Application of Targeted Gene-editing Tools to Floral Crop Improvement

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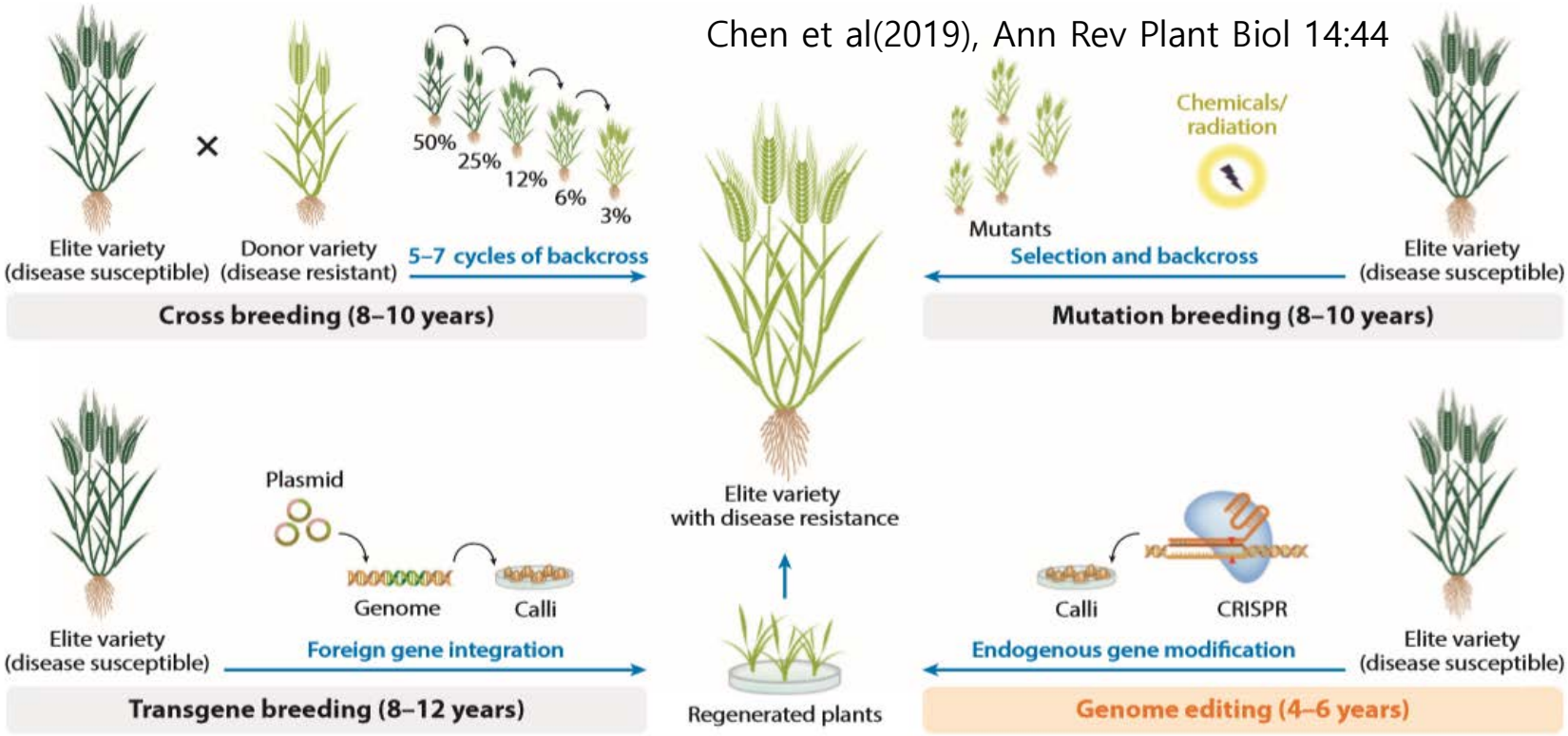
❑ 식물 육종의 기술적 과제

- **Limited genetic resources & random mutagenesis →효율적인 표적 유전자 돌연변이 유도**
- **Cheaper NGS, but many genes expressed→정확한 유전자 기능 분석**
- **Segregation hotspots→ targeted homologous recombination**
- **Making all these in NON-TRANSGENIC manner**

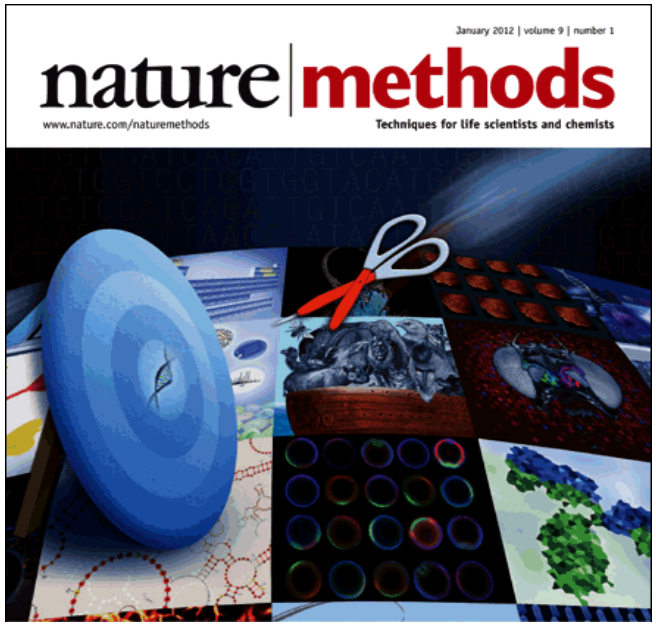
Chen et al(2019), Ann Rev Plant Biol 14:44

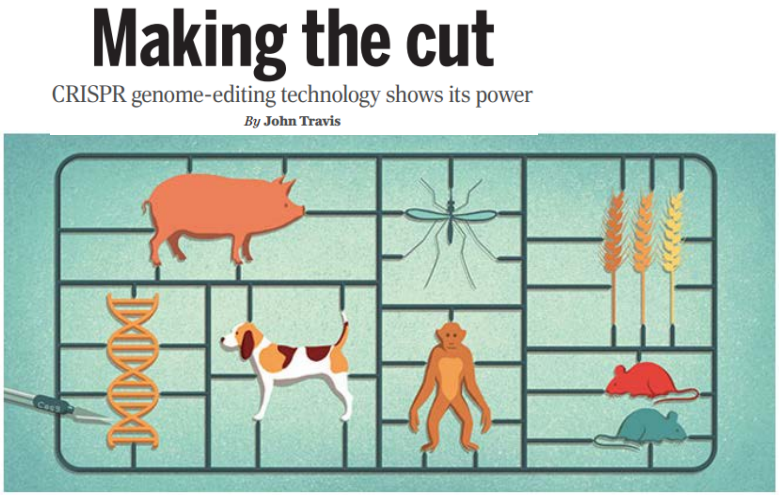


Genome editing using nuclease can do all of these

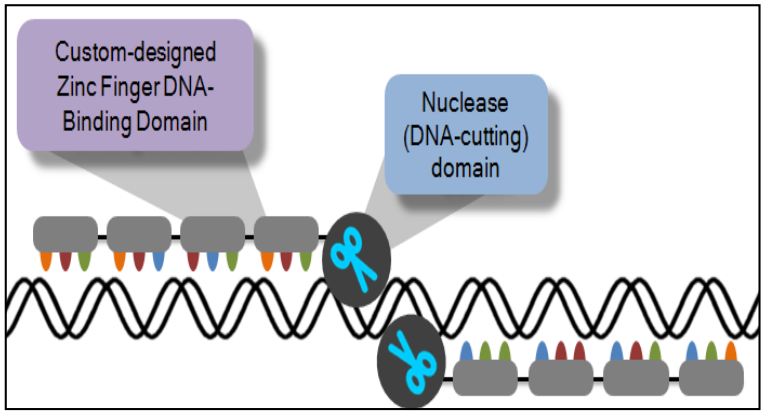
❑ 타겟 유전자 편집기술 (Genome Editing)

- Method of the year 2011
- Engineered nucleases (ENs): 유전체 특정 위치를 절단하도록 디자인된 단백질
- 제놈절단(cleavage event on genome): 표적 유전자를 자유롭게 편집

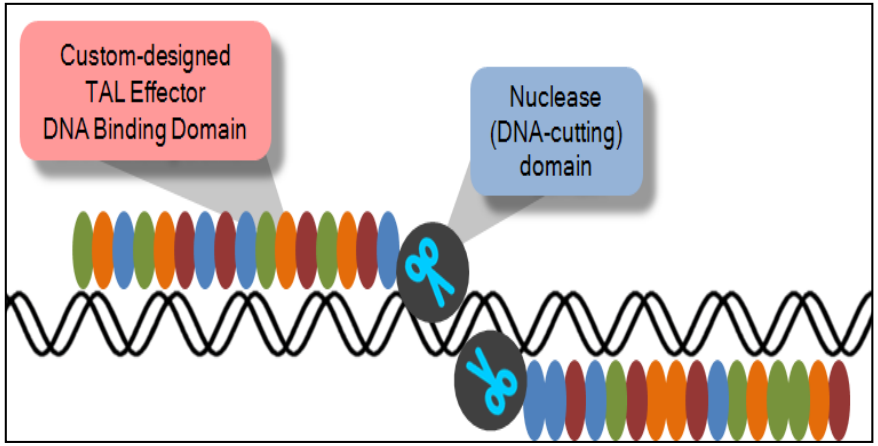




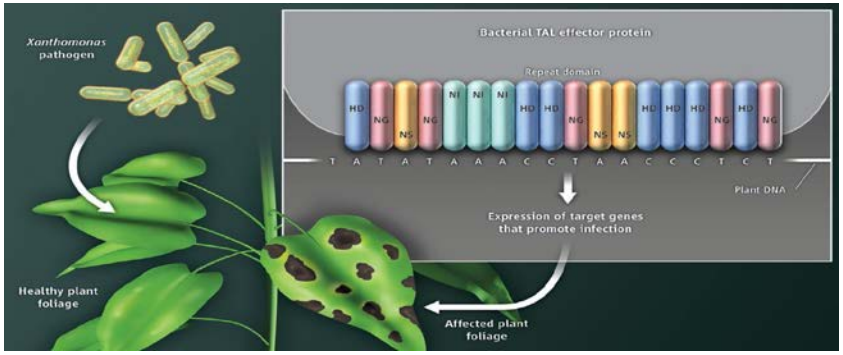
❑유전자 가위 종류와 특성-ZFN, TALEN



- Source: 진핵세포의 DNA 결합 도메인
- DNA Binding Module(3~5개로 구성):
  - 각각 모듈은 3 개의 DNA 염기 인식 & 결합→9~15bp 인식
  - 64개의 DNA triplet 조합 : 30~40개 모듈 생산



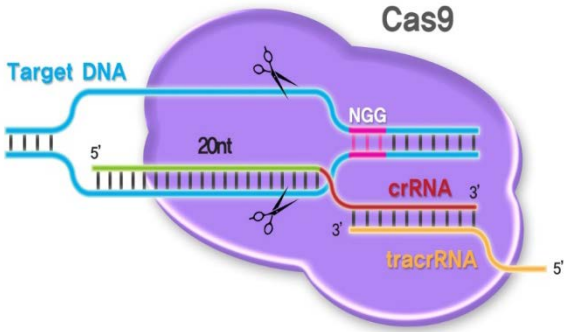
- Source: 식물 *Xanthomonas* 세균의 전사인자(TAL Effector)
- DNA Binding Module(12~20개로 구성):
  - 각각 모듈은 한 개의 DNA 염기 인식 & 결합
  - 모든 DNA 염기 모듈제작이 가능

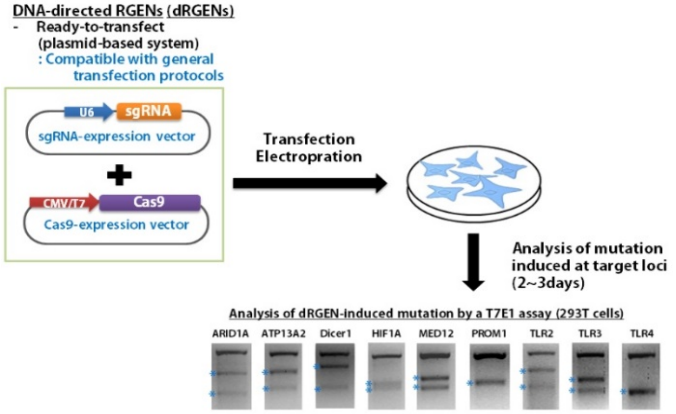


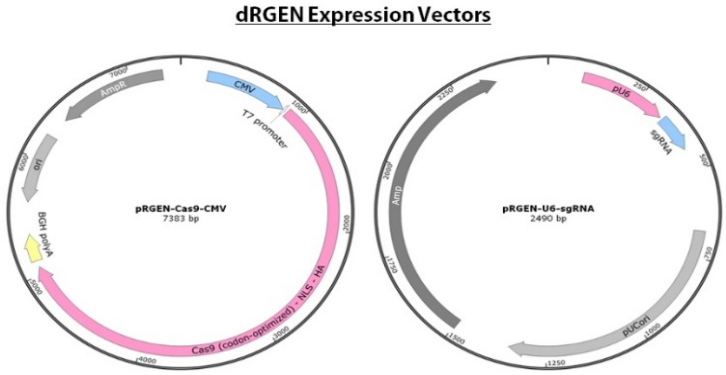
J. Boch et al. Science 2009
M.J. Moscou et al. Science 2009

❑ 유전자 가위 종류와 특성-CRISPR/Cas

- 3rd Generation Engineered Nuclease (RNA-Guided ENdonucleases, RGENs)
- 세균의 방어기작 CRISPR/Cas 시스템 이용
- 절단 기능 단백질(Cas9)과 유전자 결합 기능의 결합체→RNA만 합성교체로 맞춤형 유전자 가위 가능





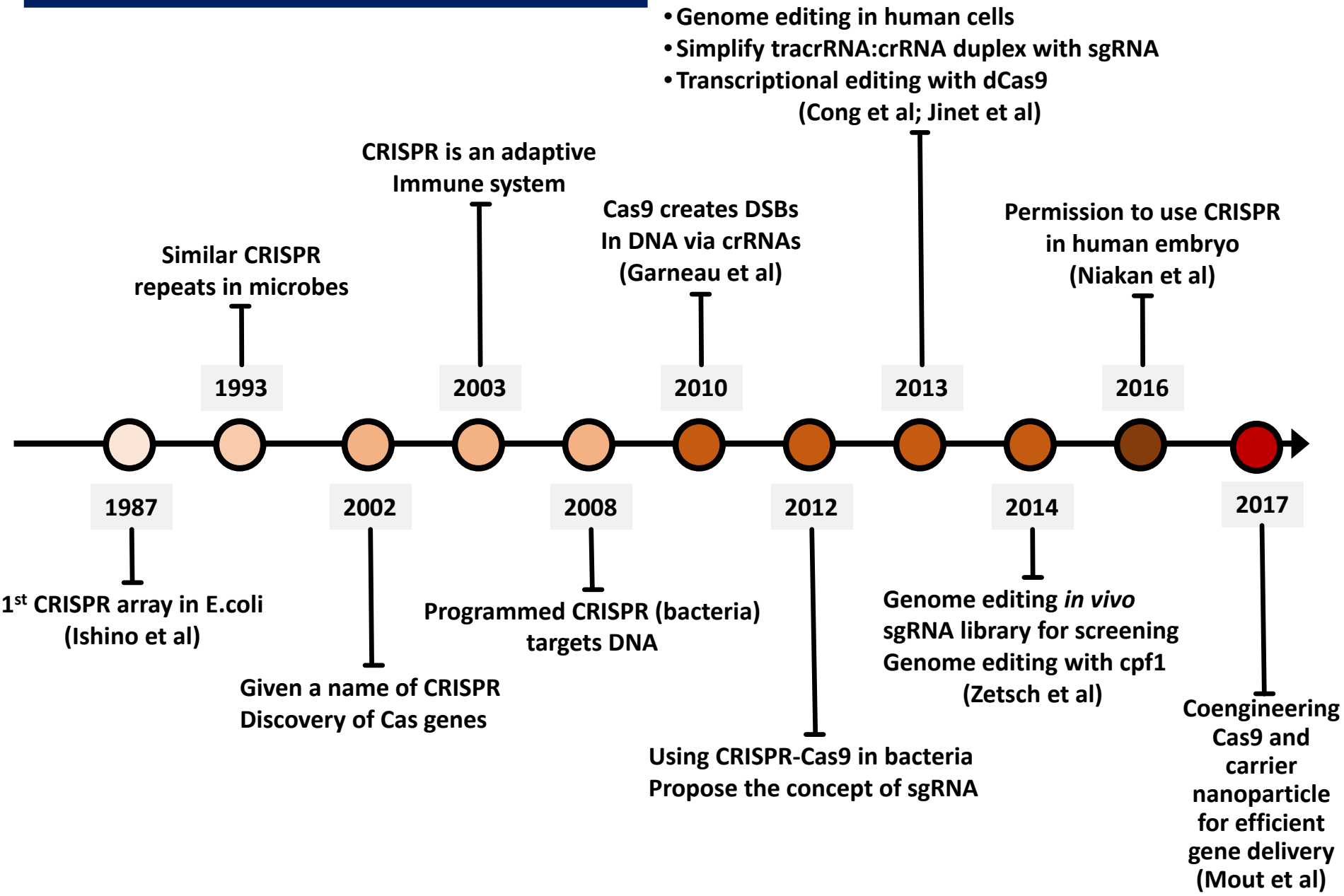


expression of Cas9 gene plasmid

target-specific guide RNA expression

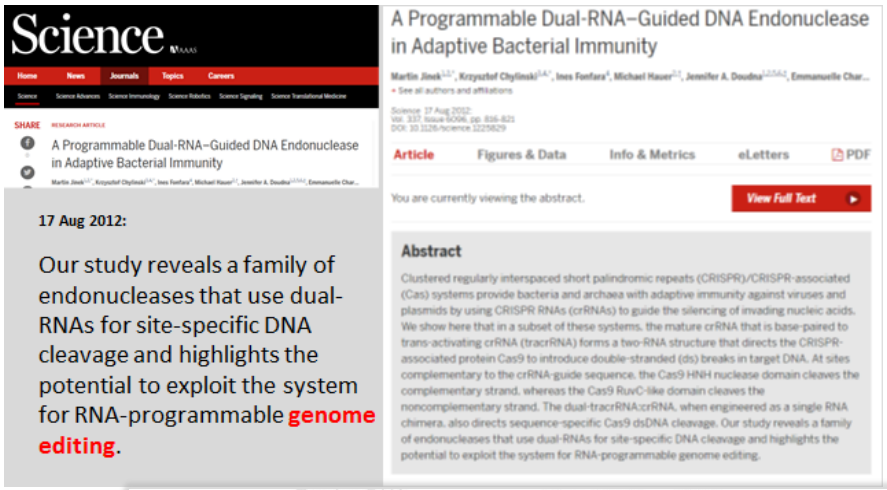
(www.toolgen.com)

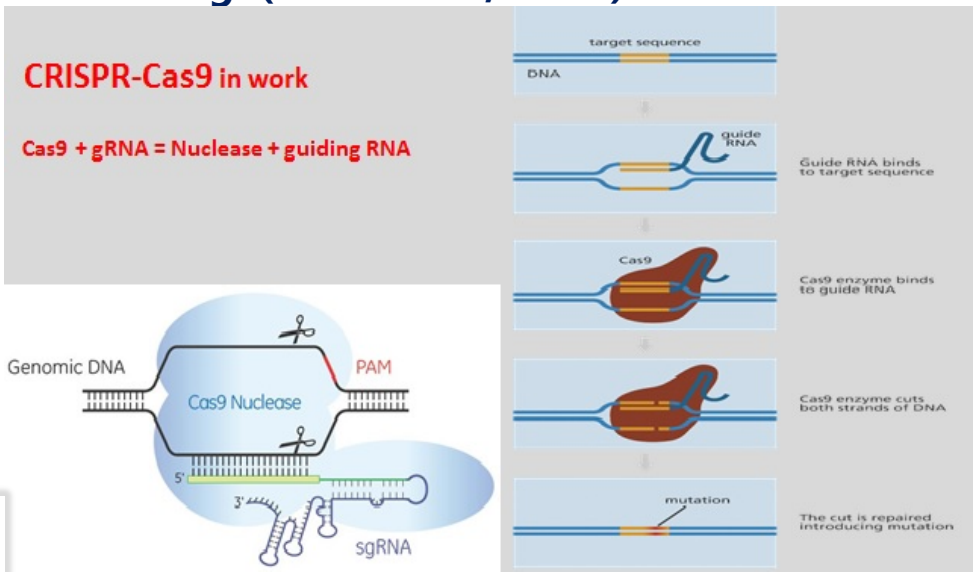
❑ Evolution of CRISPR/Cas9

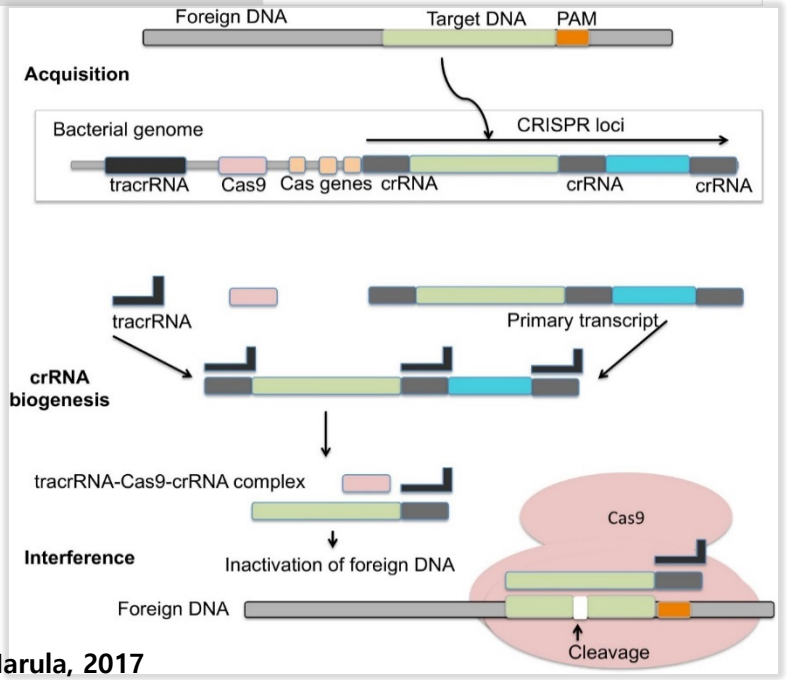


❑ CRISPR/Cas9 작용기작

- tracrRNA forms duplex with crRNA in association with Cas9 (Deltcheva et al., 2011)
- Biochemical mechanism of Cas9 mediated cleavage(Jinet et al., 2012)

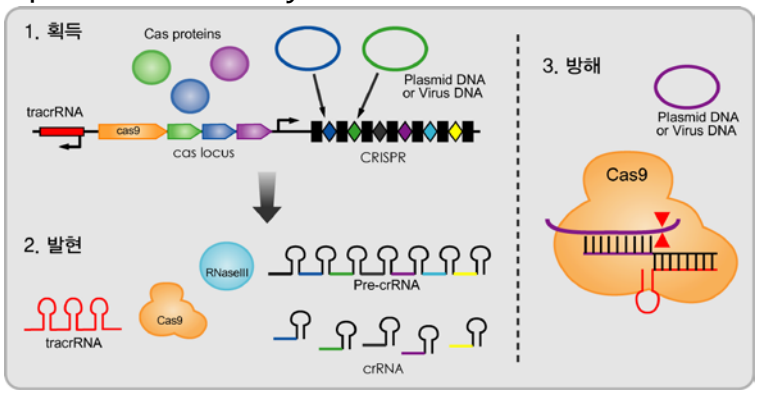






Arora & Narula, 2017

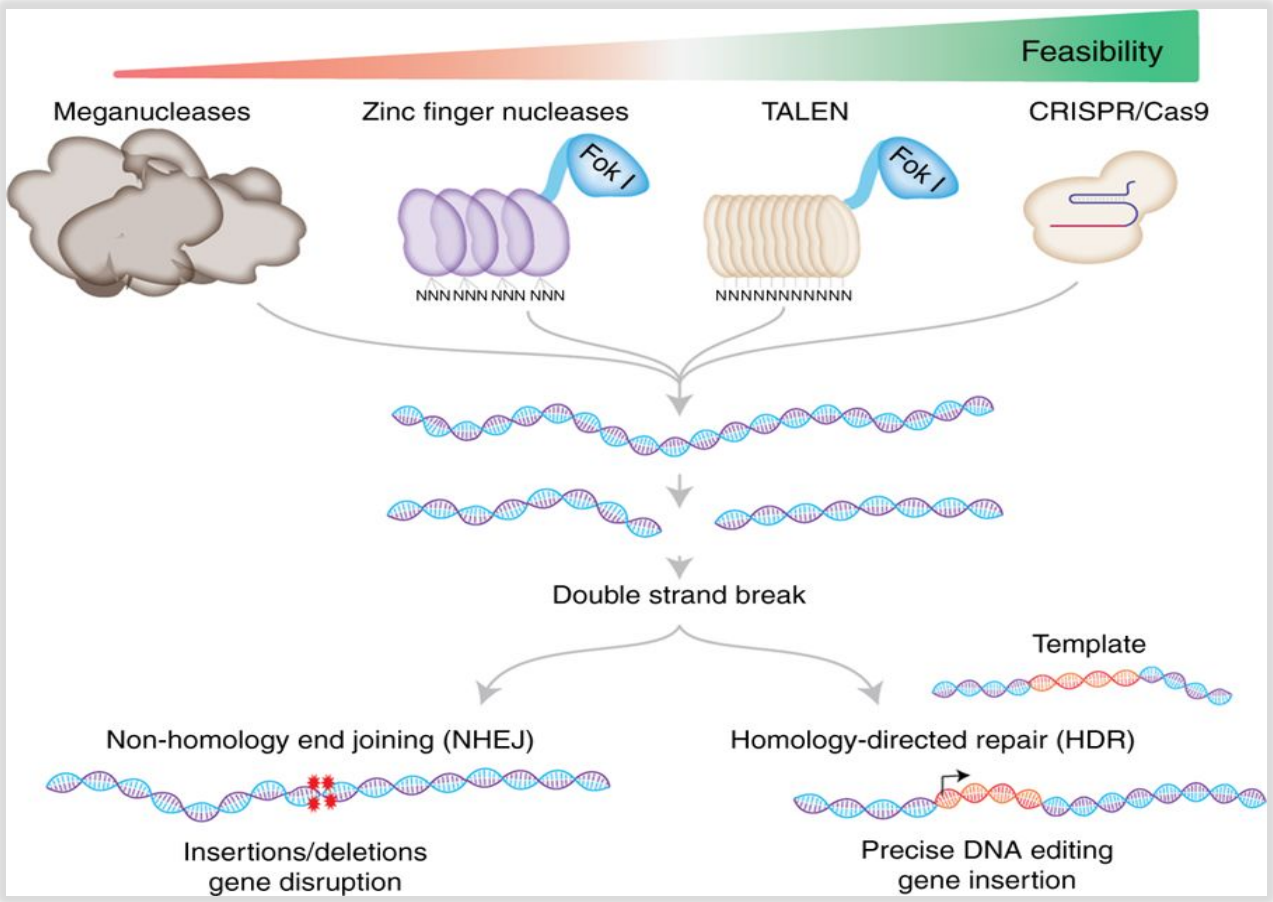
- Adapted immune system of E-coli and Archaea



Bae et al., KSBMB news (2014)

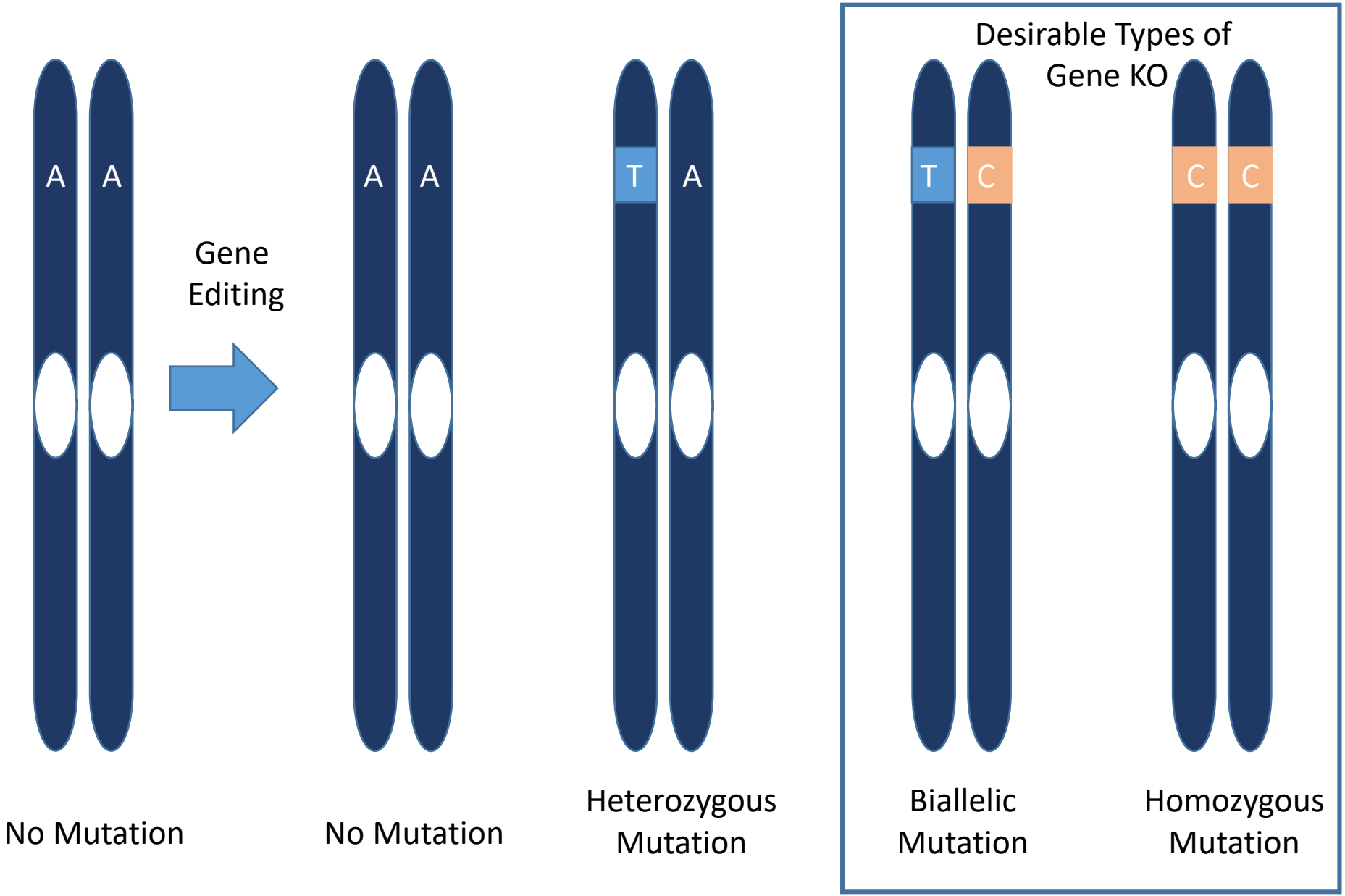
❑ 유전자가위 편집 모드

- 높은 목표 유전자 변이율 (1~49% vs. natural/random mutation rate 10^{-6})
- 변이체의 약 1/3은 대립유전자 모두 발생(biallelic or homozygous mutation)
- 모든 진핵세포에서 작동
- 선발 마커가 필요하지 않음

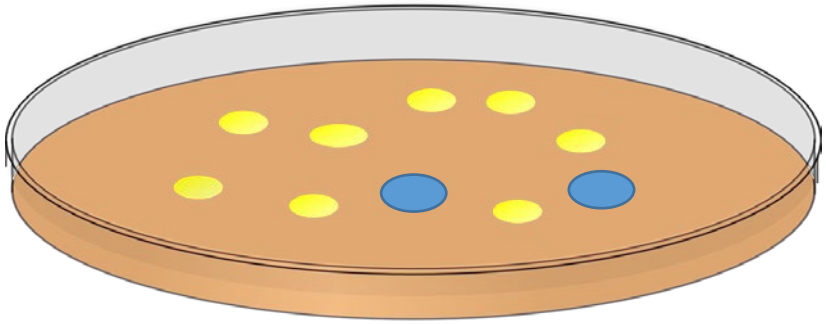


Adli(2018), Nat Commun 9:1911

❑ 4가지 형태의 변이형태(ie, diploid)



❑ Screening Strategies-교정된 세포는 어디에?

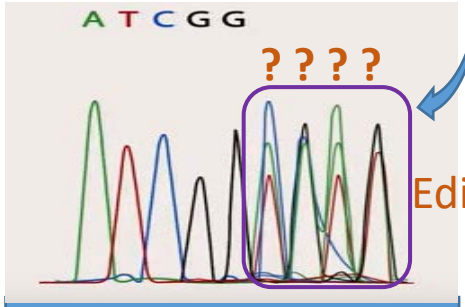


DNA Extraction of pooled cells

- PCR of gene edited
- Denatured & Re-annealed
- Denature & Randomly rehybridization
- Heteroduplex cleavage by RE

Mismatch Cleavage Assay

- Sensitive
- Not quantitative
- No Indel Seq. Info

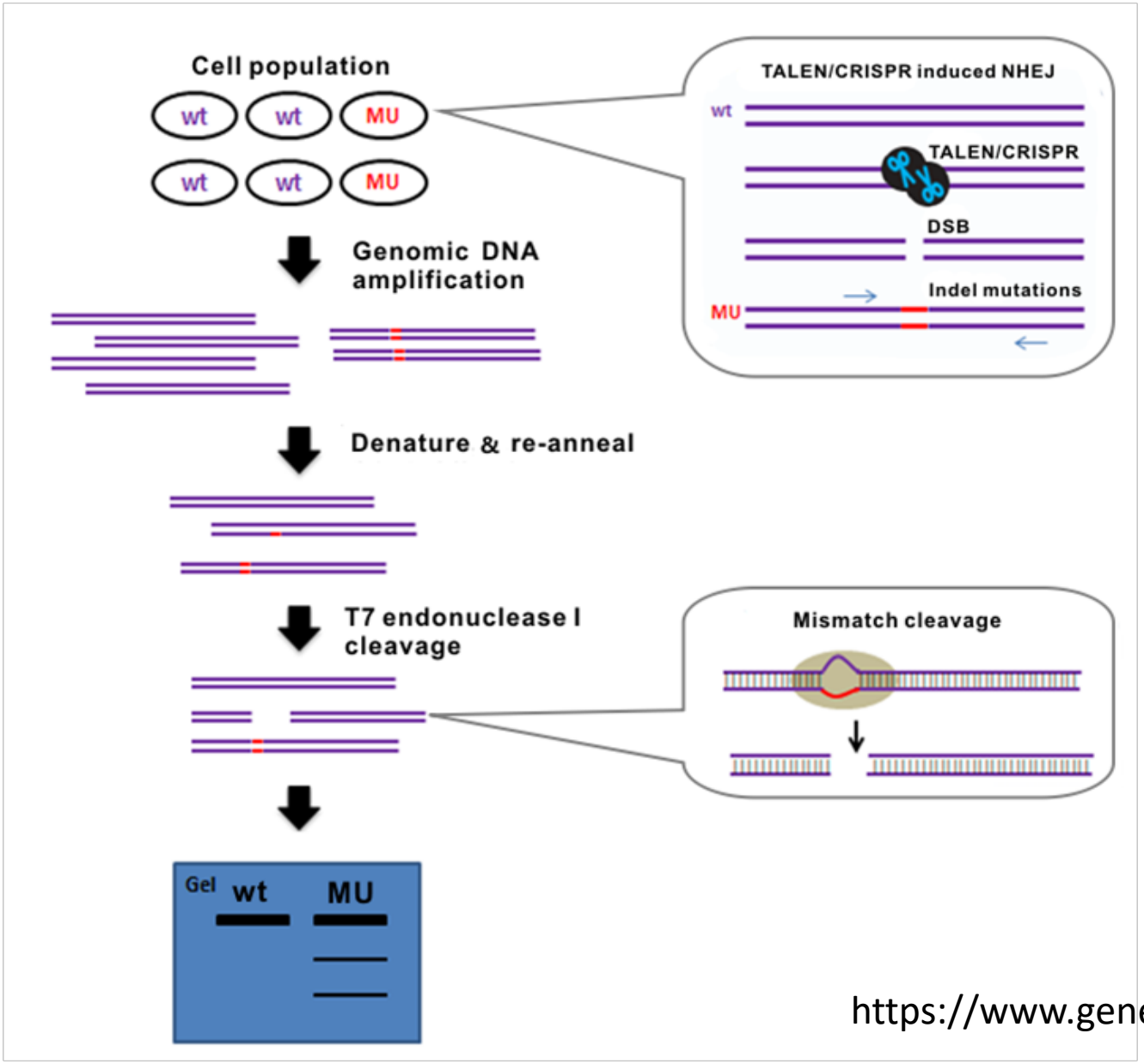


Sanger Amplicon sequencing

- Sensitive
- Quantitative
- Indel Seq. Info
- Expensive
- Complicated

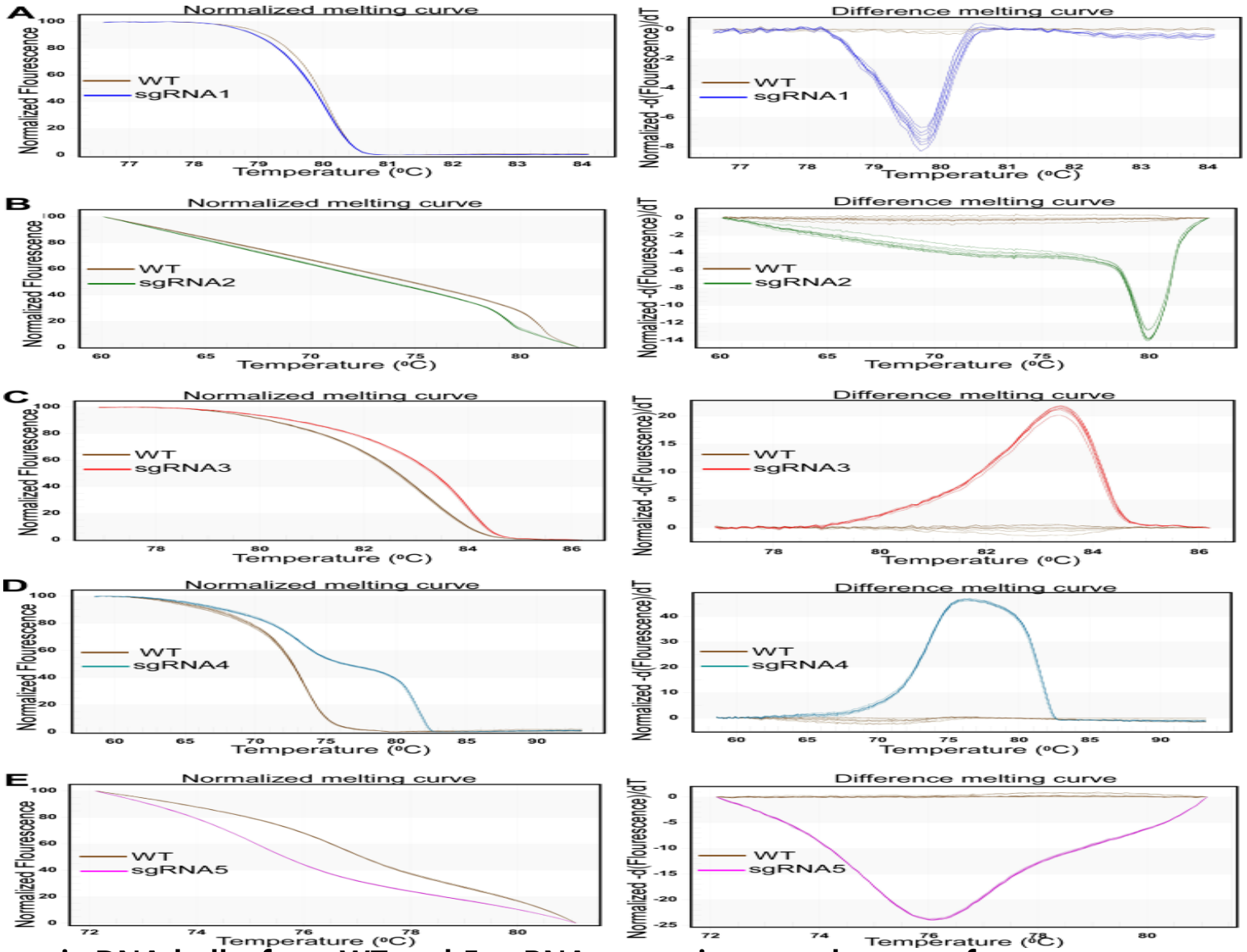
Next Generation Amplicon sequencing

❑ Mismatch Cleavage Assay-Surveyor, T7E1



https://www.genecopoeia.com

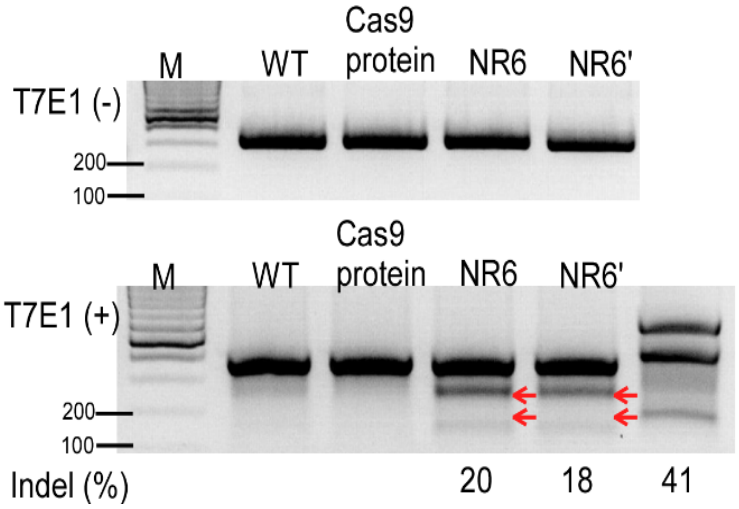
❑ HRM analysis for cell bulk screening



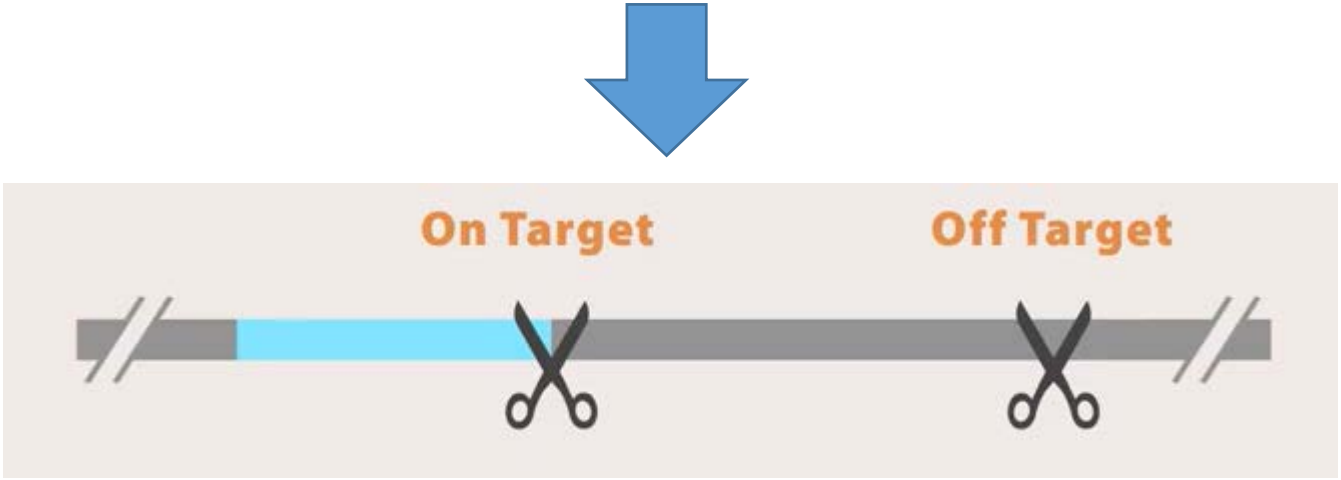
-Genomic DNA bulks from WT and 5 sgRNAs-petunia protoplast transfectants.

❑ Validation Strategies-Biallelic & Homozygous?

- Sanger sequencing: Frame shifts=Gene Knock-out



Whole genome deep sequencing



❑ Validation Strategies-Biallelic & Homozygous?

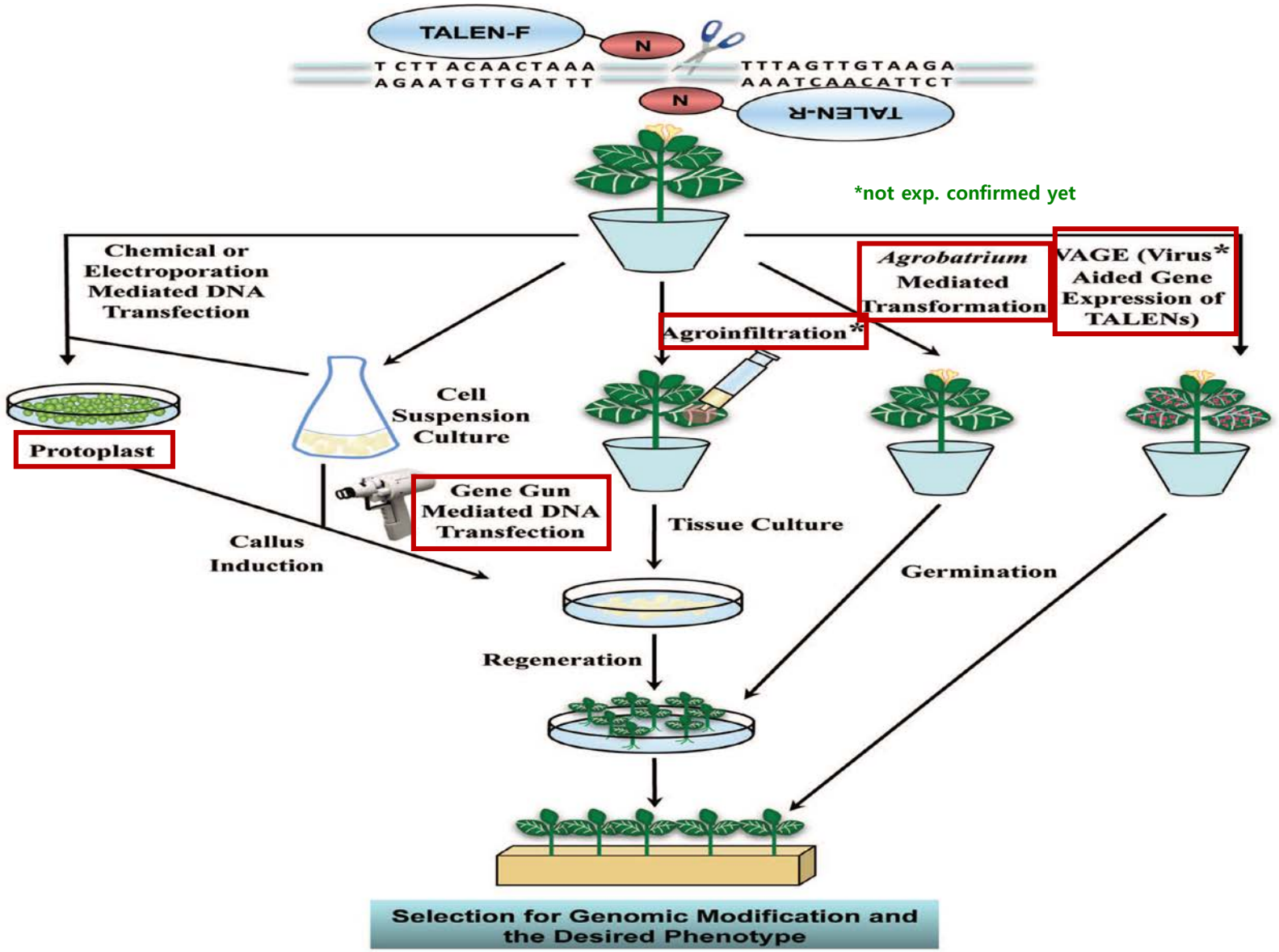
표. Estimation of mutation rate in *NR* gene sequences in non-transformed and NR-RGEN transformed Petunia protoplasts by targeted deep sequencing

Protoplast samples	Wild type-transfectants			Cas9 protein-transfectants			NR-RGEN transfectants				
	Total	Indel	Indel frequency (%)	Total	Indel	Indel frequency (%)	Total	Indel	Indel frequency (%)	Ins[a]	Del[b]
NR1	45,168	3	0.01	35,735	9	0.03	53,898	2854	5.30	1506	1348
NR2	52,699	20	0.04	52,773	24	0.05	71,103	8688	12.22	4128	4560
NR3[c]	–	–	–	–	–	–	–	–	–	–	–
NR4	29,653	4	0.01	36,003	9	0.03	40,670	4392	10.80	1537	2855
NR5[d]	34,842	4572	13.12	22,547	6118	27.13	23,754	8241	34.69	2025	6216
NR6	34,024	131	0.39	28,372	39	0.14	35,095	6256	17.83	1107	5149
Average[e]			0.11 ± 0.1			0.06 ± 0.02			11.5 ± 2	2069.5 ± 536.9	3478 ± 666.6

➡ mutations in 4 sites(4 out of 6) were confirmed at the frequency average of 14.9 ± 2.2 % by deep sequencing

Subburaj et al(2016), Plant Cell Reports 35:1535

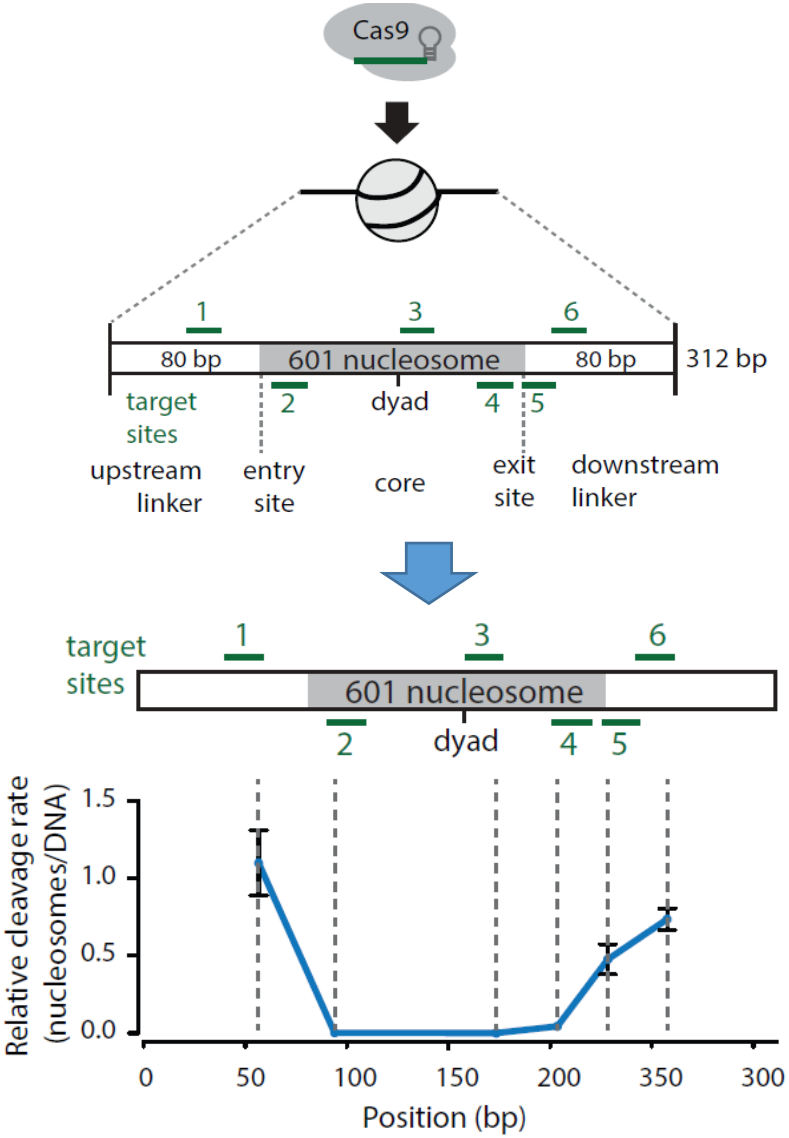
❑ 유전자가위 세포전달 방법



Mahfouz & Li(2011), GM Crops 2:99-103

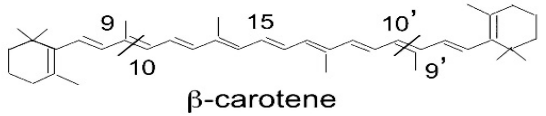
❑ 3 basic points for CRISPR design

- Exon-intron junction (*eLife* 5:e13450, 2016)
  - Propeties of DNA Sequence
  - PAM distance
  - Chromatin remodeling enzymes
- GC Content 40-80%
- Length 17-24 base pairs
- Potential off-target effects 17-24bp
  - Position of the mismatch
  - Number of mismatches
  - Sequence of gRNA
  - gRNA concentration
- Webtools
  - Cas-designer (www.rgenome.net)
  - sgRNA designer(Broad Institute)
  - Biotools

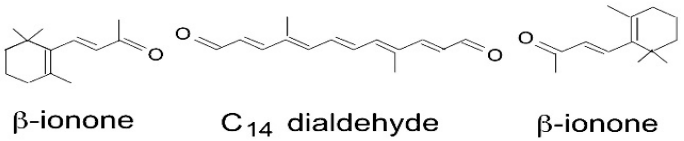


❑ 나팔꽃 *carotenoid cleavage dioxygenase 4*

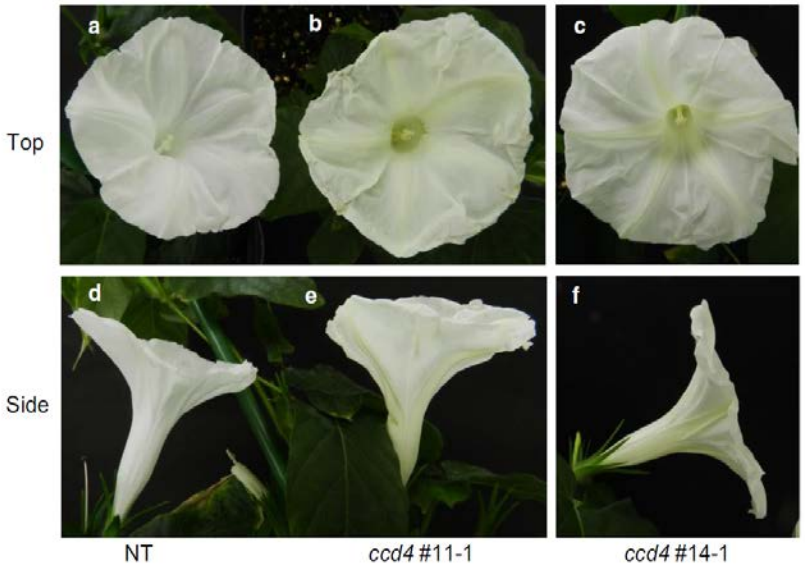
- 카로티노이드 색소 분해 및 꽃잎 축적 억제

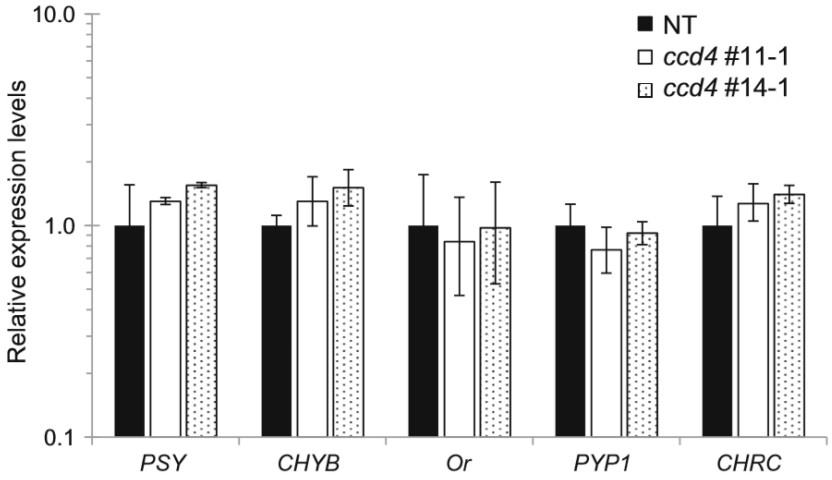


CCDs

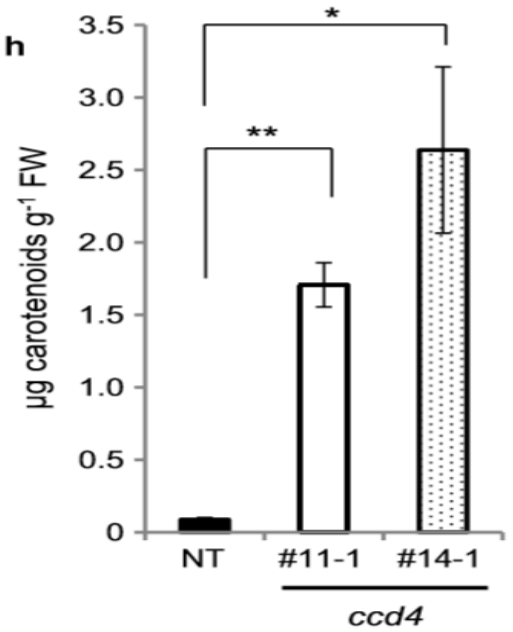


(apocarotenoid volatiles)





카로티노이드 색소 생합성 유전자의 발현차이 없음

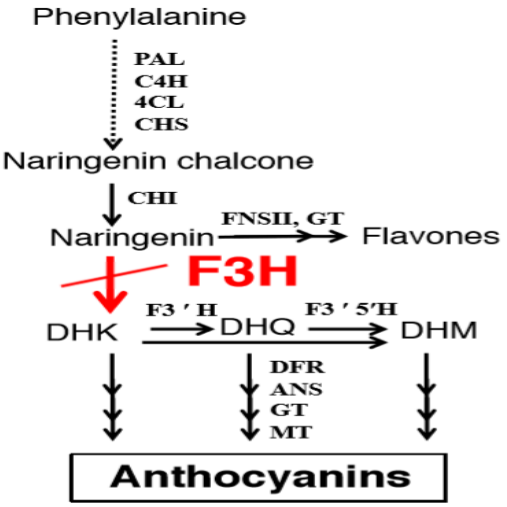


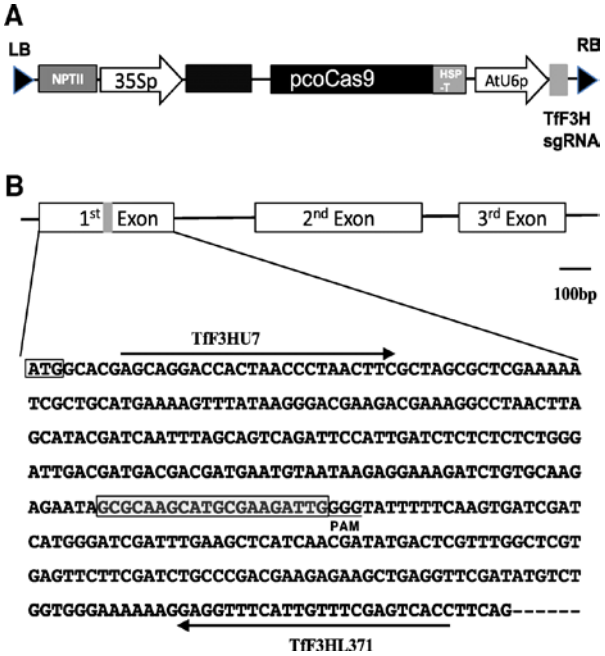
Loss-of-function→ 노란색 축적

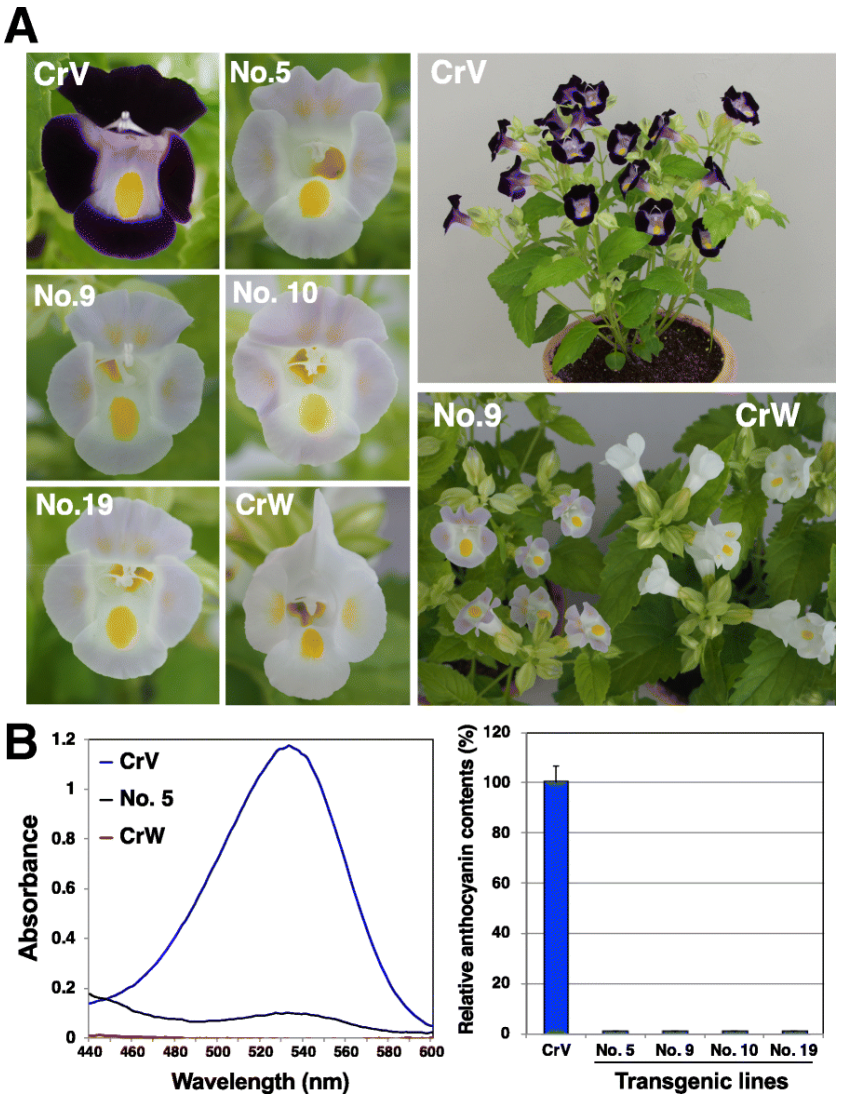
Watanabe et al (2018), Transgenic Res 27:25

❑ 토레니아 *Flavanone 3-hydroxylase (F3H)*

- 안토시아닌 생합성 유전자





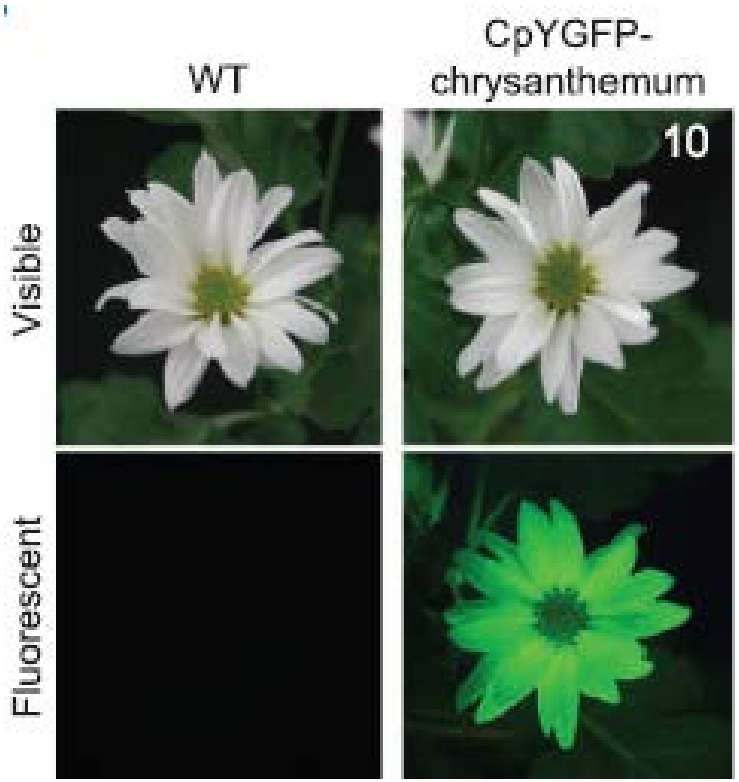


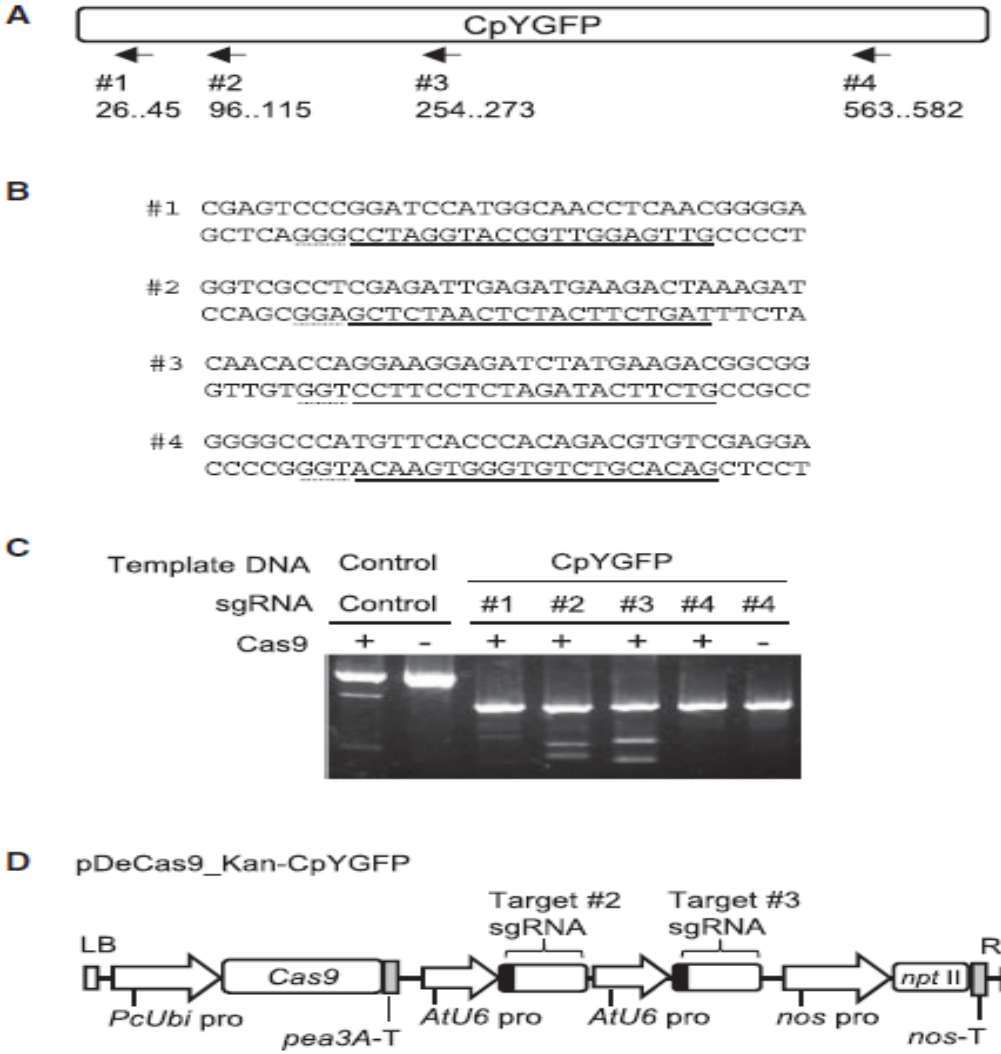
F3H-KO mutants & chimeric (mosaic) plants

Nishihara et al (2018), BMC Plant Biol 18:331

❑ Chrysanthemum Transgene (*CpYGFP*) 편집

- Marine copepod (*Chiridius poppei*) yellowish-green fluorescent protein
- *Agrobacterium tumefaciens* strain EHA105





Kishi-Kaboshi et al., Plant Cell Physiol (2017)

❑ Chrysanthemum Transgene (*CpYGFP*) 편집

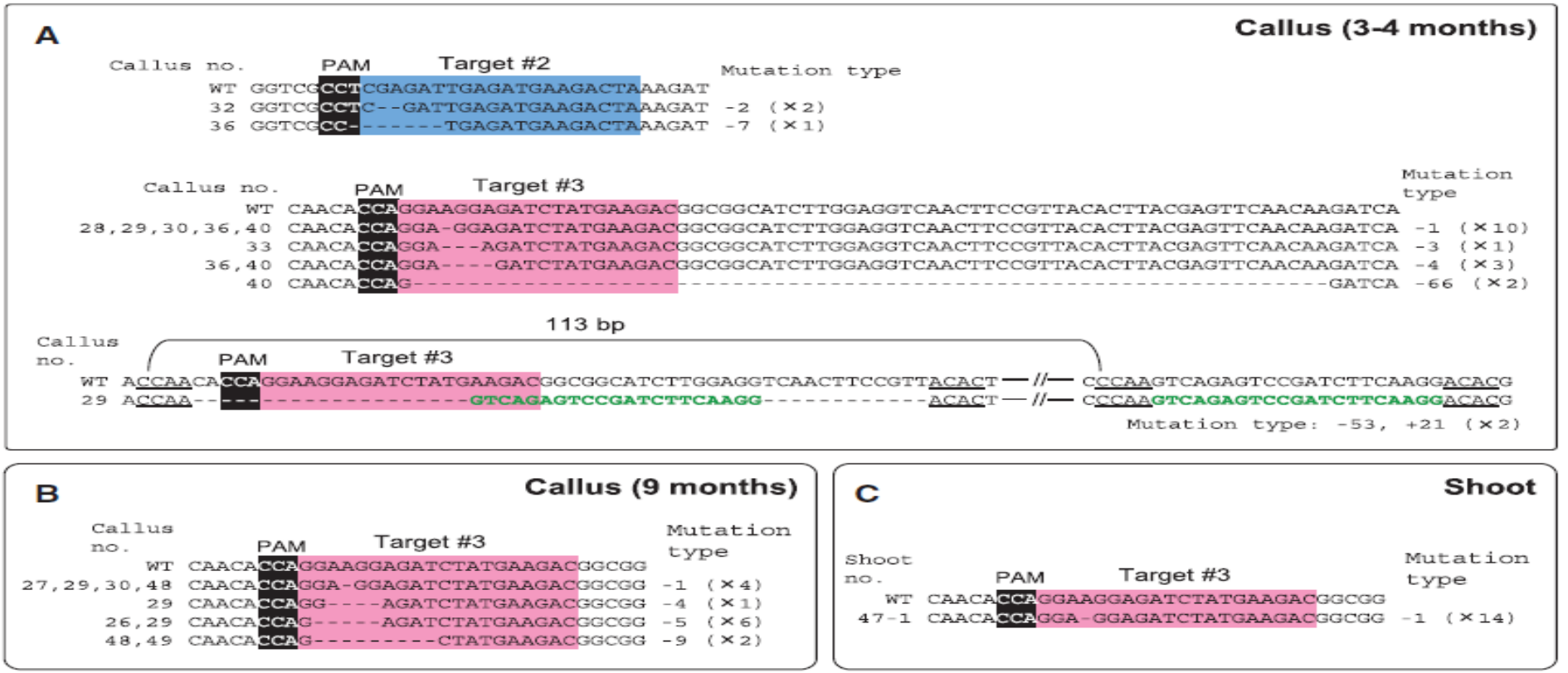


Table 2 Shoot regeneration rate and mutation frequency in CRISPR—CpYGFP-chrysanthemum

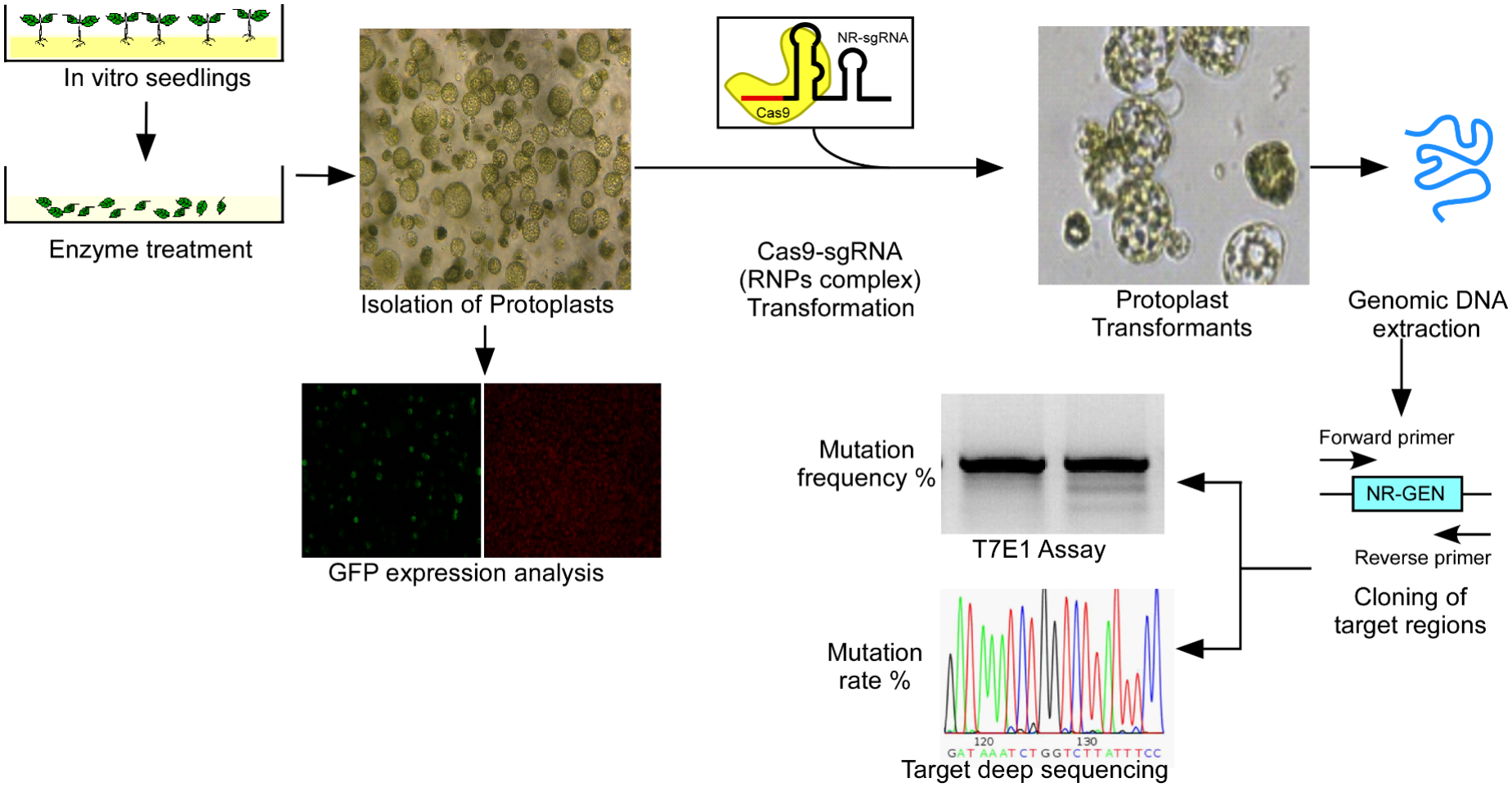
Plant used for pDeCas9_CpYGFP transformation		No. of leaf sections	No. of regenerated shoots	Shoot ID	Vector insertion	Mutation frequency[a]	Target #2	Target #3	
							No of clones	No. of clones	Mutation type
CpYGFP-chrysanthemum	#2	499	2	43-1	+	0/19 (0%)	0	0	
				46-1	+	0/16 (0%)	0	0	
	#10	499	2	47-1	+	4/39 (10.3%)	0	4	−1 (×4)
						6/40 (15%)	0	6	−1 (×6)
						4/40 (10%)	0	4	−1 (×4)
				49–1	+	0/24 (0%)	0	0	
Non-transgenic		911	74		31/38[b]	ND	ND	ND	

❑ Crop plants and genes targeted with CRISPR/Cas9

Plant species	Nuclease delivery system	Transformation method	Target gene	Trait achieved	References
O. sativa	Purified nuclease protein	PEG mediated Protoplast transfection	bacterial blight susceptibility S gene (OsSWEET14)	Blight resistance	Jiang et al., 2013
O. sativa	Plasmid vector mediation	Agrobacterium tumefaciens-mediated transformation	insertion of mutant acetolactate synthase (ALS) template at the ALS gene	Resistance to sulfonylurea herbicides	Endo et al., 2016
O. sativa	Plasmid vector mediation	Protoplast transfection	ERF transcription factor gene (OsERF922)	Resistance to blast disease	Wang et al., 2016
S. lycopersicum	Plasmid vector mediation	Agrobacterium tumefaciens-mediated transformation.	Argonaute7 (SlAGO7)	Loss of A1AGO7 leads to needle like or wiry leaflets without petioles	Brooks et al., 2014
L. sativa	Purified nuclease protein	Protoplast transfection	Brassinosteroid Insensitive 2 (BIN2) gene		Woo et al., 2015
N. tabacum	Plasmid vector mediation	Agrobacterium tumefaciens-mediated transformation.	phytoene desaturase (PDS) and PDR-type transporter (an ABC transporter involved in strigolactone transport) genes	Plantlets with etiolated leaves (PSD mutation) and more branches (PDR mutation)	Gao et al., 2015a
N. tabacum	Plasmid vector mediation	Agrobacterium mediated direct infiltration into leaves	Tomato yellow leaf curl virus (TYCLV)	Delayed or reduced accumulation of viral DNA	Ali et al., 2015
A. thaliana	Purified nuclease protein	Floral dipping method	Auxin binding protein 1 (ABP1)	No defects in auxin response and growth and development.	Gao et al., 2015b
A. thaliana, N. Benthamiana	Plasmid vector mediation	Agrobacterium tumefaciens-mediated transformation.	beet severe curly top virus (BSCTV)	Inhibition of viral DNA accumulation	Ji et al., 2015
P. trichocarpa	Plasmid vector mediation	Agrobacterium tumefaciens-mediated transformation.	Phytoene desaturase gene 8 (PtoPDS)	Albino phenotypes	Fan et al., 2015
P. trichocarpa	Plasmid vector mediation	Agrobacterium tumefaciens-mediated transformation.	4-coumarate:CoA ligase (4CL) gene	Wood discolration due to reduced lignin content	Zhou et al., 2015
B. oleracea	Plasmid vector mediation	Agrobacterium tumefaciens-mediated transformation.	gibberellin biosynthesis pathwaygene (GA4)	dwarf phenotype	Lawrenson et al., 2015

Subburaj et al., Hortic Environ Biotechnol 57:531 (2016)

❑ DNA-free RNP-mediated mutagenesis in petunia protoplast



Subburaj et al(2016), Plant Cell Reports 35:1535

❑ Plant protoplast isolation and culture protocol

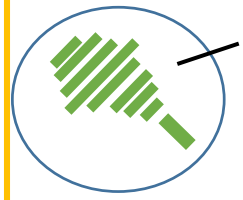
1. Select a material(plant species, tissue)

- Plant species
ex) Cucurbitaceae, Poaceae

- Tissue
ex) leaf, hypocotyl, cotyledon, callus

2. Make the slice or wound to the tissue

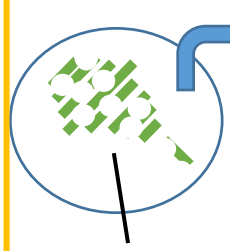
3. Immerse into enzyme solution (2h~6h ; 40~50 rpm)



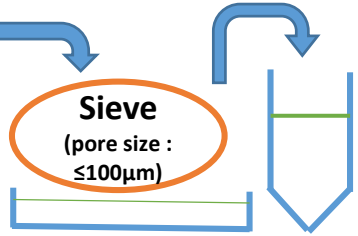
Enzyme solution
: Optimized mixture composed with different kind and gradient of cellulase, hemicellulase, pectinase for the material.

4. Purify and washing the protoplast

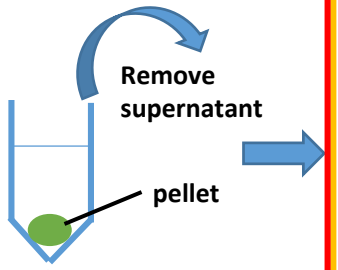
Repeat 2,3 times



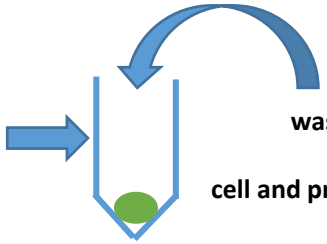
Digested material



Centrifuge
(low speed, 5 min.)



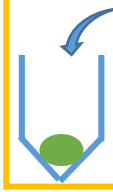
Centrifuge
(low speed, 5 min.)



washing buffer(WB)
or
cell and protoplast washing(CPW) solution
(10~20 ml)

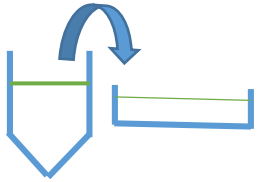
Dilution by pipetting or spinning the tube softly

5. Adjust cell density and culture the protoplast into media



culture media with the volume for adjusting cell density to 2*10 cells/ml (appropriate density for culture)

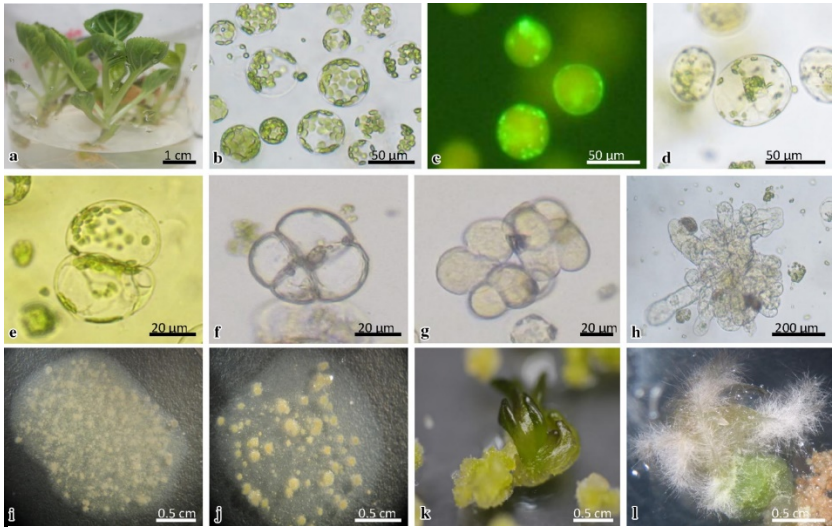
Dilution



Pour 2ml solution into petri dish

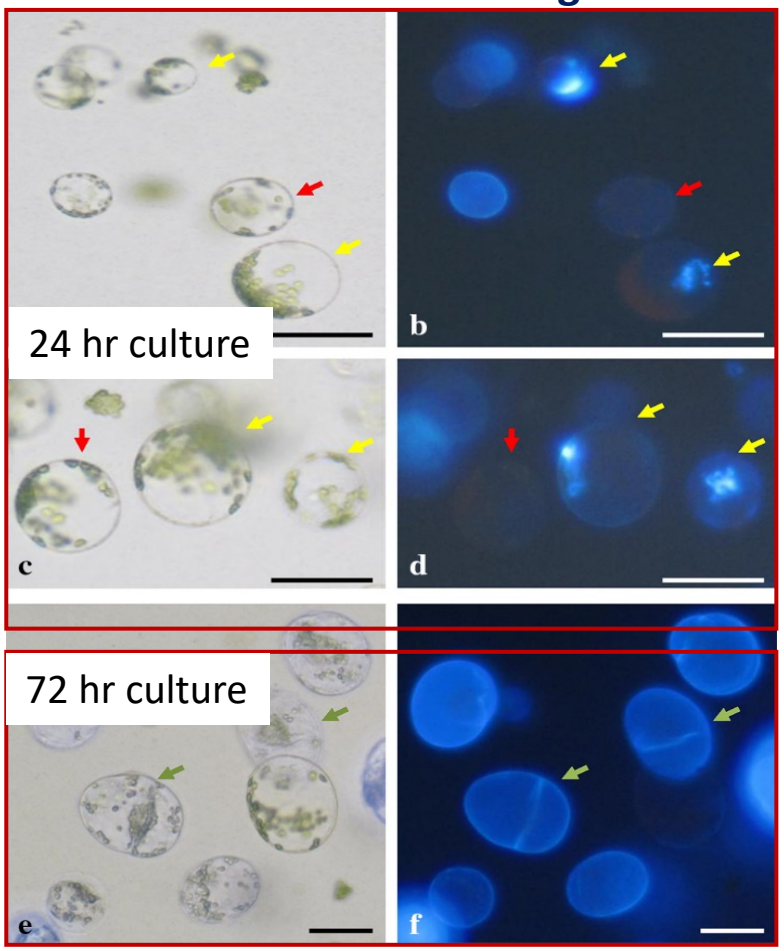
❑ Plant protoplast culture-*B. oleracea* 'capitata'

- Genotype-dependent viability and cell division
- Media-dependent regeneration (0.1 mg/L 2,4-D +0.2 mg/L zeatin+PSK)
- Cellulose regeneration completed in 72 hrs of culture



Factor	Shoot regeneration (% ± SE)	
Accession		
Kamienna Głowa	5.6 ± 0.9	b
Sława z Gołębiewa	4.4 ± 1.0	b
Oregon 123	0.0 ± 0.0	c
LM	9.6 ± 1.5	a
LM98	2.5 ± 0.9	bc
LM153	11.0 ± 1.2	a
Regeneration medium		
MS	2.9 ± 0.8	c
MS + 0.1 µM PSK-α	10.2 ± 1.1	a
B5B	1.5 ± 0.4	c
B5B + 0.1 µM PSK-α	7.5 ± 1.2	b

Calcofluor staining



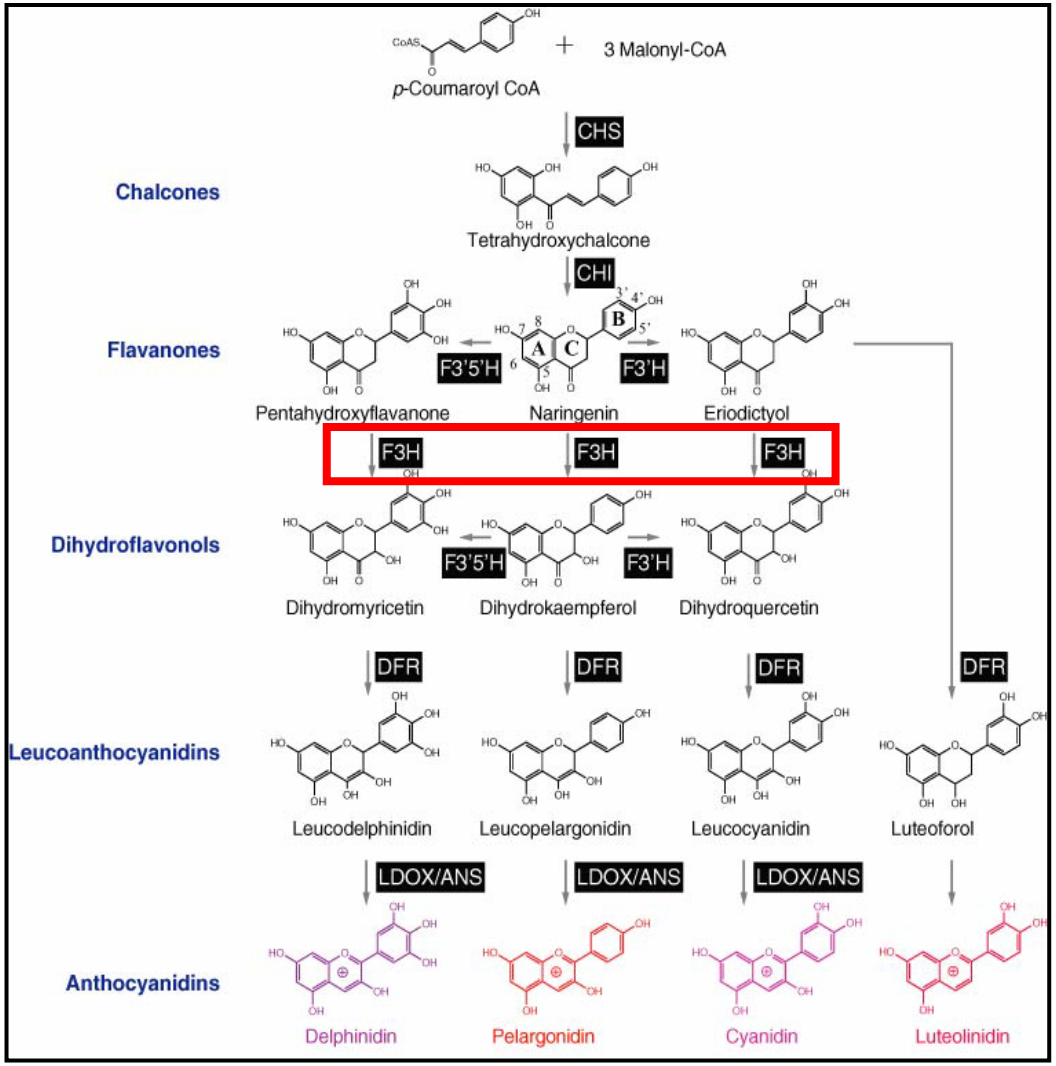
Kielkowska & Adamus(2018), J Plant Growth Reg

❑ Target flower color gene F3H in Petunia

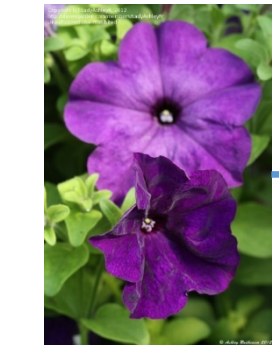
- *Petunia* has diverse flower colors as a model plant in tissue culture has the promising research value.

- The anthocyanins confer a diverse range of flower colors from pale yellow to blue-purple.
- Mutate F3H gene for modify flower color.(**F3H: Flavanone 3-hydroxylase**)

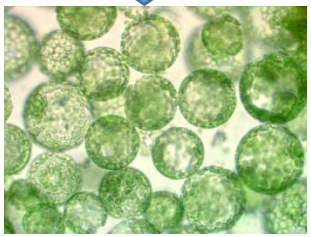
Anthocyanin Biosynthesis



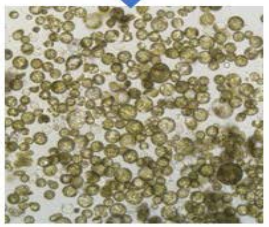
❑ Optimization of protoplast-based GFP delivery



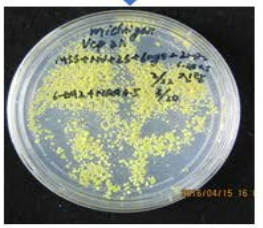
Petunia 'Madness Midnight'



In vitro seedlings (3weeks old)



Protoplast collection

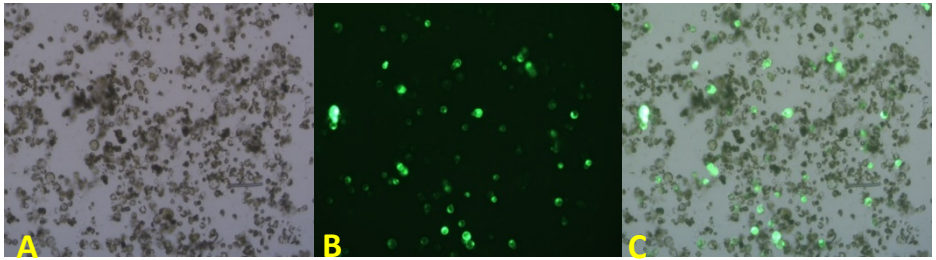


Callus induction

Enzyme digestion

Colony induction

Shoot induction



PEG-mediated plasmid PBI221:GFP transfect *petunia* protoplast in fluorescence microscope. A: Transformed protoplast in white light. B: Transformed protoplast in green light. C: combined A and B.

- 40% PEG concentration
- Incubation in room temperature for 20 min.
- GFP transformation efficiency ~40%

❑ Protoplast-derived CRISPR mutants

WT ‘Madness Midnight’ (Purple)

Mutant P4C4 [pale purplish pink (RHS 69D)]

❑ GMO vs. non-GM (DNA-free)

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유전자가위 기술 활용…`유전자교정` 사회적 논의 필요

기사입력 2015.04.01 17:42:37 | 최종수정 2015.04.01 19:32:34 28 0 0 보내기

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기사입력 2015.04.01 15:21:52 0 0 0 보내기

Trends in Biotechnology

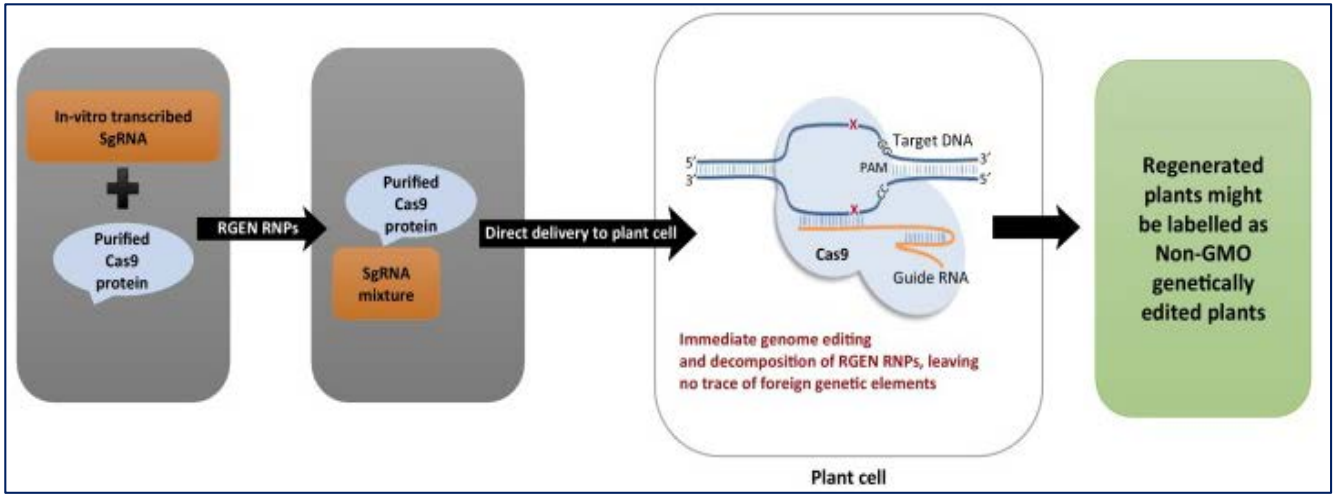
Volume 33, Issue 9, September 2015, Pages 489-491

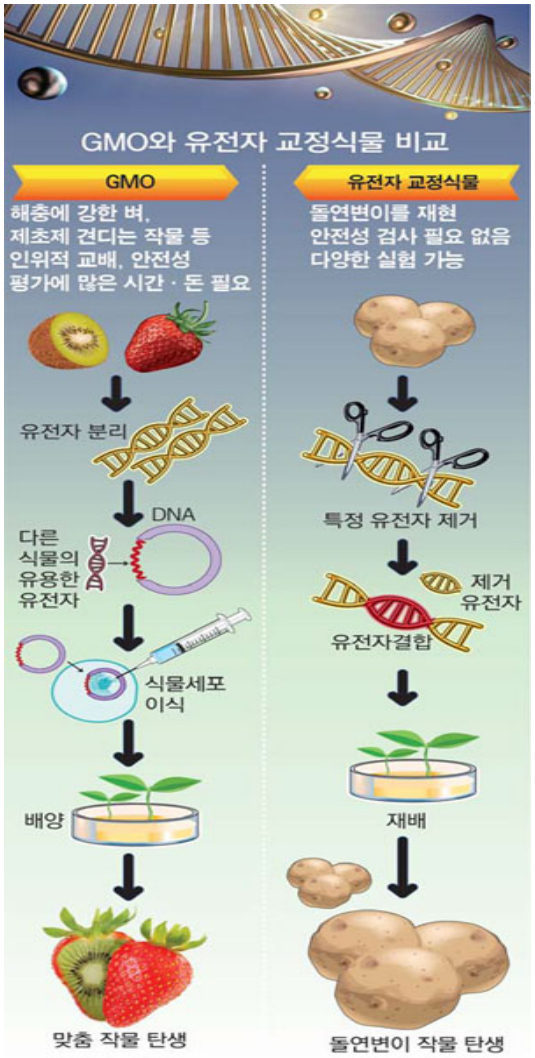
CellPress

Forum

Non-GMO genetically edited crop plants

Chidananda Nagamangala Kanchiswamy [1] ✉, Mickael Malnoy [1], Riccardo Velasco [1], Jin-Soo Kim [2, 3], Roberto Viola [1]

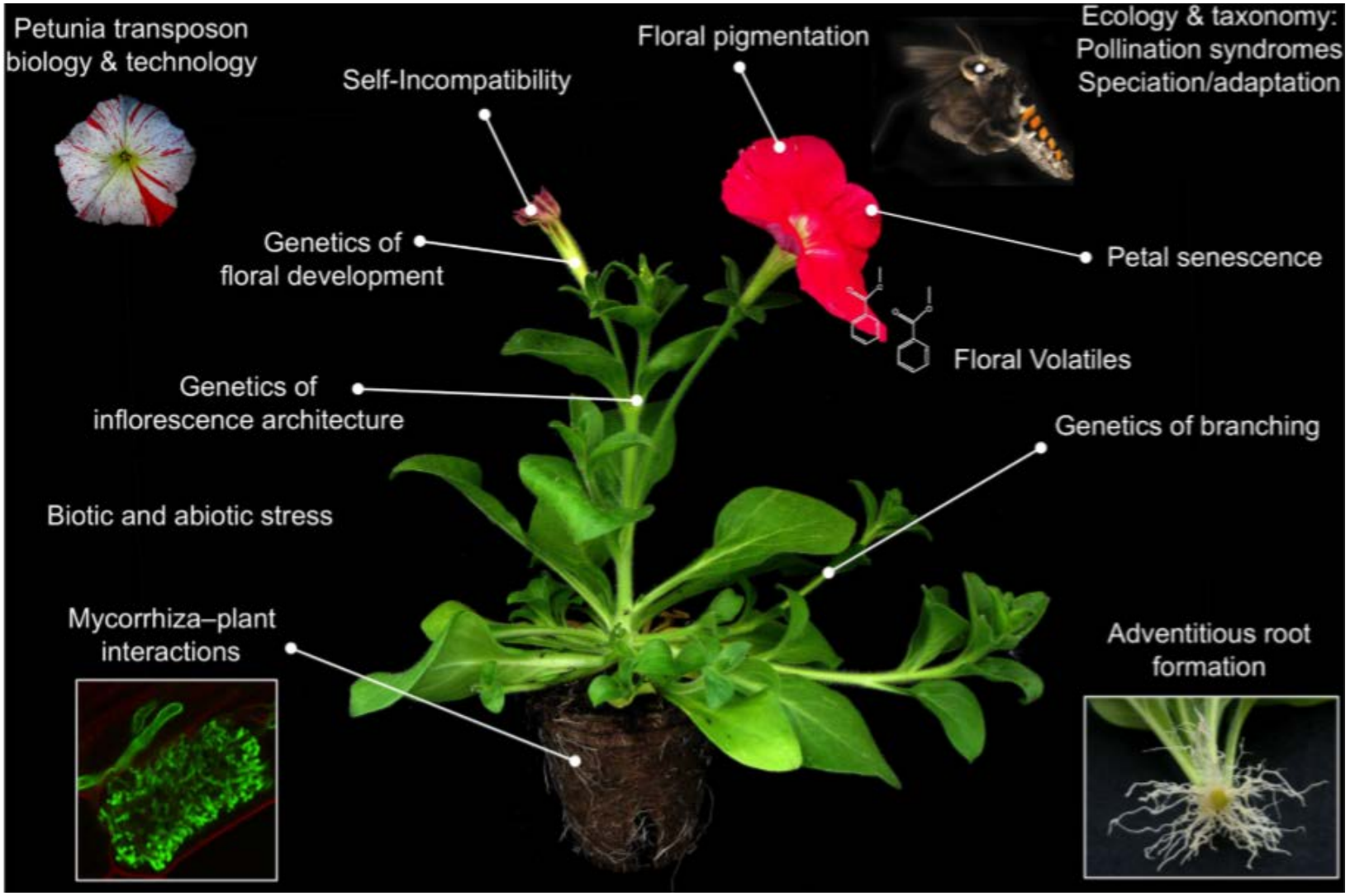




➡ Genome editing complex is degraded in the recipient cells

❑ CRISPR allows CRops for Ideal Seed PRactice!!

"A Whole Genome is now under Your Control !



Vandenbussche et al(2016), Fron Plant Sci 7:72