

Prof. Dr. Boas Pucker
PBPM-BP-06

Availability of slides

- All materials are freely available (CC BY) - after the lectures:
 - eCampus: PBPM0 - Plant Biochemistry, Physiology and Molecular Biology (LEC)
 - GitHub: <https://github.com/bpucker/teaching/PBPM>
- Questions: Feel free to ask at any time
- Feedback, comments, or questions: [pucker\[a\]uni-bonn.de](mailto:pucker[a]uni-bonn.de)



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- Specialized metabolites
- Flavonoids: flavonols, proanthocyanidins, anthocyanins
- Betalains
- Terpenoids
- Glucosinolates

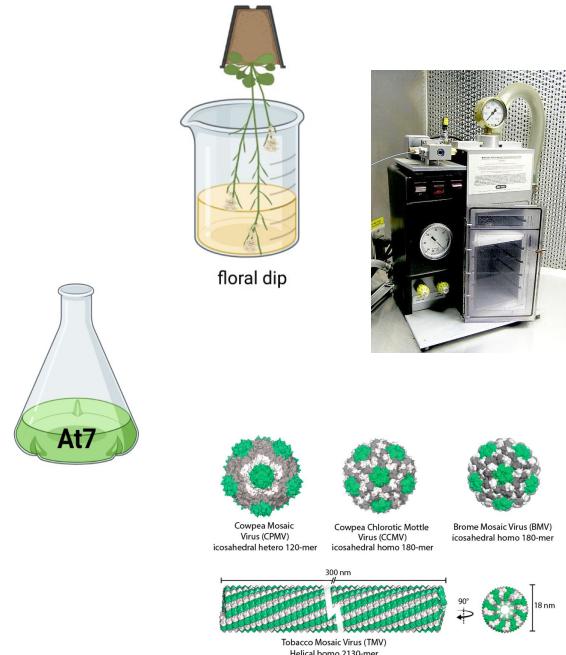
Purple tomato

- MYB+bHLH overexpression in tomato to boost anthocyanin formation
- Production of anthocyanin-rich juice
- Available in North America (not in EU)



How to transform a plant?

- Agrobacterium-mediated
- Particle gun
- Protoplast transformation
- (Viruses)

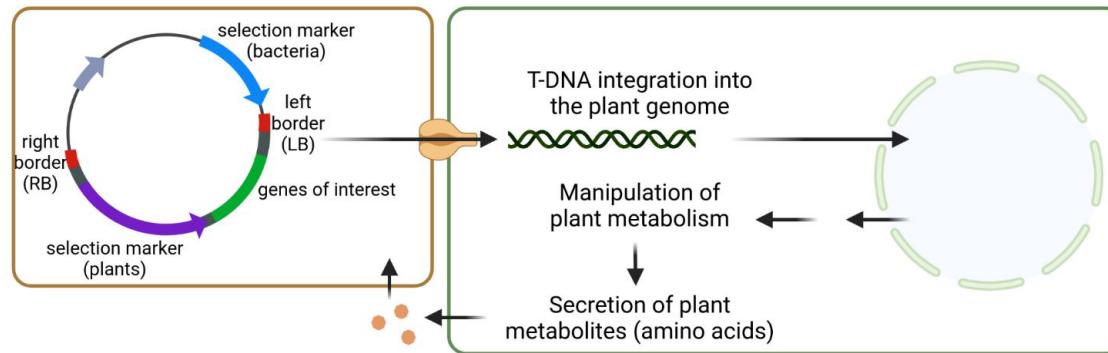


Agrobacterium tumefaciens

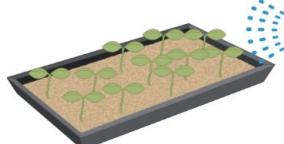
- Soil-borne, gram-negative α -proteobacterium that infects wounded plants
- Causes crown gall disease by transferring a segment of Ti (tumor-inducing) plasmid (T-DNA) into plant nuclear genomes
- T-DNA transfer mechanism resembles Type IV secretion system
- Induces host cellular reprogramming (tumor formation) and synthesis of opines



Floral dip



potentially transgenic seeds



selection of transgenic plants



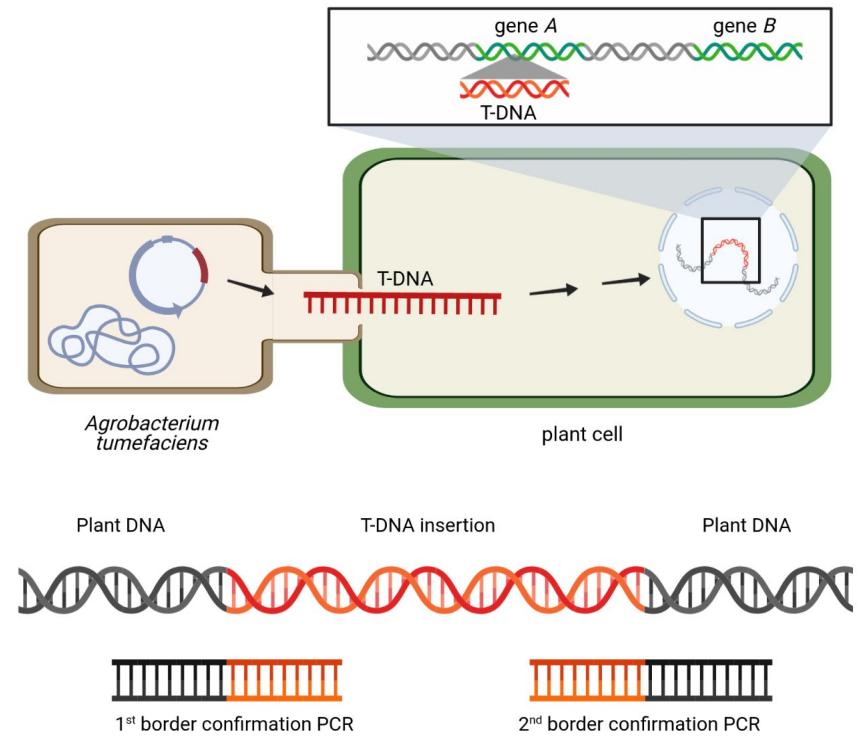
genotyping of plants



stable transgenic lines

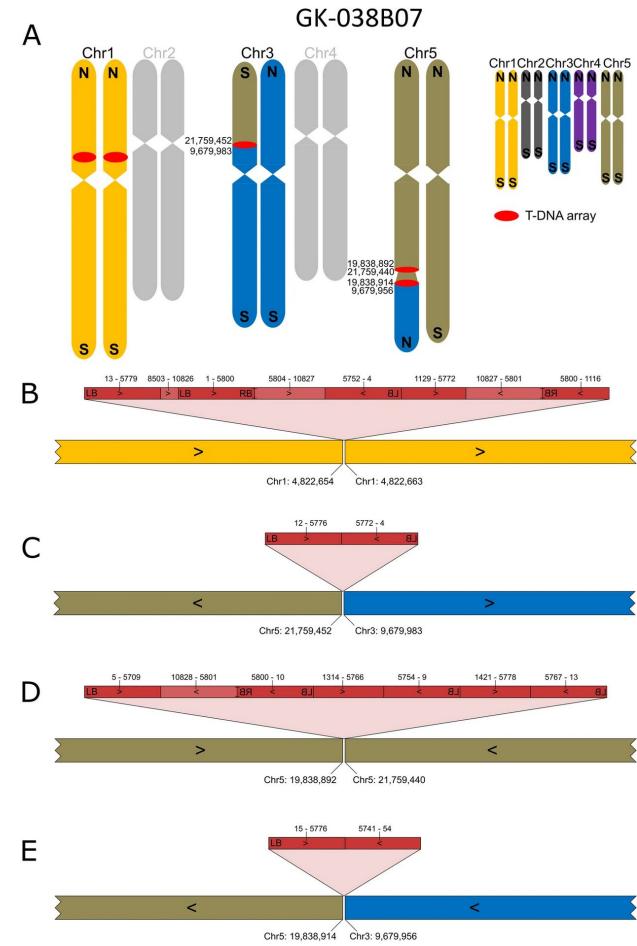
Arabidopsis T-DNA lines

- Integration of DNA in the genome is almost random (no effective homologous recombination)
- T-DNA can disrupt genes
- T-DNA carries marker for selection of transgenic plants



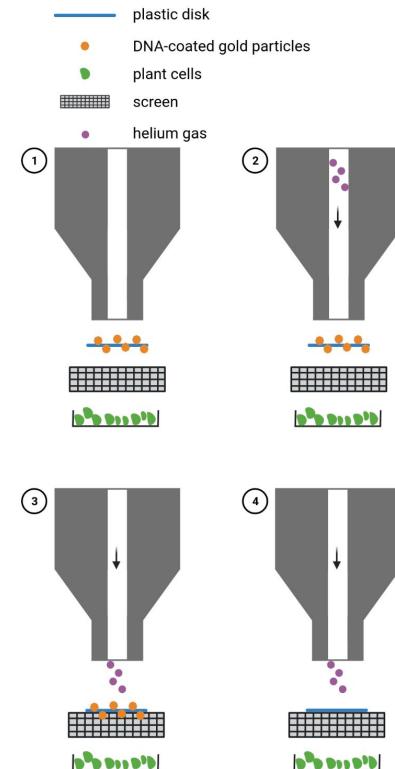
Long read genotyping

- Multiple T-DNA insertions per line are possible
- Off-targets can cause biases conclusions
- Multiple T-DNA copies inserted per locus
- T-DNA insertions can cause structural variants
- Efficient characterization with long reads possible

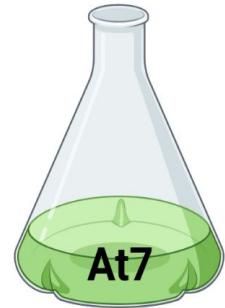


Particle gun

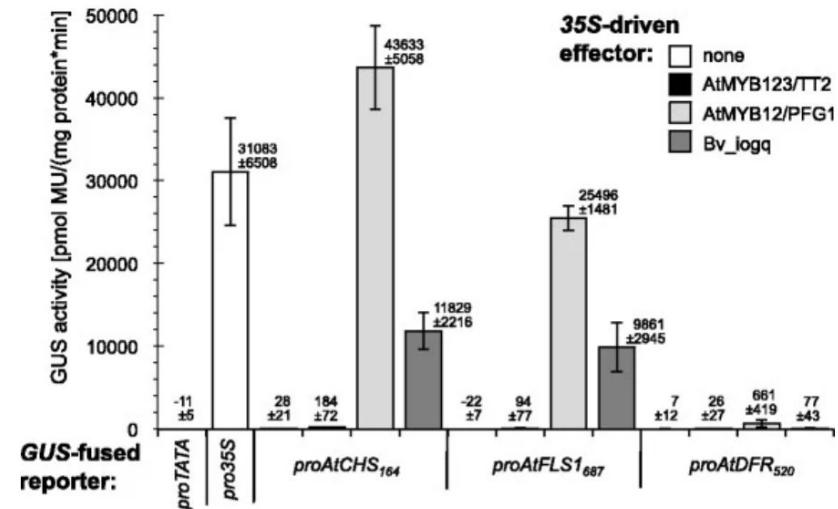
- Shooting DNA into plant cells
- Applicable to all plant species
- Optimization of conditions required



Protoplast transfection



- *Arabidopsis thaliana* suspension cell culture line At7 was established in 1995
- Removal of cell wall with enzyme cocktail (cellulase + macerase)
- Transfection of plasmid DNA into protoplast for assays
- Testing TF-promoter interactions in planta

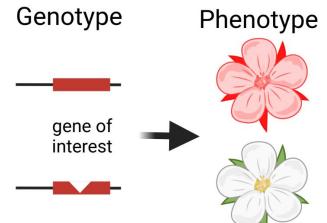


Viruses: VIGS (Virus-Induced Gene Silencing)

- Exploits plant's antiviral RNA interference (RNAi) pathway
 - dsRNA is recognized and processed into siRNAs
- Modified virus carries fragment of plant gene
- Sequence-specific knockdown, not knockout (gene expression reduced)
- Silencing is typically systemic (spreading via movement of virus and siRNAs)
- Allows rapid analysis of gene function without stable transformation

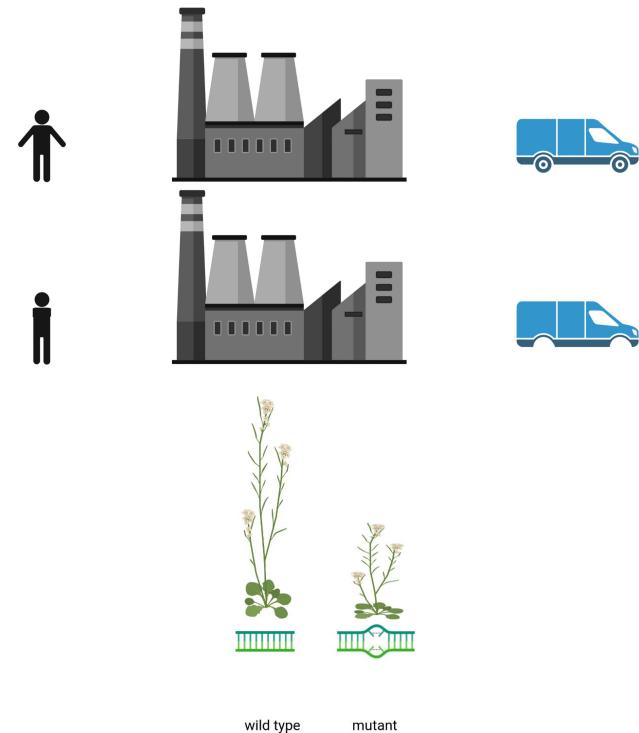
Why to transform a plant?

- Explore gene functions
- Boost nutritional value (e.g. via transcription factors)
- Conferring resistances (against herbicides)
- Enhancing resilience (against abiotic stress)



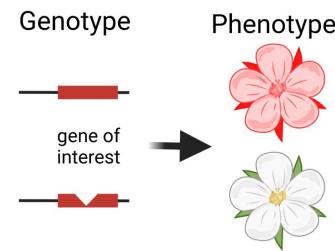
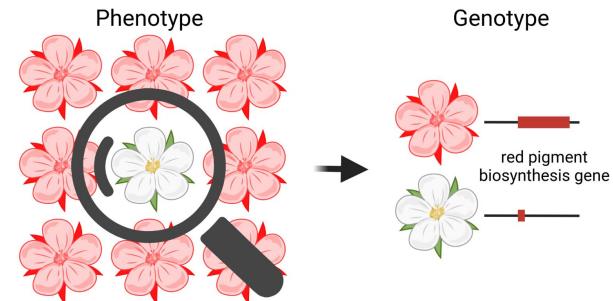
Exploring gene functions

- How would you find out what a car factory worker is doing?
- Bind/remove hands and arms and see how the product differs from the normal product
- Transfer to gene: knock-out a gene and see how the plant looks different compared to a wild type



Forward and reverse genetics

- Forward genetics = Interesting phenotype is observed and responsible gene is identified in the next step
 - Examples: transposon mutagenesis, ethyl methanesulfonate (EMS), radiation
- Reverse genetics = Finding the function of a known gene through targeted mutation of this gene
 - Examples: Transfer-DNA (T-DNA) collections, CRISPR-Cas



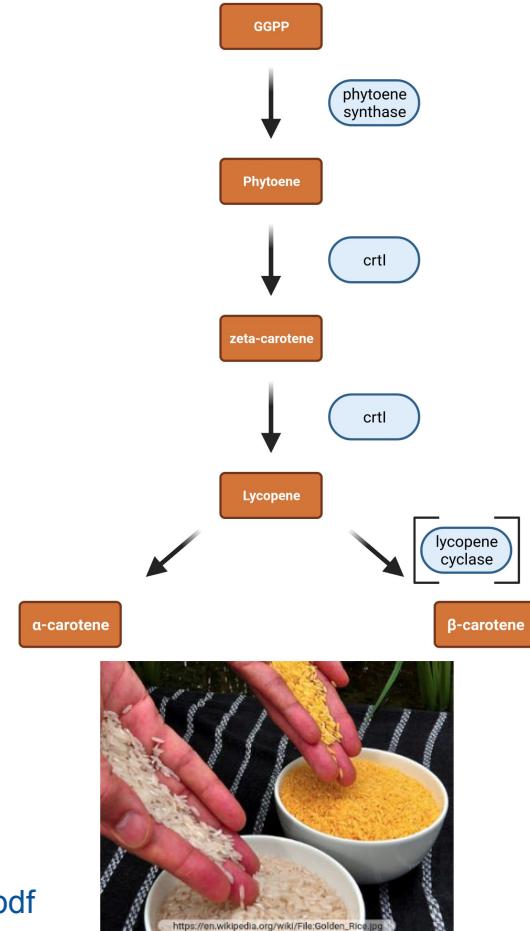
Example: boosting petunia pigmentation?

- Discovery of antisense transcripts by expressing *Chalcone Synthase (CHS)* heterologously in petunia
- Pigmentation was not enhanced, but reduced by RNA-mediated gene silencing (50x reduced *CHS* transcript)



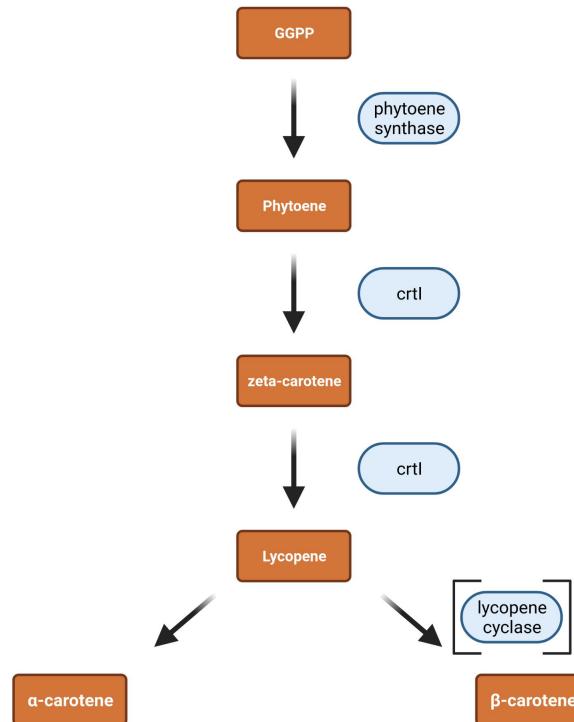
https://commons.wikimedia.org/wiki/File:Petunia_Hybrid_Bi_Color.jpg

- Vitamin A deficiency causes numerous illnesses (in developing countries)
- Rice was engineered to increase the β -carotene content (provitamin A):
 - psy = phytoene synthase (*Narcissus pseudonarcissus*)
 - crtI = phytoene desaturase (*Erwinia uredovora*)
 - lcy = leucopene cyclase (already present)



More details about vitamin A: <https://github.com/bpucker/teaching/blob/master/VitaminA.pdf>

- General considerations:
 - Optimization of gene expression in tissues (endosperm)
 - Stability of system over time
 - Genomic integration locus should not influence other traits
- Golden Rice 2:
 - Selection of effective enzymes (maize psy is more efficient)
 - >20x increase in carotenoid production



Transgenic plants: examples

- **Flavr Savr tomato** (anti-mush tomato): Down-regulation of polygalacturonase expression by antisense RNA (prevents cell-wall degradation)
- **Amflora potato**: down-regulation of starch synthase leads to only branched amylopectin (α -1,4 + α -1,6 glycosidic bonds); better for production of paper, textiles, and adhesives
- **HB4 wheat**: integration of *HB4* gene from sunflower using *Agrobacterium tumefaciens*; TF which increases drought resilience



Herbicide resistances



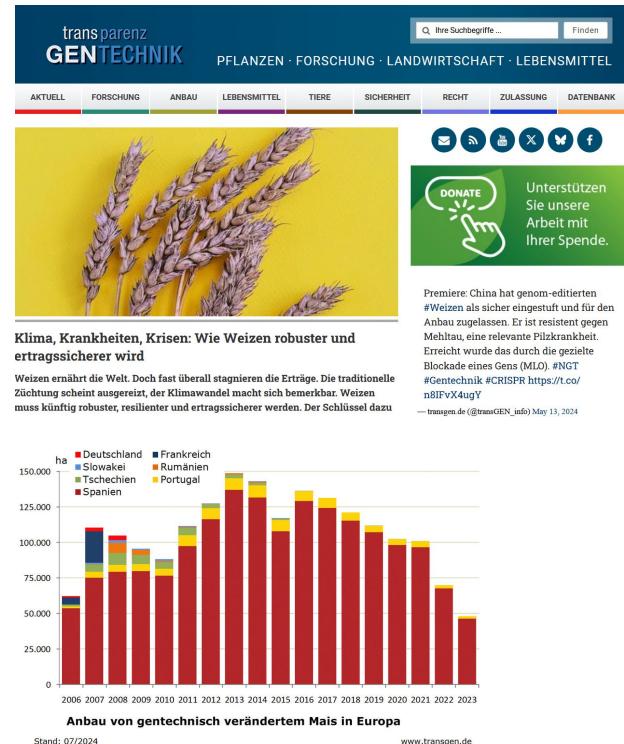
- Roundup Ready Soy (Monsanto/Bayer):
 - Glyphosate normally inhibits EPSPS, blocking aromatic amino acid synthesis
 - Resistance via bacterial EPSPS gene not inhibited by glyphosate
 - EPSPS = 5-enolpyruvylshikimate-3-phosphate synthase
- Phosphinothricin / Glufosinate (Basta):
 - Natural product from *Streptomyces viridochromogenes*; inhibits glutamine synthetase
 - Resistance via BAR/PAT gene → detoxifies herbicide by N-acetylation
- Both herbicides: cheap, used for decades, non-selective
- Soy, cotton, and maize with herbicide resistance (cultivation since 1996)
- Current debate: Glyphosate; no solid evidence for danger to health

Insect repellence (Bt maize)

- *Bacillus thuringiensis* produces Cry (Bt) proteins toxic to insect larvae
- Alkaline insect gut conditions activate toxin that lyses epithelial cells
- Safe for mammals, because Bt toxins are degraded in acidic stomach
- Bt toxins are plasmid-encoded (exchanged between bacterial strains)
- >170 Bt toxins exist, each with different insect specificities
- Bt toxins degrade quickly in the environment
- Some larvae are inaccessible to sprays (e.g., European corn borer)
- GMOs produce Bt toxins themselves via bt genes (internal pest protection)



- Informative regarding presented examples and other approaches in research and application:
www.transgen.de
- More examples of useful transgenic plant applications
- Molecular biological techniques such as CRISPR-Cas technology
- Current statistics about cultivation

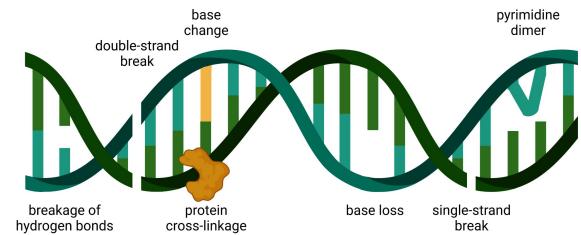
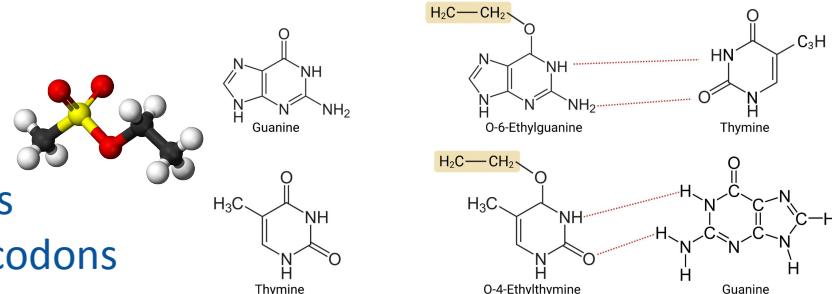


Importance of mutagenesis

- Chemical: EMS mutagenesis
 - EMS = ethyl methanesulfonate
 - Produces a high number of point mutations
 - Genes often disrupted by premature stop codons

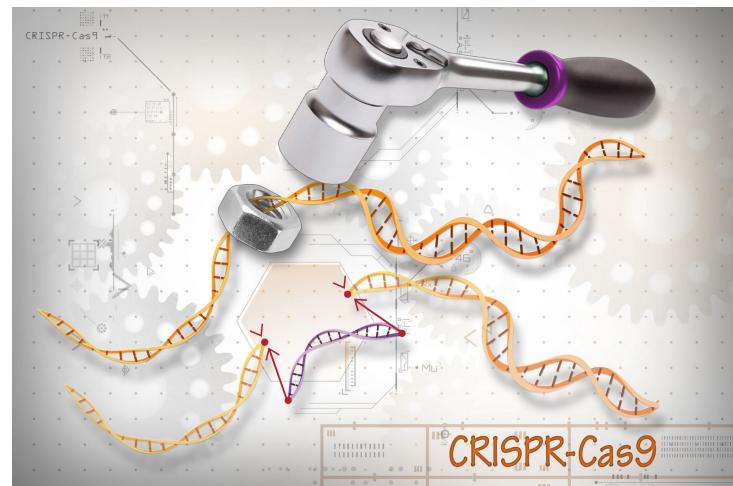
- Physical: Radioactive radiation
 - Generates a high number of mutations
 - Various types of mutations possible

- Biological: Transposons
 - Activation of transposons (“jumping genes”)
 - Example in maize: Barbara McClintock



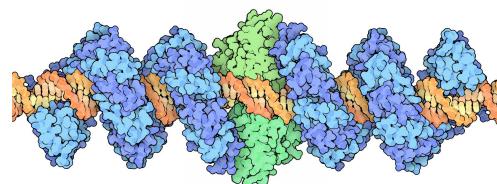
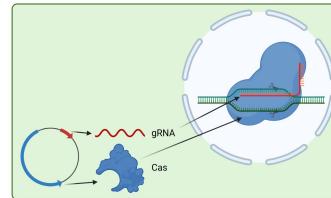
https://en.wikipedia.org/wiki/File:Corn_3different_types.jpg

- Targeted modification of single bases
- Indistinguishable from spontaneous mutations
- Modification of bases (methylation) possible

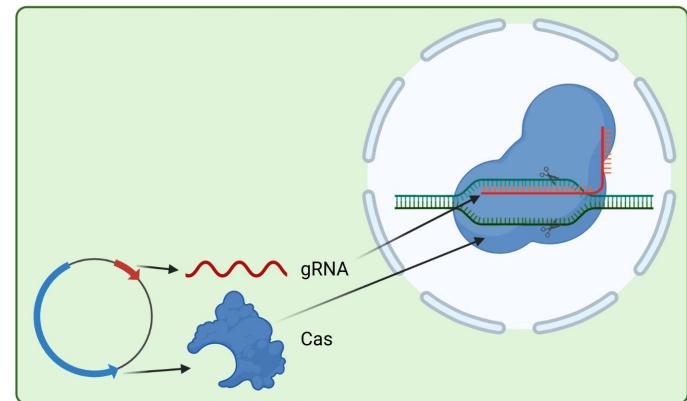


Genome editing methods

- CRISPR-Cas9
- dCas
- TALENs

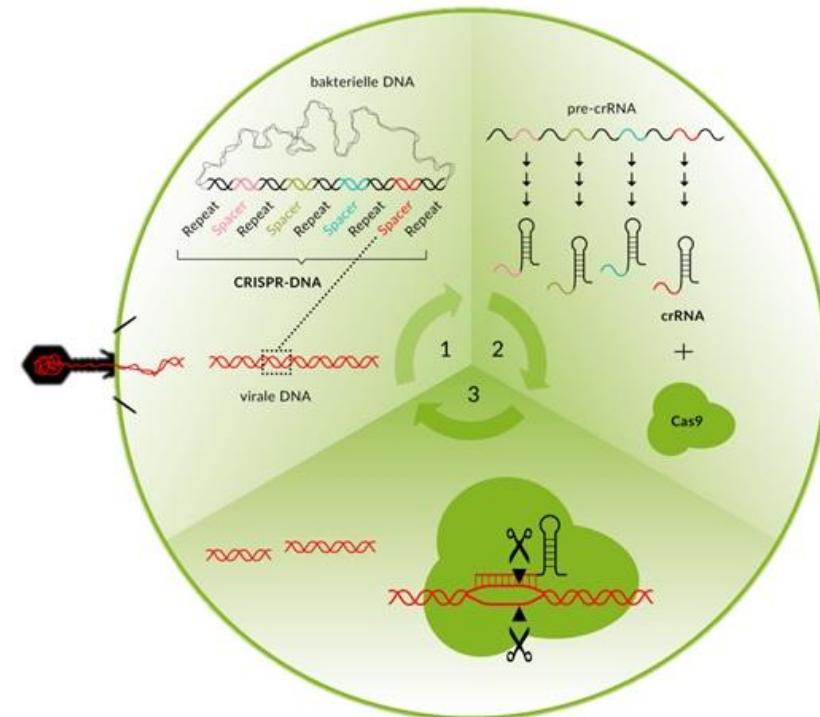


- CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats
- Cas9 = CRISPR-associated (endonuclease)
- Discovered by Emmanuelle Charpentier and Jennifer Doudna in 2012 (Nobel Prize in Chemistry: 2020)
- Targeted modification in (plant) genomes

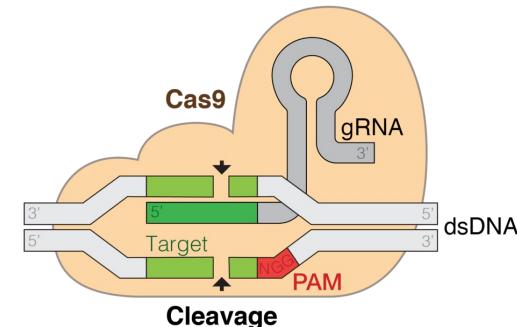


'Emmanuelle Charpentier and Jennifer A. Doudna have discovered one of gene technology's sharpest tools: the CRISPR/Cas9 genetic scissors. Using these, researchers can change the DNA of animals, plants and microorganisms with extremely high precision. This technology has had a revolutionary impact on the life sciences, is contributing to new cancer therapies and may make the dream of curing inherited diseases come true.' <https://www.nobelprize.org/prizes/chemistry/2020/press-release/>

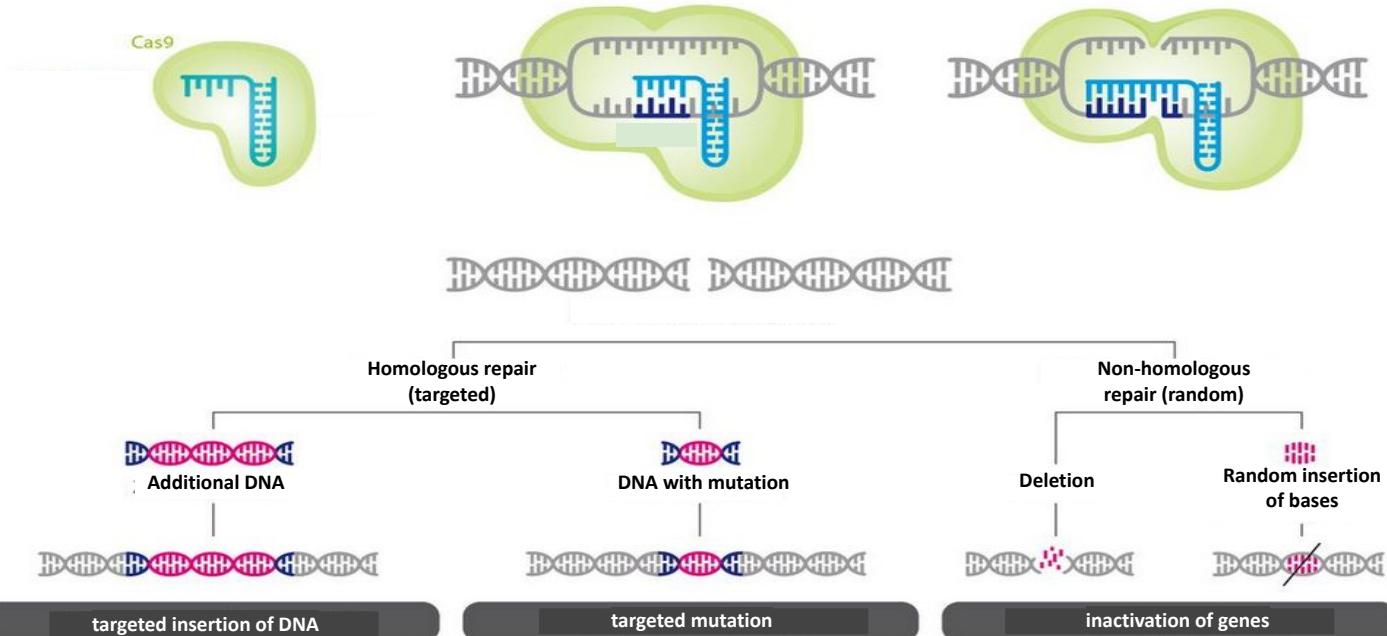
- CRISPR/Cas is bacterial immune system
- Fragments of bacteriophage DNA are stored in bacterial genome to enable rapid defense response upon infection
- PAM (Protospacer Adjacent Motif): short sequence of 2–6 defined nucleotides that allows discrimination between bacterial DNA (no PAM) and bacteriophage DNA (with PAM)



- Guide RNA (gRNA) recognizes highly specific site in genome
 - crRNA (CRISPR RNA) + tracrRNA
- gRNA directs Cas9 endonuclease to the correct location
- Cas9 cuts near the single-guide RNA binding site
- Cell usually repairs break using non-homologous end joining (NHEJ), which is error-prone
- Results in deletions/insertions causing a frameshift, leading to a KO of affected gene

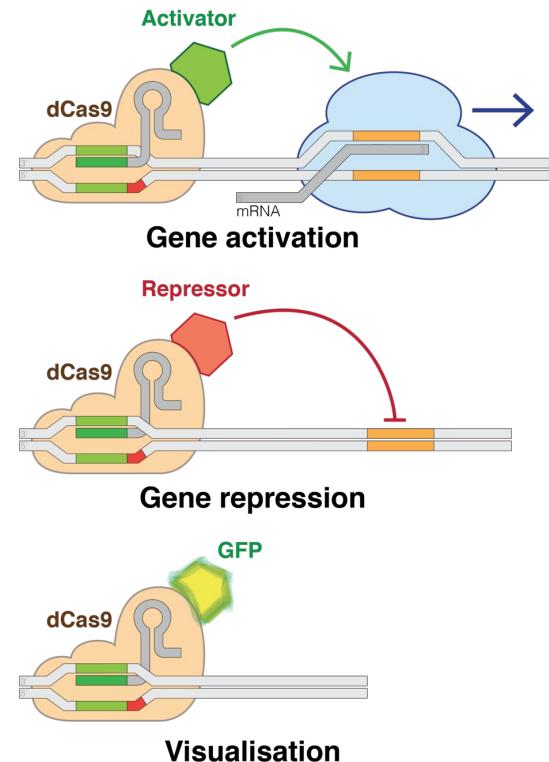


DNA modification with CRISPR/Cas9



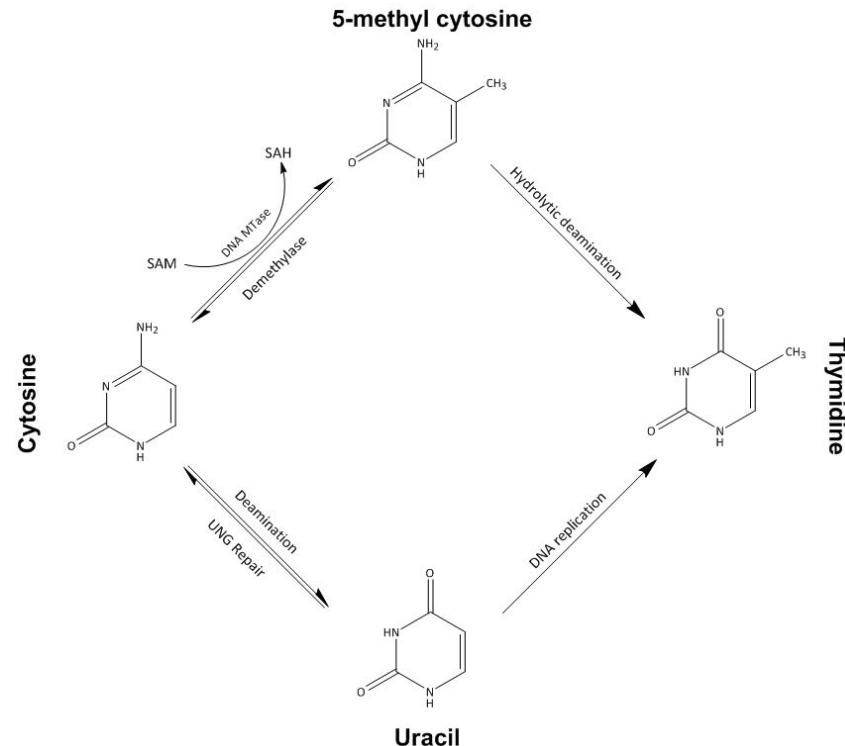
Dead Cas (dCas)

- dCas fused to transcriptional activators or repressors
- dCas can be linked to enzymes that modify DNA methylation or histone marks
- dCas can be used as a sequence-specific binding platform
- Multiple sgRNAs guide dCas to several loci at once
- dCas can be delivered transiently (no foreign DNA)

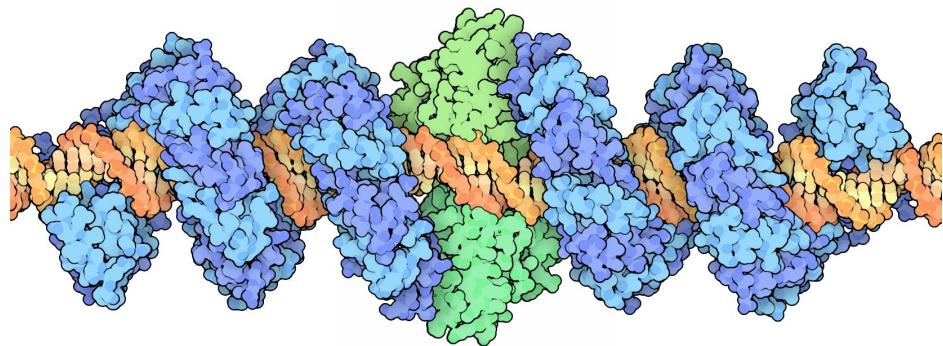


Cas modifications

- Cas fused deaminases enables single-nucleotide changes (C→T or A→G)
- Cas fused to reverse transcriptase allows targeted insertions, deletions, and all 12 base conversions
- Engineered Cas proteins recognize different PAM sequences

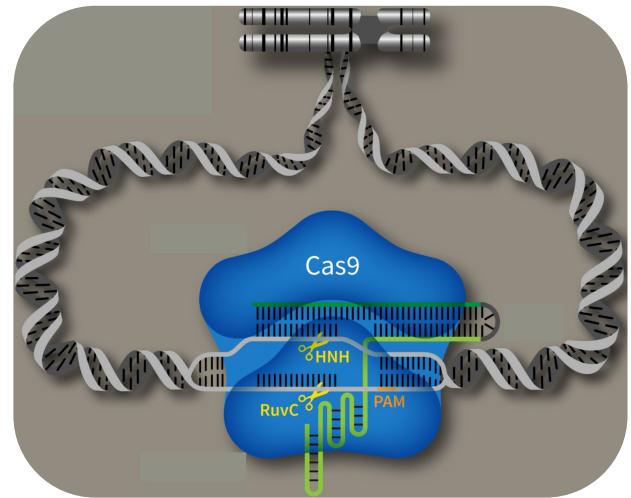


- TALEN = Transcription Activator-Like Effector Nucleases
- TAL = DNA binding domain
 - highly repeated 33-34 amino acids
 - 12th + 13th amino acid determine specificity
- Nuclease to cut DNA at binding site



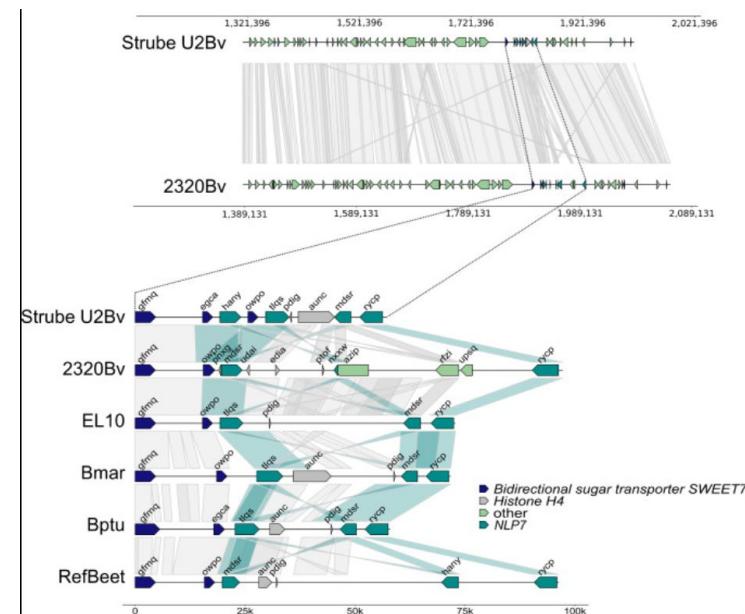
Advantages of CRIPR/Cas

- Universally applicable, not limited to specific organisms
- Targeted modification, with no random integration
- BUT: recombinant constructs for sgRNAs + Cas need to be cloned and transformed into plant cells!
- Vector carrying sgRNA and Cas information can be removed — only mutation remains; no foreign DNA in final modified organism

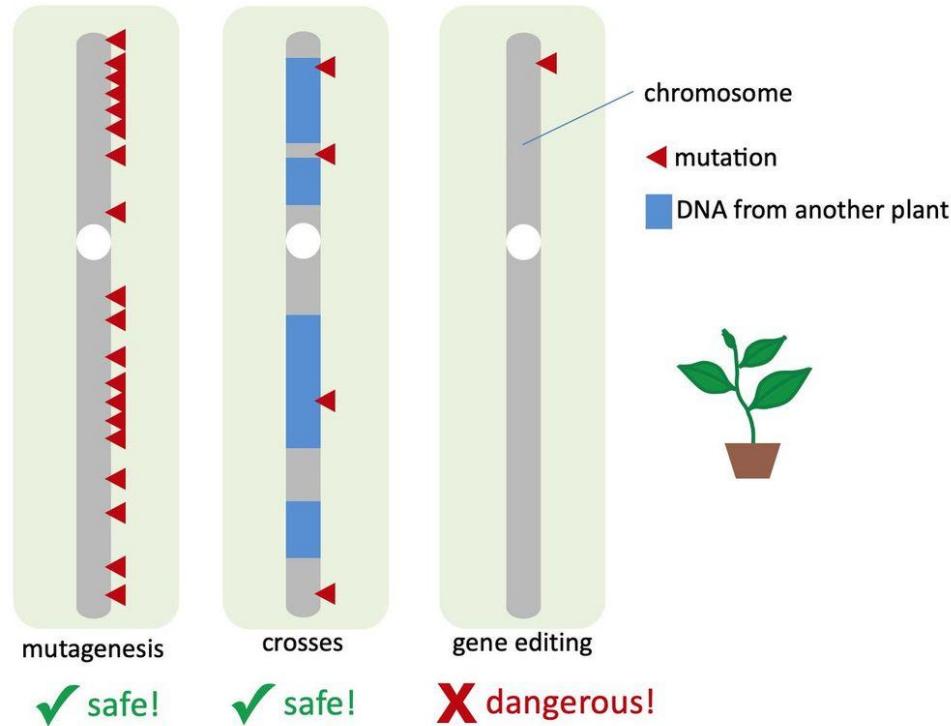


Importance of crop wild relatives

- Wild relatives of crops can harbour promising resistance/tolerance genes
- Pangenomics enable identification of such genes
- Genetic engineering or (time consuming) breeding allow transfer into crops
- Example: nematode-tolerant sugar beet lines



Legal situation in the EU



Genome editing in the EU

- NGT1 plants:
 - Targeted mutagenesis using CRISPR/Cas or TALEN (up to 20 bp); also includes cisgenic plants
 - Intended to be exempt from most GMO regulations
 - Field trial locations do not need to be disclosed
 - No labeling requirement for food or feed

- NGT2 plants:
 - Modifications >20 bp (more strictly regulated)
 - Patentability is currently a matter of debate

- NGT plants are indistinguishable from natural mutations



The screenshot shows the homepage of the transparenz GENTECHNIK website. The header features the logo "transparenz GENTECHNIK" and the categories "PFLANZEN · FORSCHUNG · LANDWIRTSCHAFT · LEBENSMITTEL". Below the header is a navigation bar with links for "AKTUELL", "FORSCHUNG", "ANBAU", "LEBENSMITTEL", "TIERE", "SICHERHEIT", "RECHT", "ZULASSUNG", and "DATENBANK". To the right of the navigation bar are social media icons and a search bar. A large blue banner in the center contains the European Union flag's twelve yellow stars arranged in a circle around a stylized DNA double helix and a small green plant. To the right of the banner is a green button with a hand icon that says "DONATE Unterstützen Sie unsere Arbeit mit Ihrer Spende.". Below the banner, there is a red "Themen" section with links to "EU-POLITIK (Regulierung)", "Konfliktfeld: Gentechnik", "Regulierung Genome Editing / NGT-Pflanzen", and "Genome Editing, CRISPR/Cas". At the bottom, there is a "Scenario starten" button with a magnifying glass icon, followed by a link to "Klickstrecke: Verfahren der Pflanzenzüchtung: Kreuzungszüchtung, Mutationszüchtung, Genome Editing, Cisgenetik, klassische Gentechnik" and a link to "Mehr Information zu den verschiedenen Stationen des Szenarios".

Glowing plants: Light Bio

- Genes from bioluminescent mushroom *Neonothopanus nambi*
- Bioluminescence via metabolic recycling: caffeic acid converted into luciferin, and then back again (no external substrate)
- Glow is visible in dark environments
- USDA approved GMO petunia for sale (approx. US\$29 per plant)
- Applications: studying metabolic cycles or sustainable lighting



Petunia

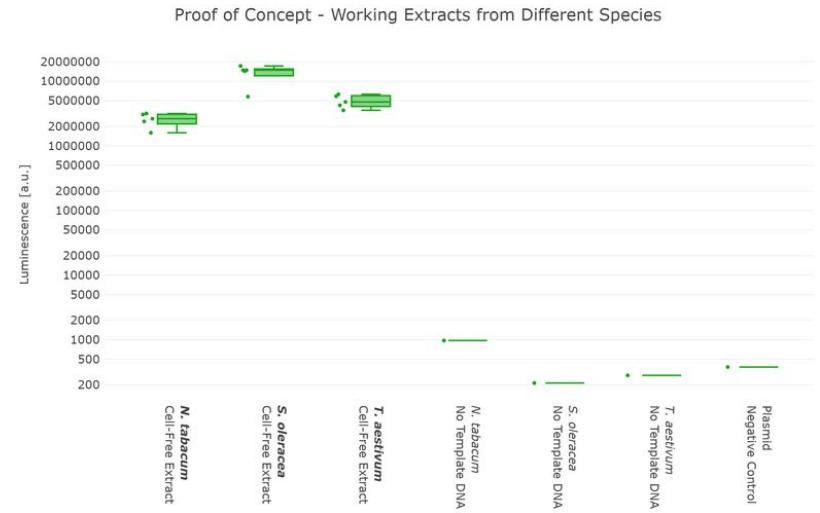
- iGEM = international Genetically Engineered Machine competition
- Student teams conduct synthetic biology projects
- Yearly competition with presentation of completed projects
- Numerous special prizes available



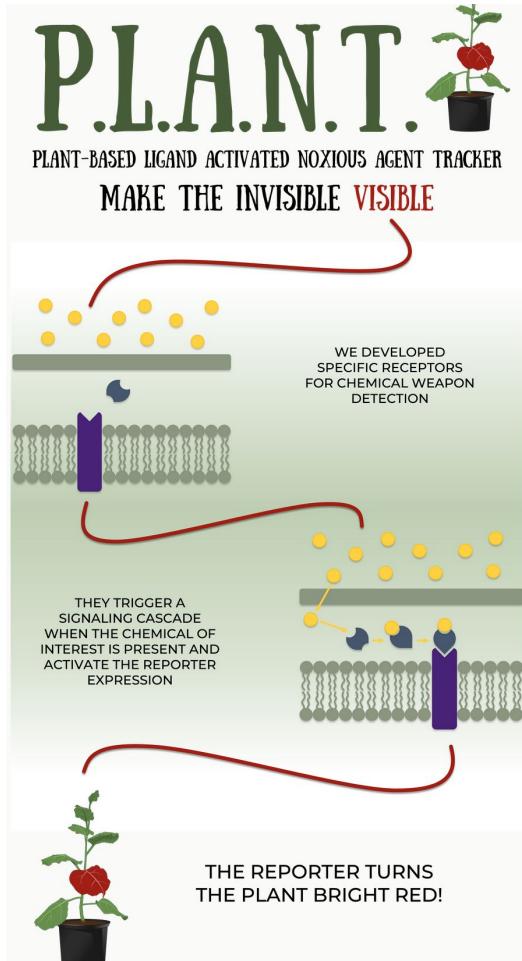


- Harvard 2010: iGarden
- Cambridge-JIC 2016: toolkit for chloroplast transformation
- Marburg 2021: OpenPlast
- Bielefeld-CeBiTec 2021: P.L.A.N.T.: Plant-based Ligand Activated Noxious agent Tracker - make the invisible visible
- SynBio2024: BlueBloom - blue color as biomarker

- Establishing cell-free systems from chloroplasts
- Rapid characterization of BioBricks
- Range of plant species covered (*T. aestivum*, *Q. robur*, *N. tabacum*, *S. oleracea*)

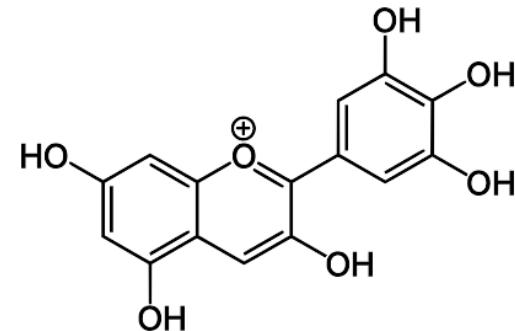


- Plant-based Ligand Activated Noxious agent Tracker
- Hydroculture allow controlled exposure of plants to compounds
- RUBY and Anthos serve as reporter (red pigments)

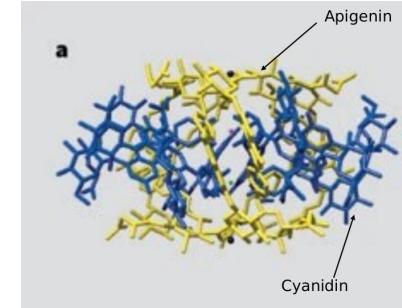




- Objective: developing markers for plant infections based on blue color (rare in plants)
- Delphinidin-based or protocyanin-based solutions



Delphinidin



Protocyanin

Shiono, M., Matsugaki, N. & Takeda, K. Structure of the blue cornflower pigment. *Nature* **436**, 791 (2005). <https://doi.org/10.1038/436791a>

- Transformation methods
- Transformation examples: purple tomato, golden rice
- Genome editing: CRISPR/Cas9
- Legal implications (EU)
- Plant SynBio (iGEM)

Time for questions!

Questions

1. Which plant transformation methods exist?
2. What is a T-DNA?
3. Why is DNA transferred into a plant?
4. Which mutagenesis methods are frequently applied in plant sciences?
5. Which tools can be used for genome editing?
6. Which method is preferred by breeders in the EU: mutagenesis or genome editing?
7. Which plant biology projects have been conducted in iGEM?