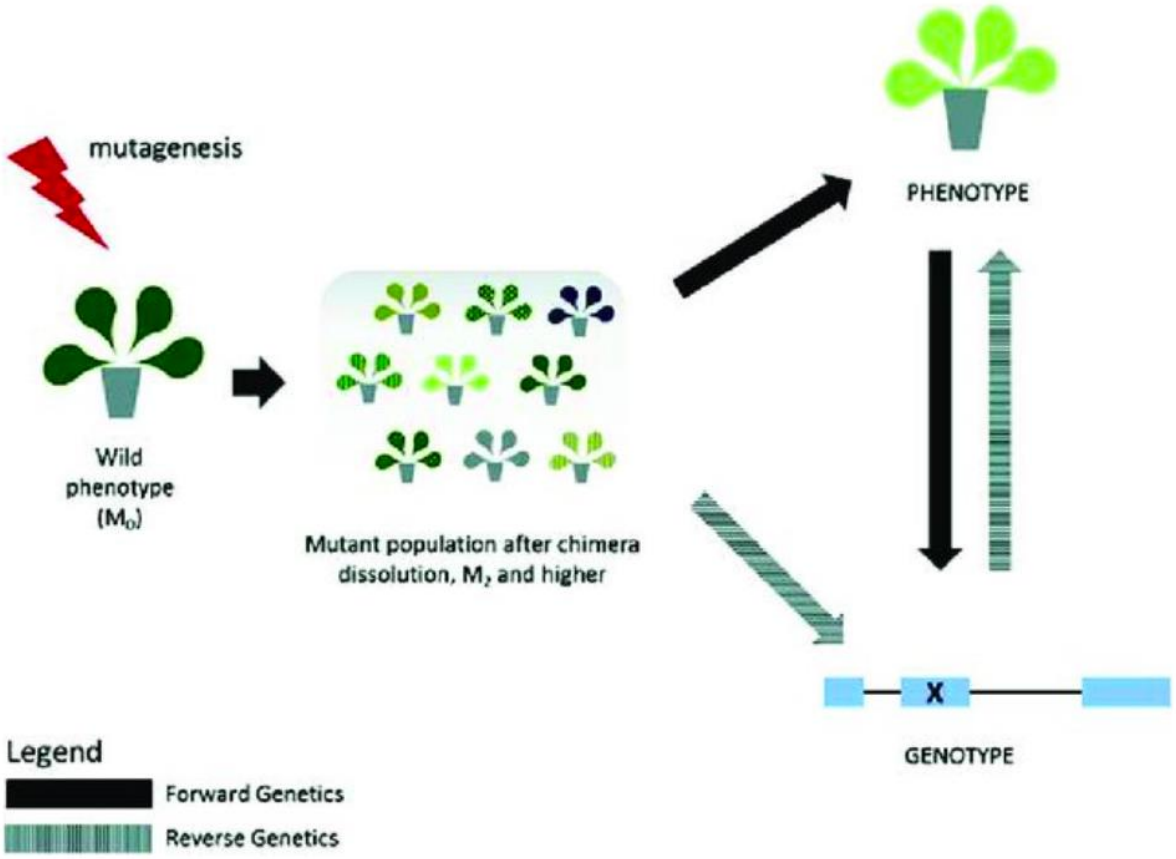
Conventional Breeding takes very long time

Generation	Donor genome (%)
F_1	50
BC_1	25
BC_2	12.5
BC_3	6.75
⋮	
BC_n	$100 \times (1/2)^{n+1}$

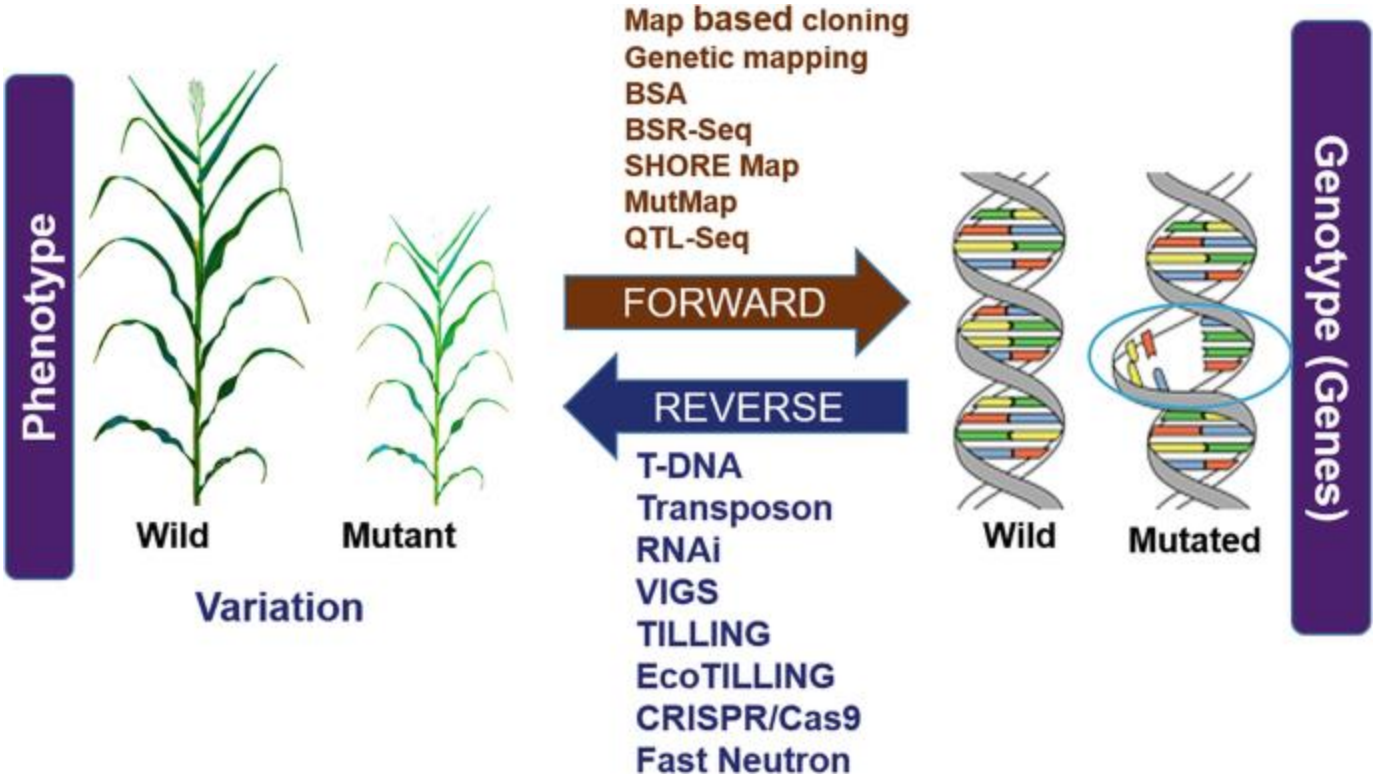


Recurrent backcrossing with the recipient reduces the donor parent genome in each generation by one half

Understanding Gene function through Mutagenesis



Understanding Gene Function –
by Forward and Reverse genetics…..



Forward and Reverse genetics

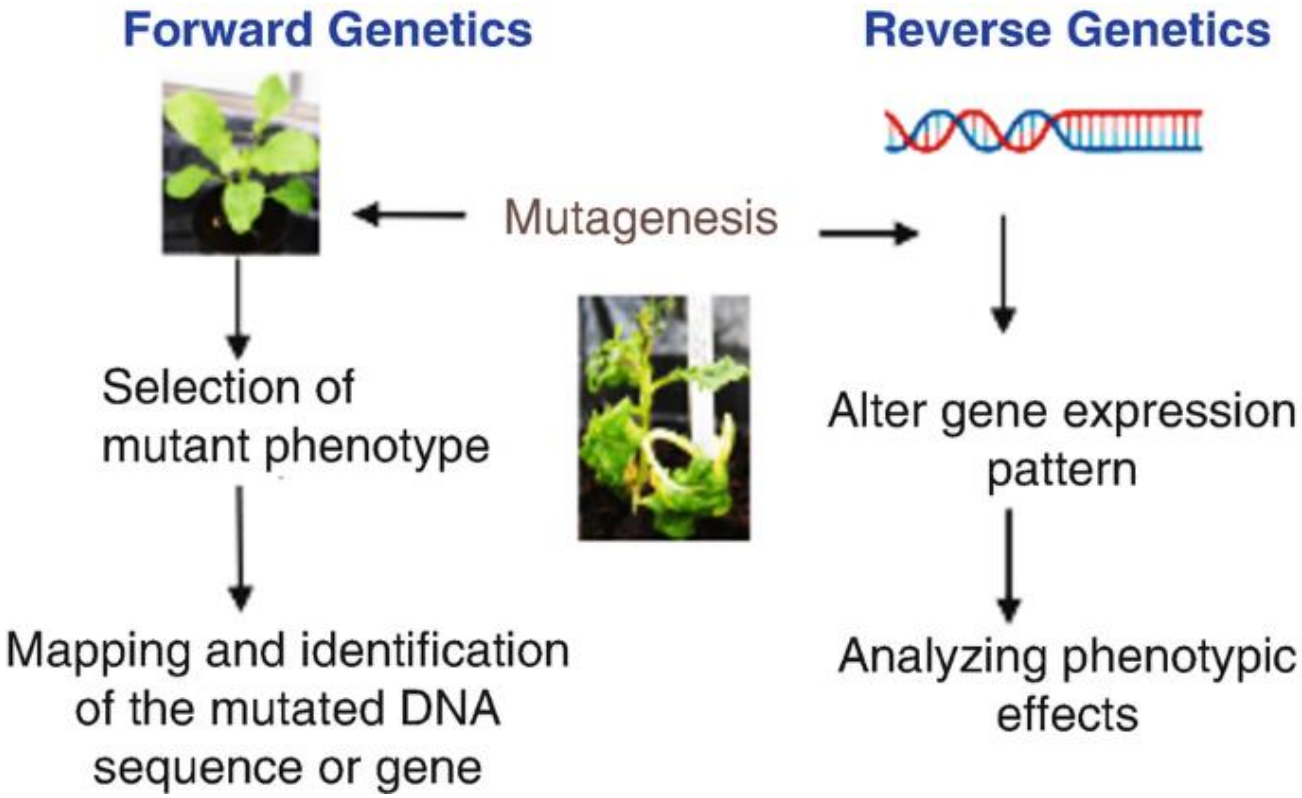
Reverse genetics

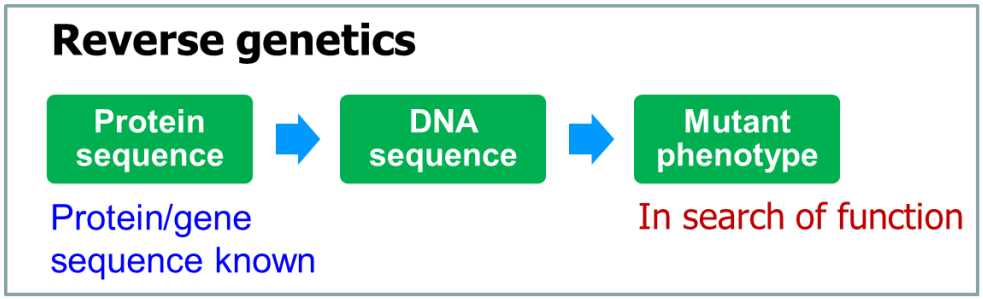
You know the gene sequence,
looking for its function/trait !!!!

Forward genetics

You have a new phenotype or mutant
(due to loss of function or gain of function)
looking for gene sequence !!!!

Forward and Reverse genetics

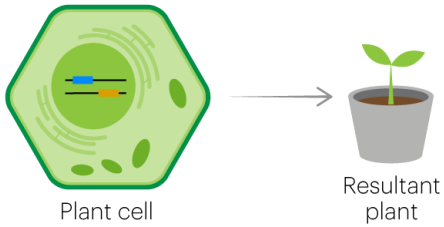




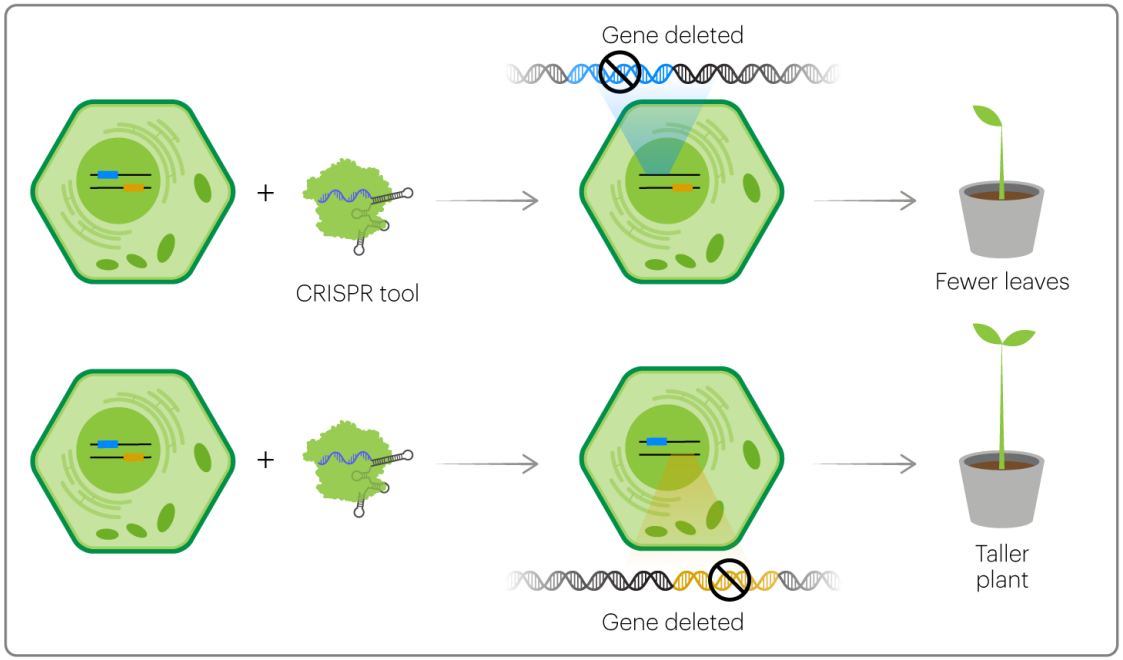
Reverse Genetics to understand gene function

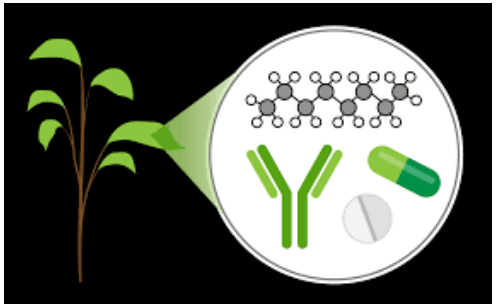
1. Delete a gene **(Knock out)**
2. Over-expression
3. Suppression **(Knock down)**
   - i) Antisense
   - ii) RNAi
   - iii) Transient transgenics: VIGS

Breaking genes to determine what they do (Knock out)

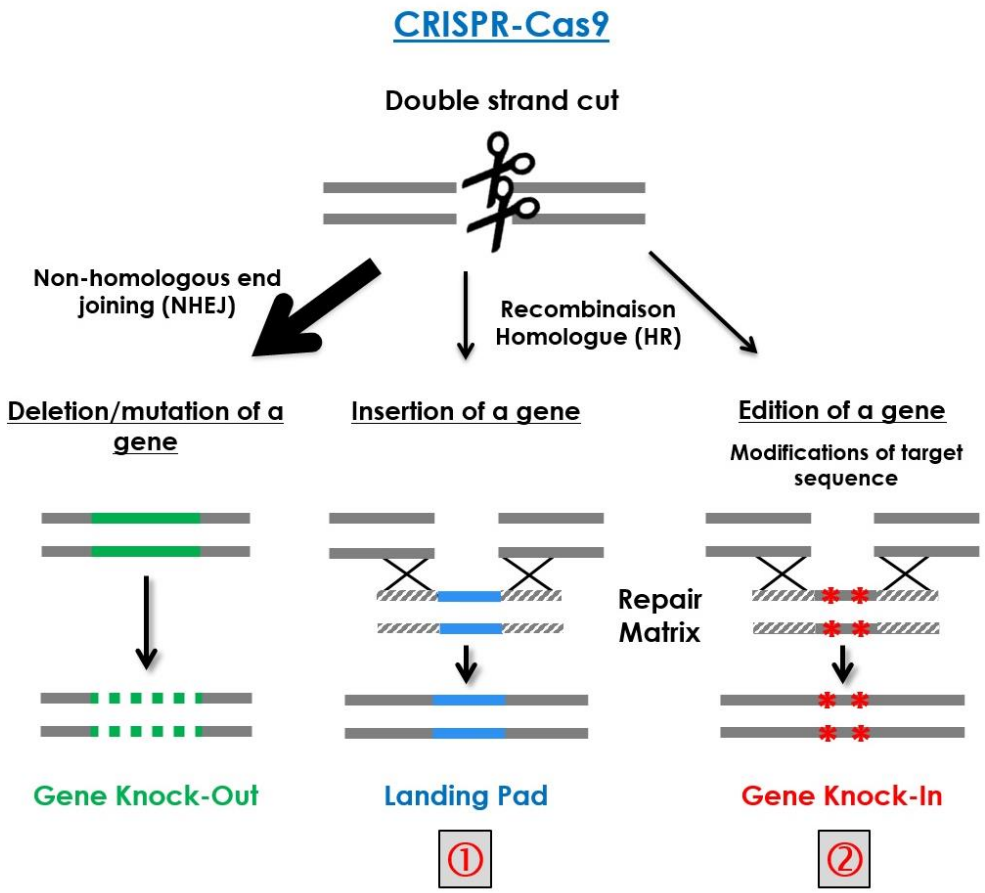


Breaking genes to determine what they do





Targeted Knock-out by CRISPR-Cas



The CRISPR system allows to **generate double-strand DNA cuts in the genome**. These cuts can be repaired in two ways:

1) Non-homologous end joining (NHEJ), the most frequent technique used to **efficiently generate a "knock-out" in a gene** in order to render it nonfunctional.

2) Homology-directed repair (HR), a less frequent technique used effectively to provide a repair template whose extremities present sections of homology with the target DNA.

Helps in the **insertion of a gene**, precisely positioned at the desired location (**landing pad**) on the genome.

It is possible to **insert a repair template** with small but very precise modifications to the sequence. This template becomes a model/dressing for the repair. With this technique (**called "knock-in**"), it is possible to introduce small modifications in the genome."

CRISPR-Cas9, is based on the use of a **protein – Cas9, an enzyme** used by bacteria to protect themselves against viral aggression. On introducing, this **enzyme acts like a pair of scissors**, cutting the DNA and then repairing it. This cut – or genome editing – is **carried out within specific sections** that are "recognized" by a particular section of RNA (guide RNA).

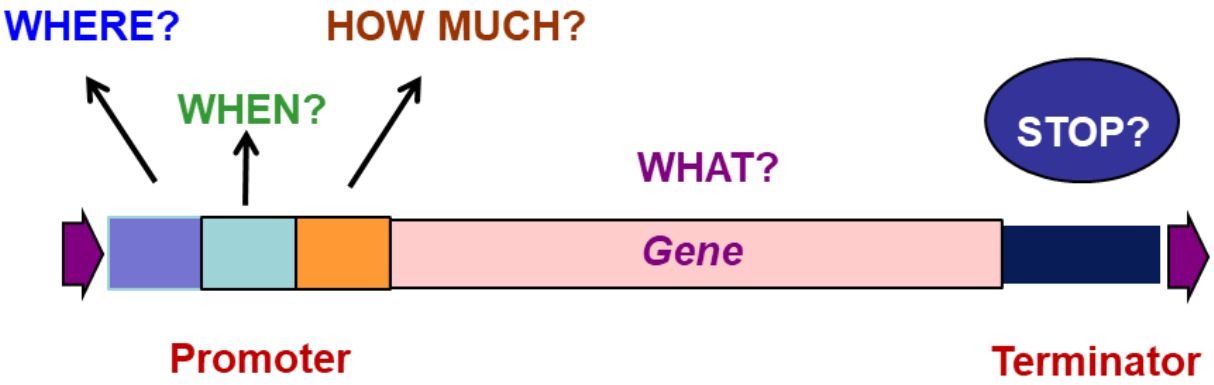
Turn-on or Turn-off genes to know what they do

(Over-expression and Suppression)

Modulate the gene activity by

- **Decrease** ↓ Suppression **(Antisense/RNAi/VIGS)**
- **Increase** ↑↑↑ Over-expression

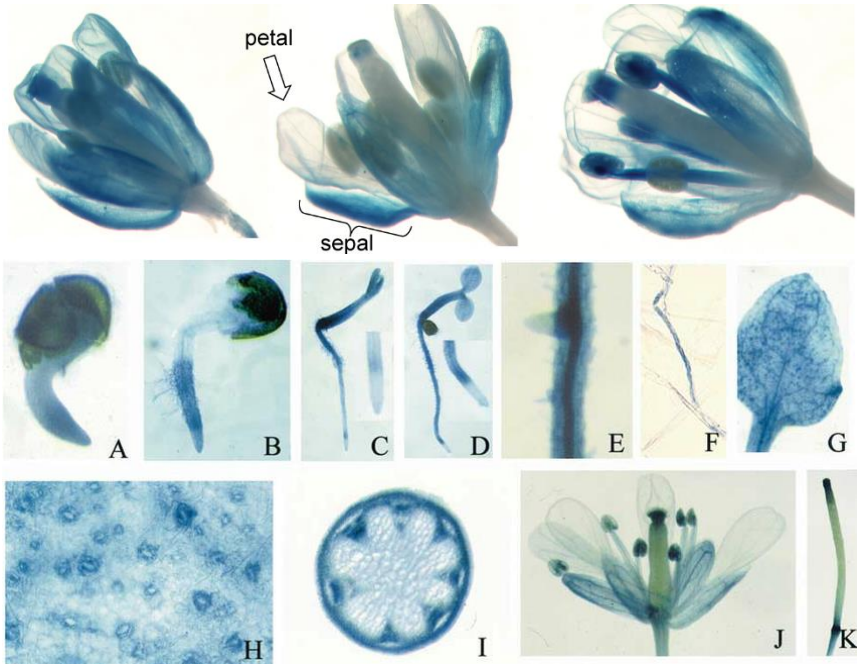
Over-express a gene using a promoter of choice

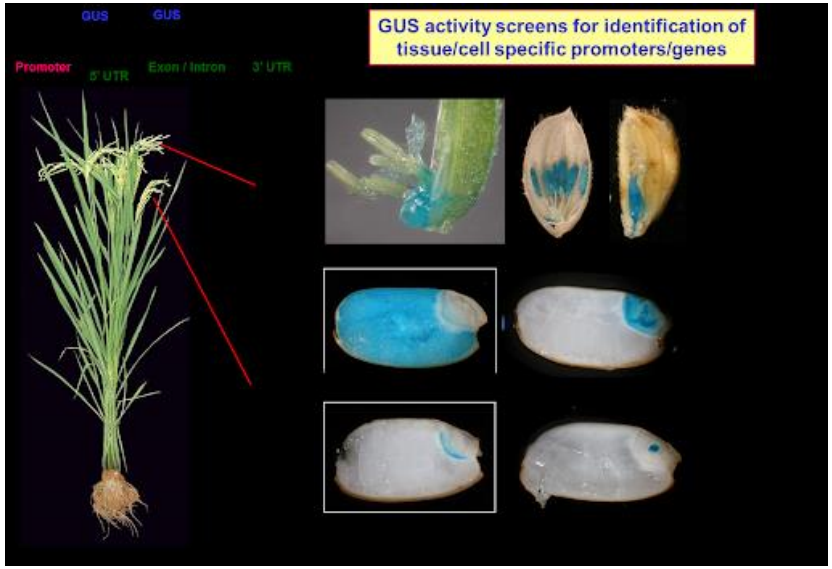


Promoter regulates **level (strength)** and **pattern (timing and tissue type)** of gene expression

- Analyze the over-expressed plants to see if any change in phenotypes
- Relate the phenotype change with biological processes

Analyze the over-expressed plants to see your gene activity



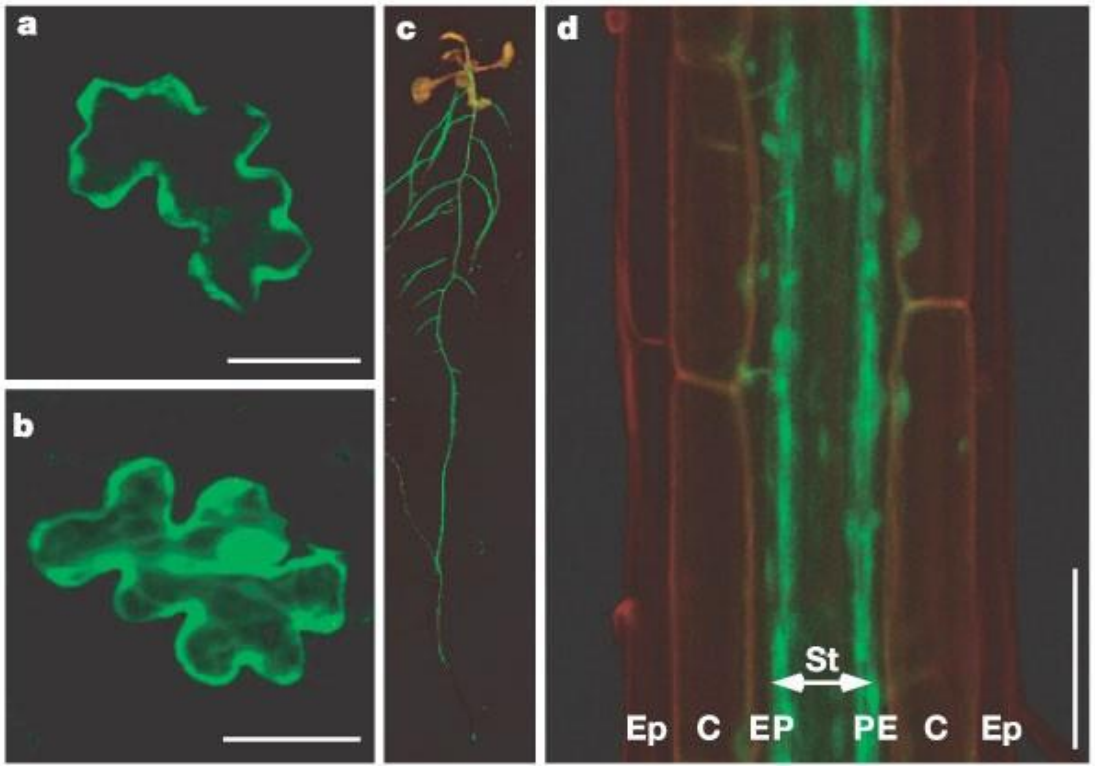


ETH zürich
Department of Biology
Institute of Molecular Plant Biology

Tissue-specific promoter library for cassava

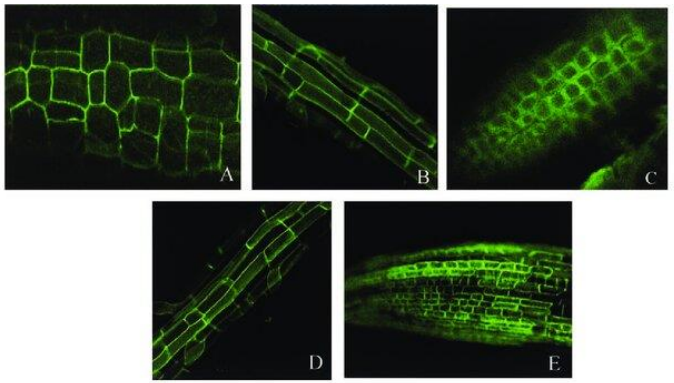
Generating a tissue-specific promoter library for cassava.
Images show transgenic cassava with different promoters driving a *GUS* reporter gene (expression stained in blue).

Analyze the over-expressed plants to see your gene activity

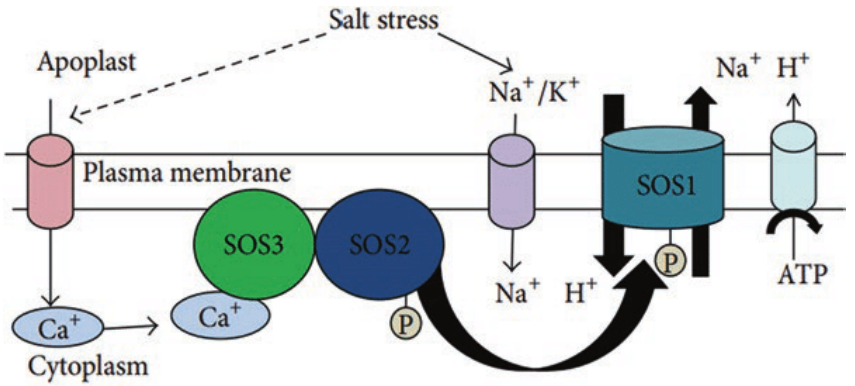


Subcellular localization of BOR1 and cell-type-specific localization of *BOR1* expression in Arabidopsis.

Task: Know the function of SOS1 gene
by over-expression and knock out in a model plant

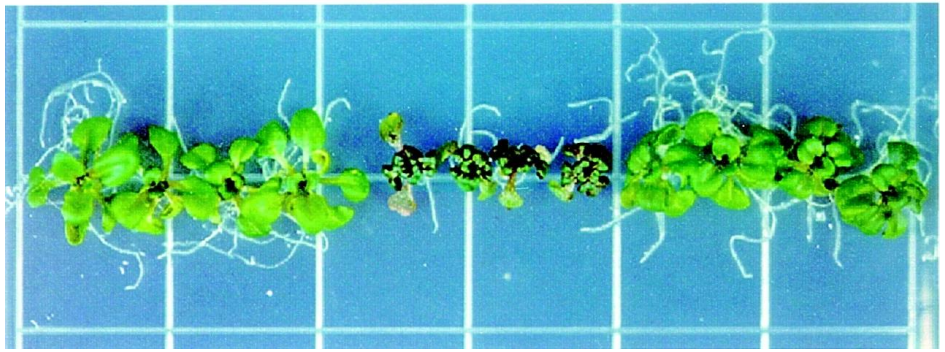


Subcellular Localization of the **SOS1-GFP fusion protein** in root cells of Arabidopsis plants overexpressing SOS1-GFP.



***SOS1* Encodes a Putative Na^+/H^+ Antiporter**

WT *sos1-1* *35S-SOS1 sos1-1*



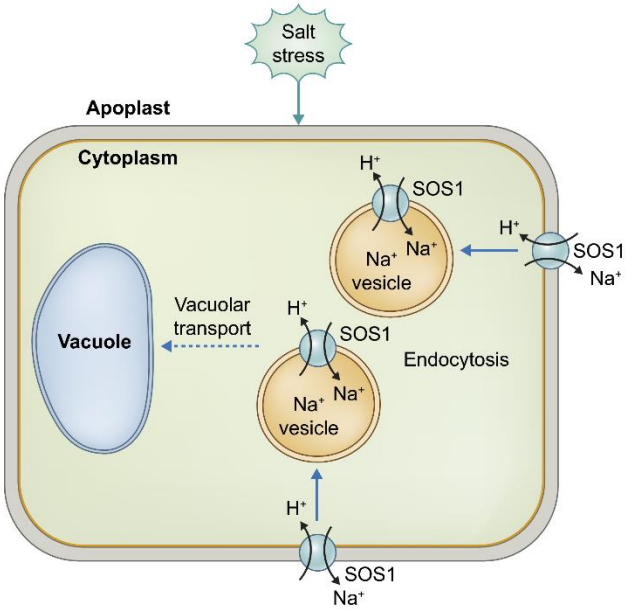
Complementation of *sos1* by 35S-*SOS1*: Seedlings in nutrient medium supplemented with **100 mM NaCl** after **10 days of treatment**.

(*Left*) **Wild-type plants (WT)**. (*Center*) ***sos1-1* mutant plants.** (*Right*) **Transgenic *sos1-1* plants containing the wild-type *SOS1* gene** under control of the strong constitutive promoter from cauliflower mosaic virus 35S.

SOS1 gene codes for a Plasma Membrane Na^+/H^+ exchanger

Soil salinity is a major problem in agriculture. Most plant/crop species are glycophytes, which are not salt-tolerant and are adversely affected by high salt. Salt stress is commonly caused by high concentrations of sodium (Na^+) and chloride ions in soil. Na^+ diffuse into plant cells and high concentrations of Na^+ in the cytoplasm disrupt the uptake of other ions such as potassium (K^+) into plant cells, which adversely impacts K^+ nutrition, catalytic activities of cytosolic enzymes, photosynthesis, and metabolism.

Three mechanisms function cooperatively to prevent the accumulation of Na^+ in the cytoplasm, i.e., restriction of Na^+ influx, active Na^+ efflux, and compartmentalization of Na^+ in the vacuole. The Na^+ efflux is carried out by SOS1 gene encoded plasma membrane Na^+/H^+ antiporters. Proton motive force created by H^+-ATPases would drive Na^+ efflux from plant cells through plasma membrane Na^+/H^+ antiporters.



In *Arabidopsis thaliana*, the *SOS1* (*S*alt *O*verly *S*ensitive *1*) locus is essential for Na^+ and K^+ homeostasis, and *sos1* mutations render plants more sensitive to growth inhibition by high Na^+ and low K^+ environments. *SOS1* gene expression in plants is up-regulated in response to NaCl stress.

Understanding Gene Function

Gene sequence known, function not known?

To determine the function of a specific gene, **we ask many fundamental questions** such as

- the gene expression **pattern**,
- **localization** of specific proteins,
- **phenotypes** of the plants when a gene is ***over-expressed*** or ***knocked down*** or ***suppressed***

Turn-off genes to know what they do (Suppression)

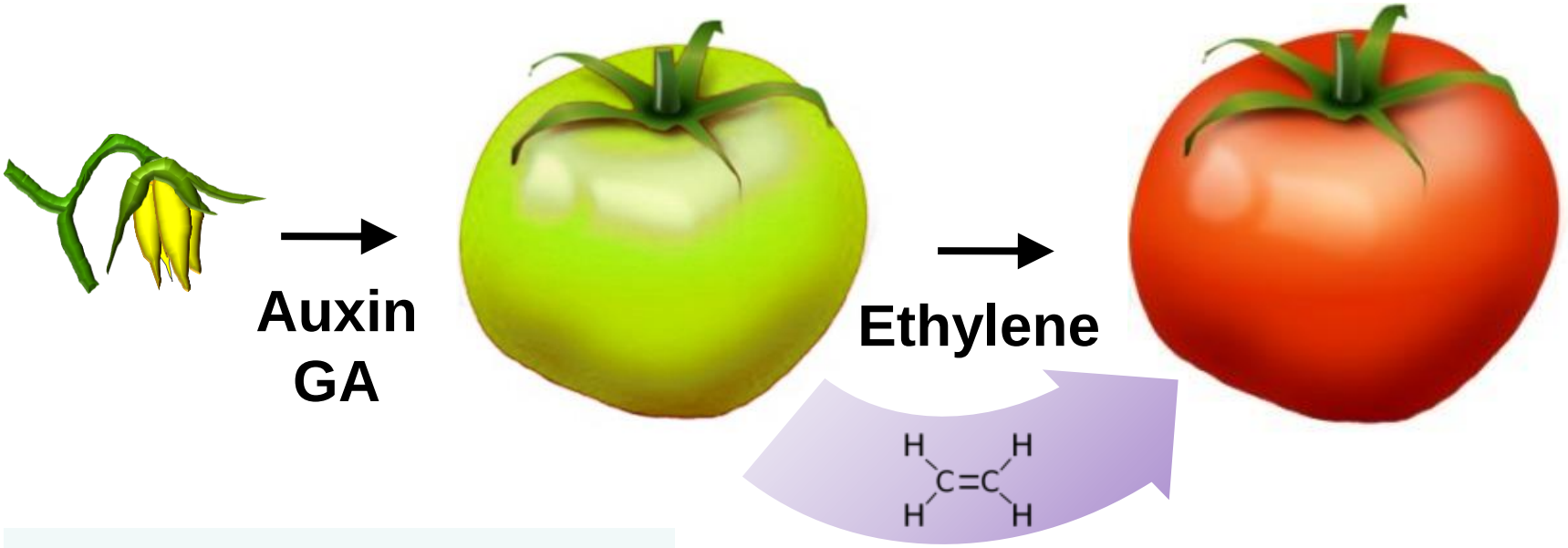
Suppress a gene expression (interfere with transcription)

- Antisense RNA
- RNA interference (RNAi)
- VIGS

Antisense RNA

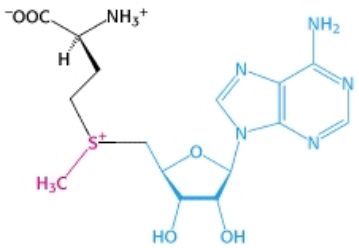
- Introduce full length complementary RNA
- Forms double-stranded RNA in cells

Fruit ripening is induced by ethylene



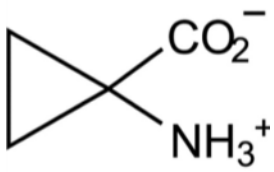
Ethylene is a gaseous hormone that promotes fruit softening and flavor and color development

Molecular approaches to limit ethylene synthesis



S-adenosyl methionine

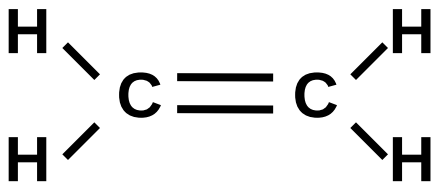
ACC synthase →



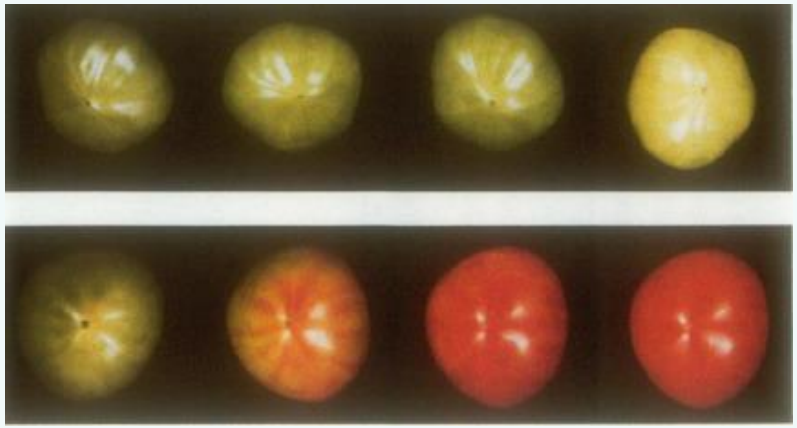
ACC

(1-aminocyclopropane-1-carboxylic acid)

ACC oxidase →



Ethylene

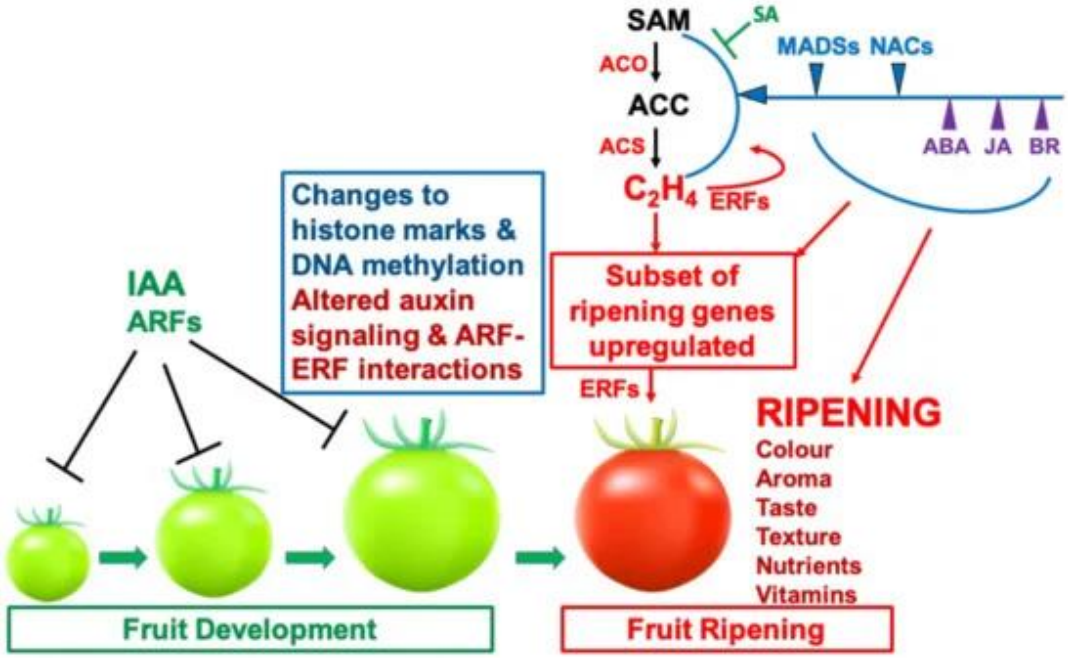


Antisense ACC synthase

Control

Introduction of antisense constructs **to interfere with expression of biosynthesis enzymes (such as ACC synthase)** is an effective way to control ethylene production.

Molecular approaches to limit ethylene synthesis



Fruit softening during ripening is, at least partly, due to cell wall changes catalysed by wall-modifying enzymes, there are at least 10 such cell wall-modifying enzymes that are expressed during ripening.

The ripening hormone, ethylene is known to initiate, modulate and co-ordinate the expression of various genes involved in the ripening process.

The burst in ethylene production is the key event for the onset of ripening in fruits. Therefore ethylene is held accountable for the tons of post-harvest losses due to over-ripening and subsequently resulting in fruit rotting. Delayed ripening tomatoes could be generated by silencing or suppressing 1-aminocyclopropane-1-carboxylate (ACC) synthase (*ACS*) gene during the course of ripening using RNAi technology. The **RNAi-ACS construct designed to target *ACS* homologs**, effectively **repressed the ethylene production in tomato fruits**. Fruits from such lines exhibited delayed ripening and extended shelf life for ~45 days, with improved juice quality.

Gene suppression by RNAi

- Double-stranded RNA able to disrupt gene expression

  Cells have machinery that destroy double-stranded RNA: viruses/cDNA

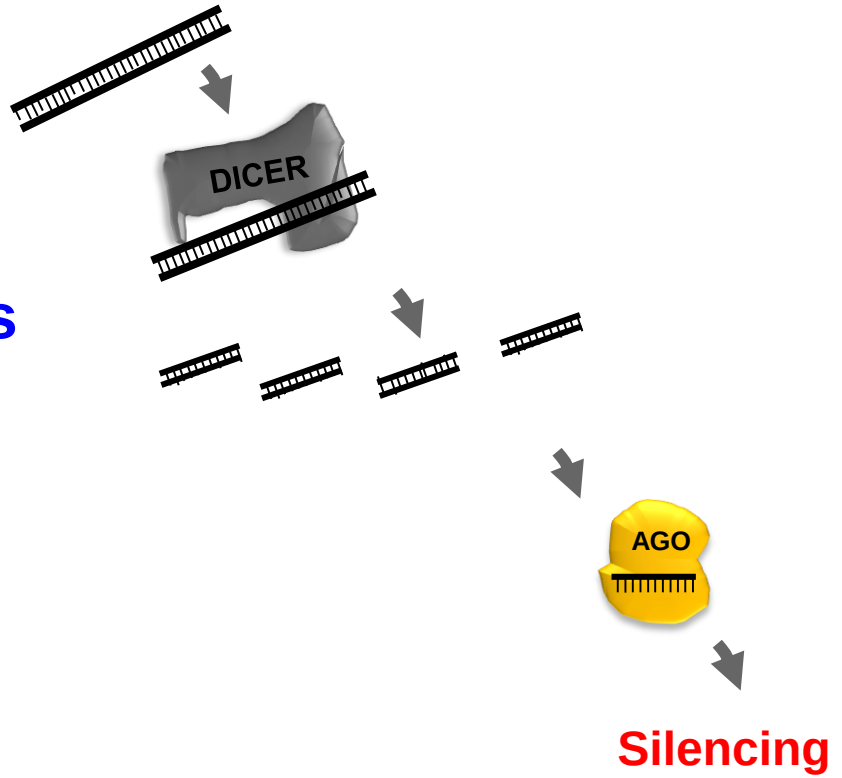
- Appears to be basis for the RNA interference

  when double-stranded RNA introduced into cells

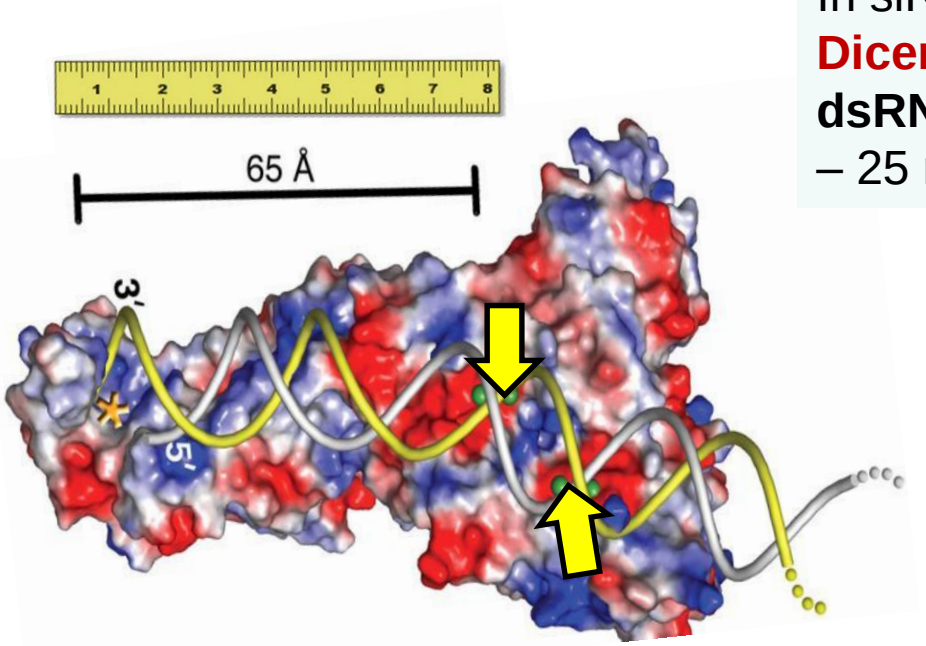
The core of RNA silencing: Dicers and Argonautes

RNA silencing uses a set of core reactions in which **double-stranded RNA (dsRNA)** is processed by **Dicer** or **Dicer-like proteins** into **short RNA duplexes**.

These small RNAs subsequently associate with **ARGONAUTE** proteins to confer silencing.



Dicer and Dicer-like proteins

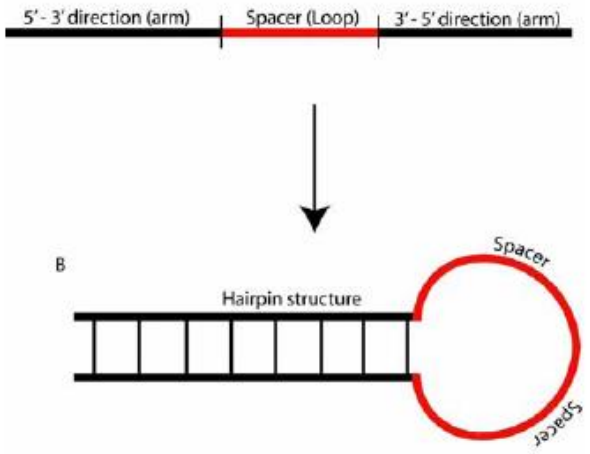


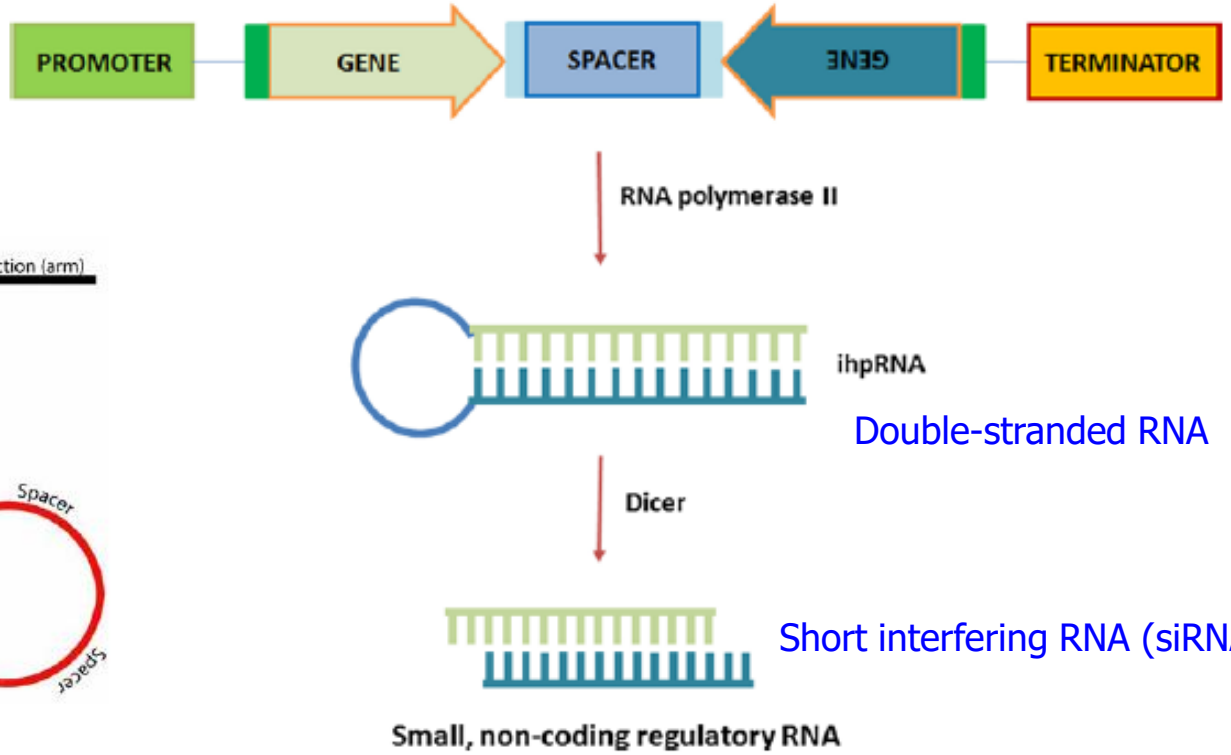
In siRNA and miRNA biogenesis, **Dicer or Dicer-like (DCL) proteins cleave long dsRNA** or **foldback (hairpin) RNA** into ~ 21 – 25 nt fragments.

Dicer's structure **allows it to measure the RNA** it is cleaving. Like a cook who "dices" a carrot, **Dicer chops RNA into uniformly-sized pieces**.

From MacRae, I.J., Zhou, K., Li, F., Repic, A., Brooks, A.N., Cande, W.., Adams, P.D., and Doudna, J.A. (2006) Structural basis for double-stranded RNA processing by Dicer. Science 311: 195 -198. Reprinted with permission from AAAS. Photo credit: Heidi

Silencing a gene by introducing its inverted-repeat (IR) sequences





RNA Interference-based Pesticides and Antiviral agents

Microbial overproduction systems for dsRNA for applications in Agriculture and Aquaculture

Research Institute for Bioscience Products & Fine Chemicals, **Ajinomoto Co. Inc.**, Kanagawa, Japan
Institute for Open Innovation, **Kobe University**, Japan
Research and Development Center for Precision Medicine, **University of Tsukuba**, Japan