PlantEd COST Action CA18111 Genome Editing in Plants

INA3 INSTITUTE OF APPLIED BIOSCIENCES ΙΝΣΤΙΤΟΥΤΟ ΕΦΑΡΜΟΣΜΕΝΩΝ ΒΙΟΕΠΙΣΤΗΜΩΝ CERTH

Zinc-Finger Nucleases as Genome Editing Tools for Specific and Efficient Plant Breeding

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cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

A NEW EARTH

- Earth's climate is changing at a very fast pace. Changes in weather conditions threaten food production

The worst drought for half a century in the U.S. has destroyed one-sixth of the country's expected corn crop

- Global food system

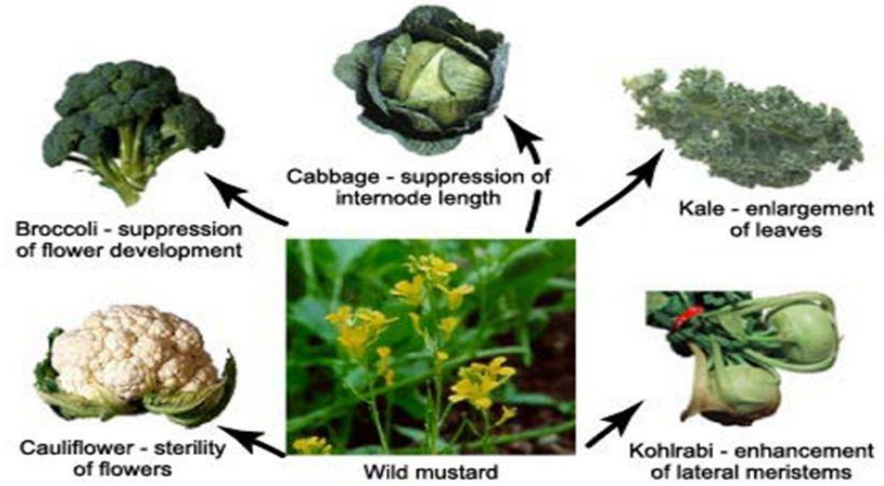
- In 2050, we will need to feed 10 billion people
- In 2050, we will need 50% more energy and 40% more water to feed the planet Earth
- Energy, fuel and industrial raw material production are becoming increasingly dependent on **agricultural output** in the transition to a sustainable, bio-based society

NEW EARTH REQUIRES NEW BIOTECHNOLOGICAL TOOLS, WHICH ARE **EFFICIENT**, **SUFFICIENT** AND **SPECIFIC**

Atropos, a goddess of fate, cutting the thread of life

Accumulation of natural mutations in DNA→ Biodiversity

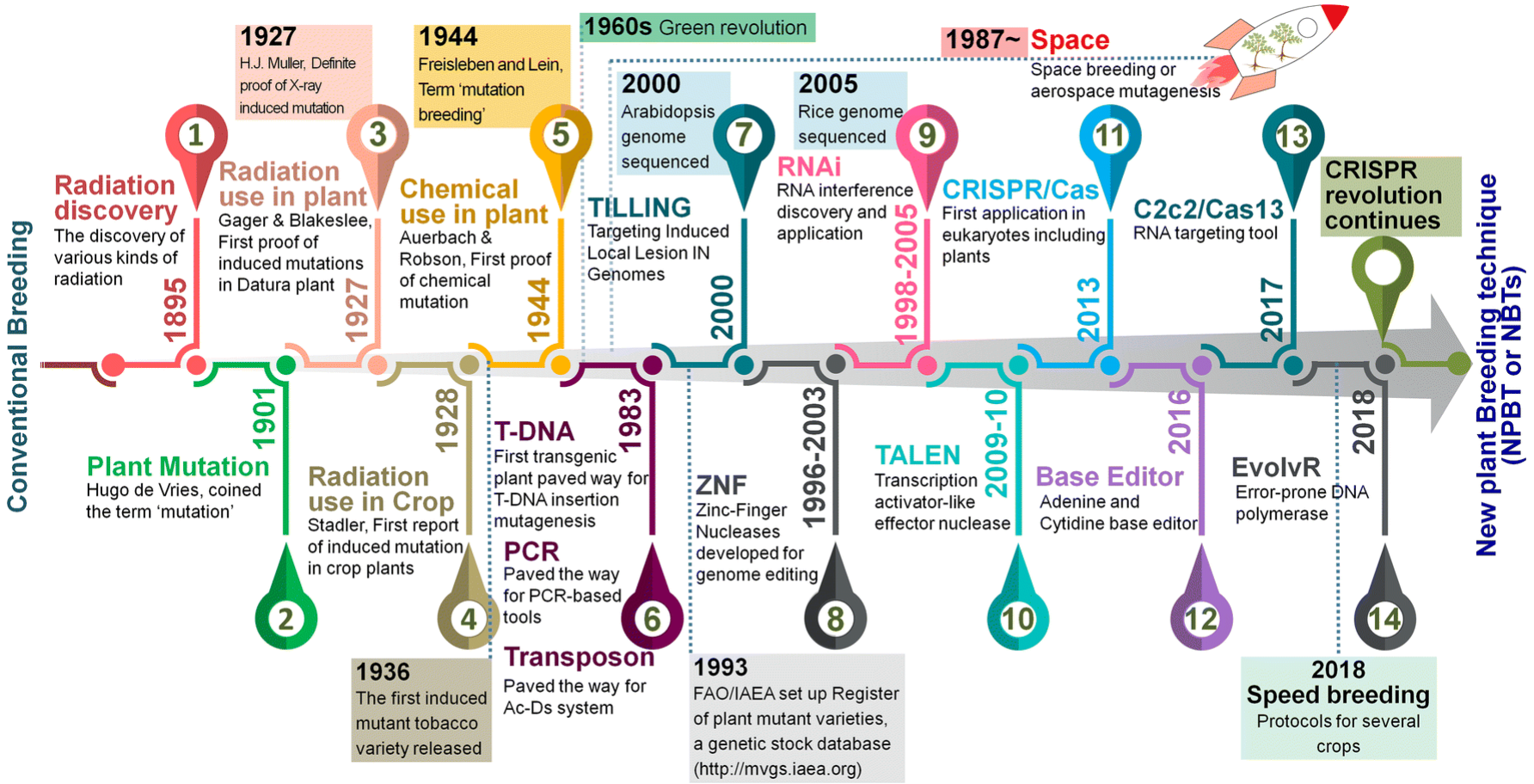
Problem: *plants do not adapt fast, natural mutations occur randomly at low frequency and development of new varieties is a lengthy process (~9 years)*



Broccoli, cauliflower, cabbages, Brussels sprouts and kale are all varieties of a single species of wild mustard established by selective breeding.

Genetic modifications by Nature

EVOLUTION OF PLANT MUTAGENESIS



Shelake, R.M., Pramanik, D. & Kim, JY. Plant Biotechnol Rep (2019).

Exploring uncharted territory

the technology is what you make it to be

OUR AIMS:

1. Non-transgenic approach

2. High-throughput

3. Beyond proof-of-principle approach

4. Coding region disruption

ZFN application in tomato

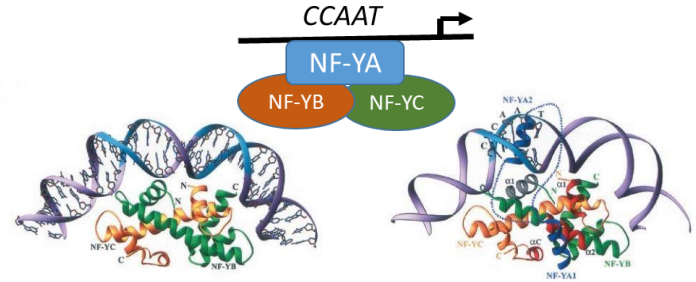
Inspiration

LEC1 is embryonic regulator that mediates the switch between embryo and vegetative development.

Arabidopsis mutants are desiccation intolerant, have trichomes on cotyledons and exhibit precocious meristem activation

The target gene

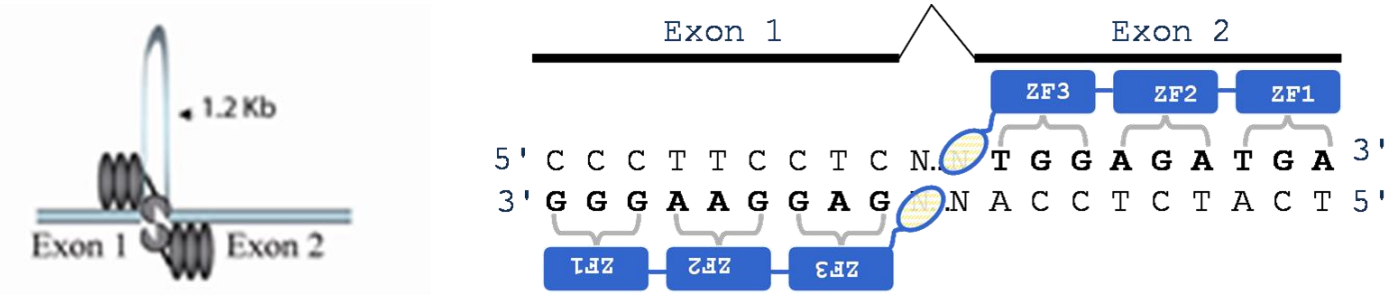
- **Lec1-like** (NF-YB6) in tomato
- It encodes the subunit B of the trimeric NF-Y transcription factor, which binds the CCAAT DNA motif



- The target gene is **essential** in embryo development & **is not characterized** in tomato

DNA recognition by L1L4-ZFPs

Can this ZFN design function in vivo?



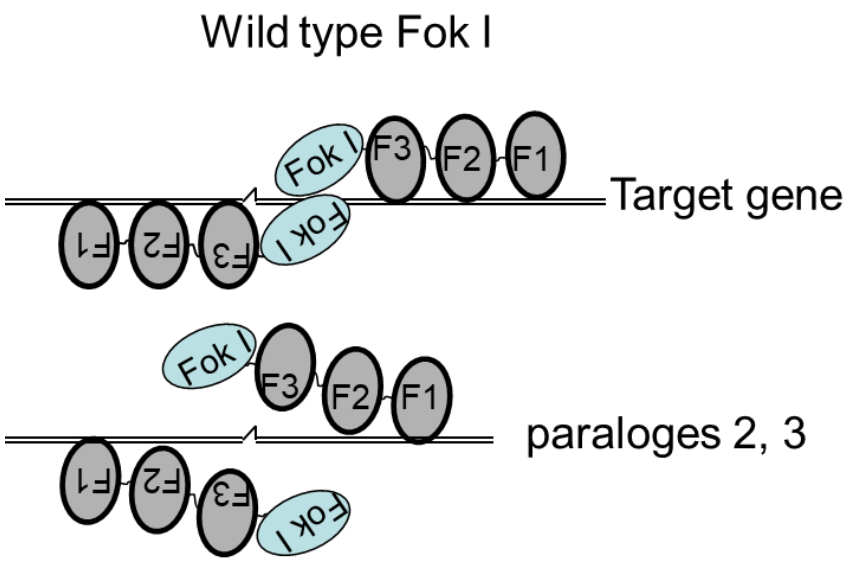
Methodologically speaking this is the first **in vivo** testing whether ZFNs can efficiently edit the genome when their DNA binding sites are spaced by an intron.

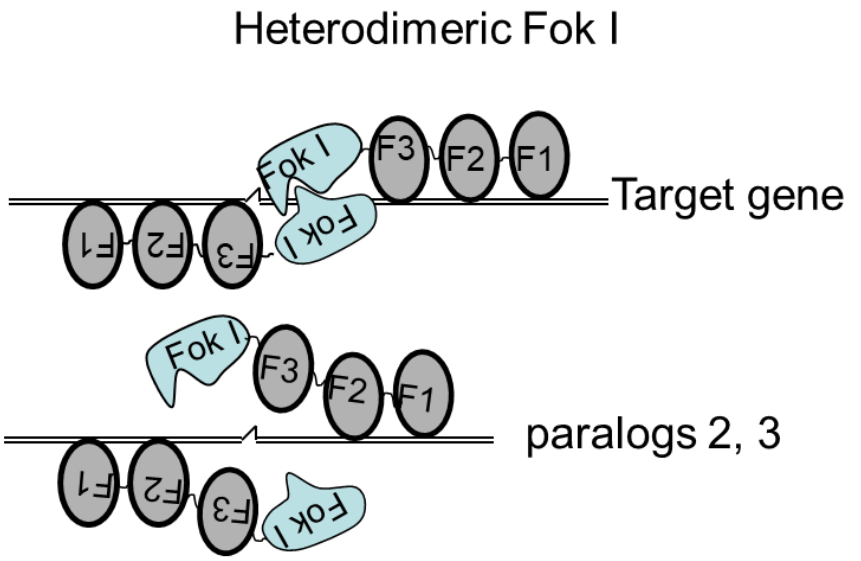
Nuclear DNA in eukaryotes is assembled into chromatin by nucleoprotein complexes

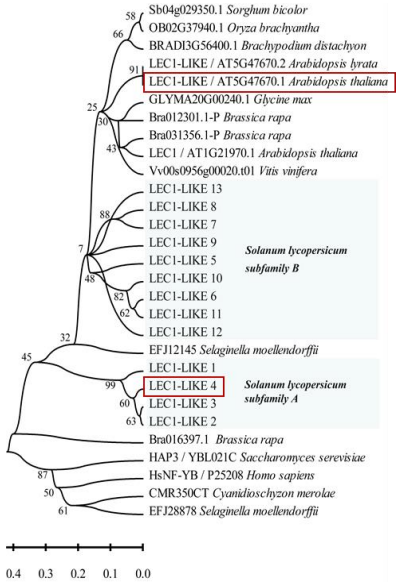
Processes that involve DNA looping: DNA replication and repair, site-specific recombination, transcription regulation and DNA cleavage by many restriction endonucleases

If it works, it may have implications for other FokI endonuclease-based targeted genome editing technologies (e.g TALENs)

Scheme of the ZFN constructs to bind the nine base pairs of DNA







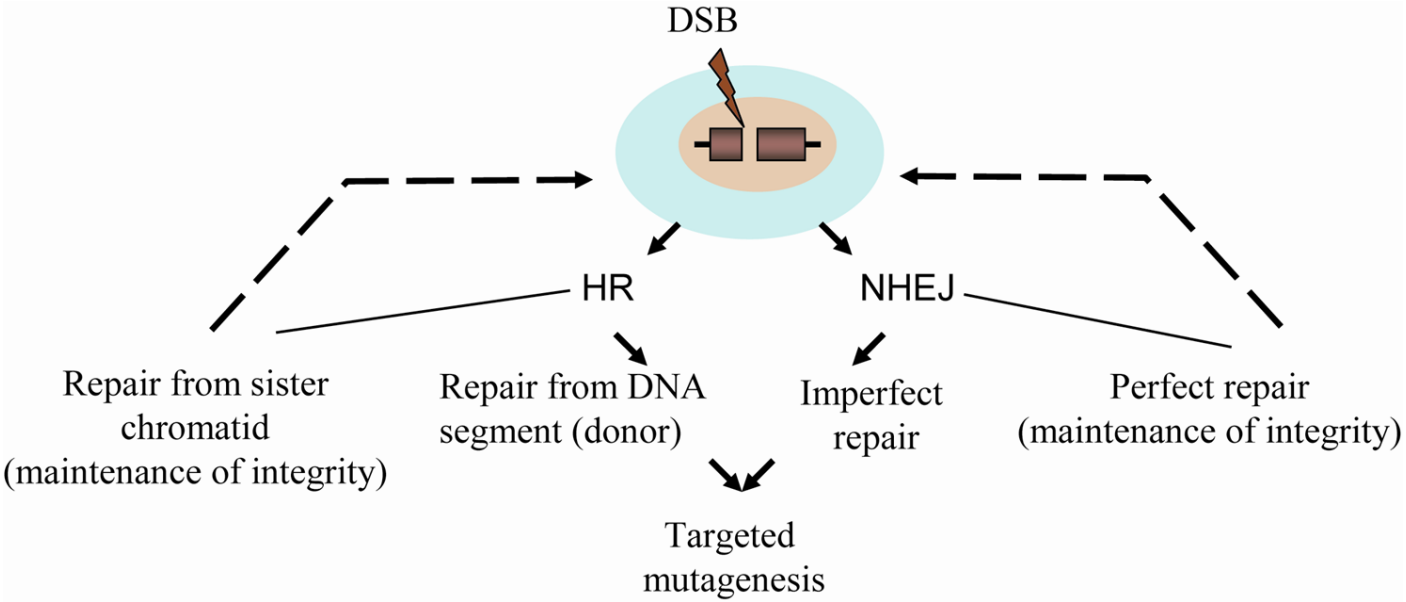
-Miller JC et al., 2007. Nature Biotechnology

-Szczepek M et al., 2007. Nature Biotechnology

The tomato L1L gene family (13 paralogs)

Hilioti Z, Ganopoulos I, Bossis I, Tsaftaris A, 2014. ***LEC1-LIKE paralog transcription factor: how to survive extinction and fit in NF-Y protein complex****. Gene 543: 220-233.*

Repair of DNA Double-stand breaks can be either recombinogenic or mutagenic



Adjustments: To induce mutations via **NHEJ**, manipulate the system (seeds) and the conditions (post-delivery of ZFNs)

Suboptimal conditions favor NHEJ

Design a strategy

The **seed system** is multicellular, available any time, contains the embryo buffering of deleterious mutations and enabling the system to be more tolerant than the single protoplast cells

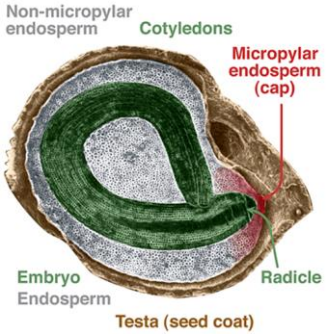
- *L1L4*-ZFNs: in a plasmid DNA (**plant expression vector**)

-Delivery method: **electroporation of seeds**

-Transient expression of ZFNs, w/out selection

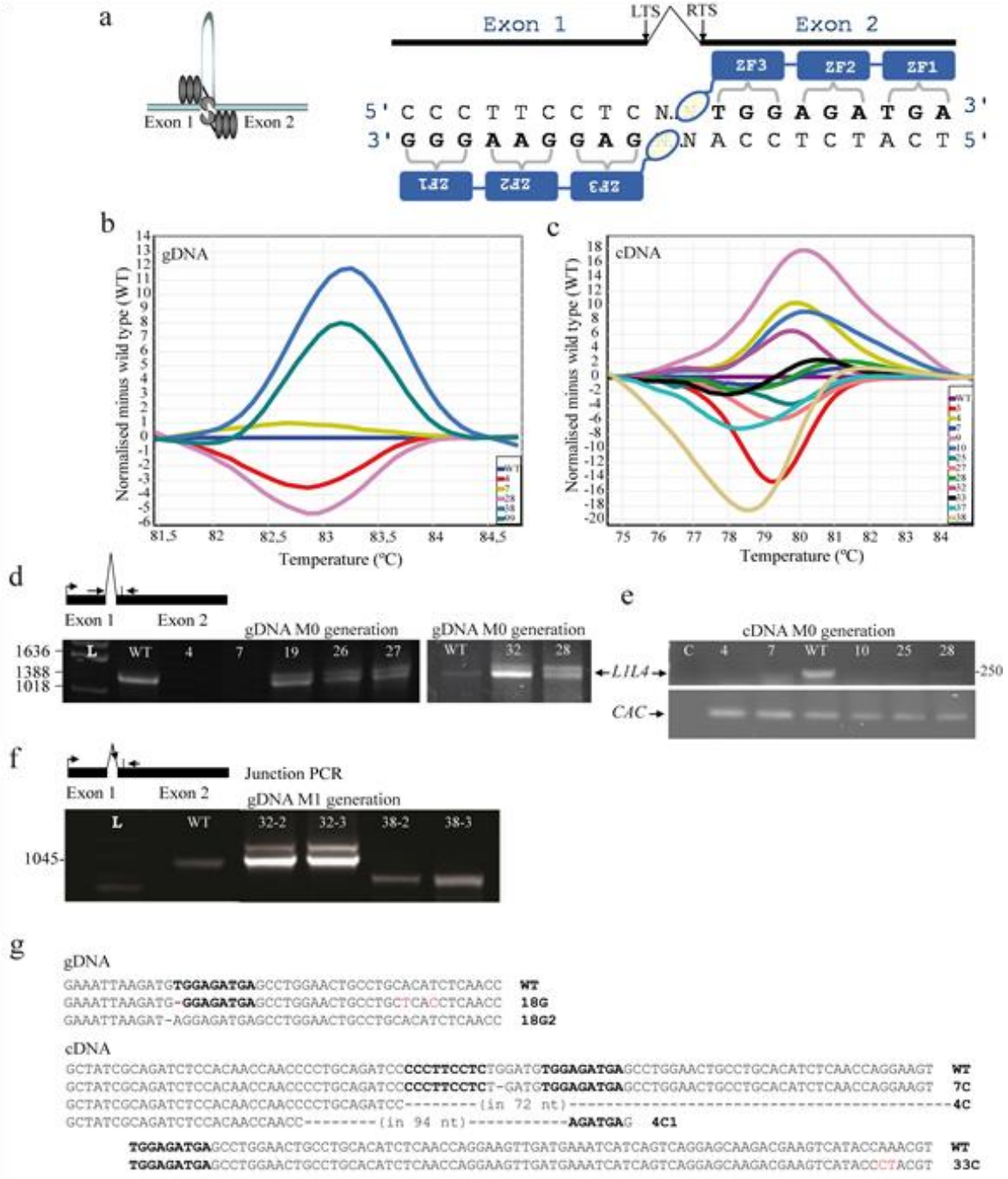
-Leave O/N under suboptimal conditions

-Transfer to soil next day



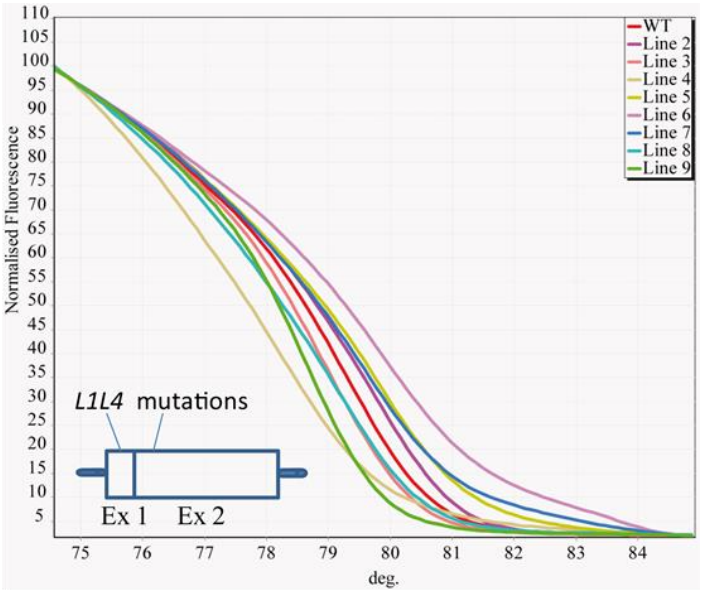
Hilioti Z, 2018: Non-transgenic Approach to Deliver ZFNs in Seeds for Targeted Genome Engineering. In: Liu J. (eds) Zinc Finger Proteins. Methods Mol Biol, vol 1867

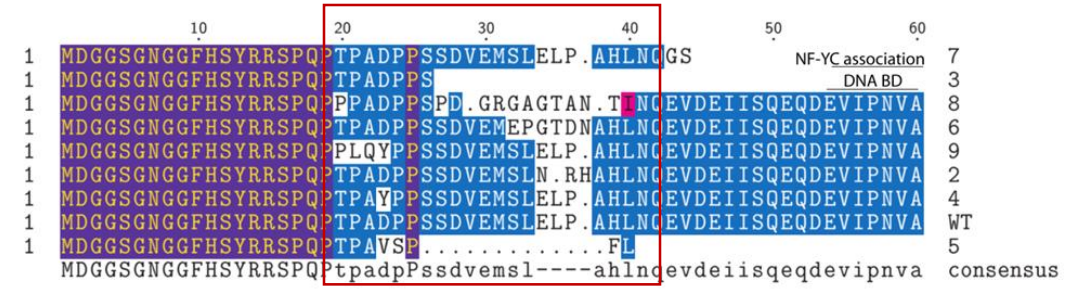
Screening plants for the presence of mutations
i) at the target site and
ii) off-target sites

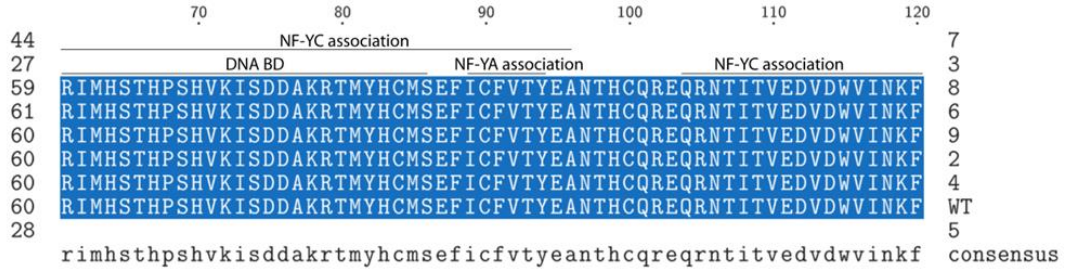


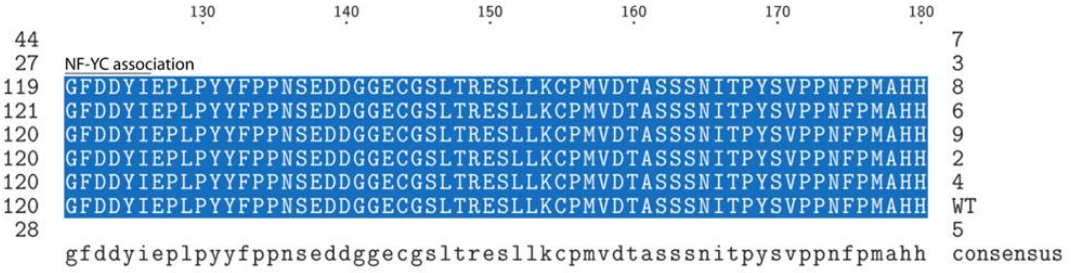
Hilioti Z, Ganopoulos I, Ajith S, Bossis I, Tsaftaris A, 2016: ***A novel arrangement of zinc finger nuclease system for in vivo targeted genome engineering: the tomato LEC1-LIKE4 gene case.*** *Plant Cell Reports 35 (11): 2241-2255.*

From Genome Editing to basic research









M4 generation of plants

Mutations in DNA correspond to a region between the residues 20 and 38 of L1L4 protein, which suggests that this region is under relaxed selection and mutations can be tolerated

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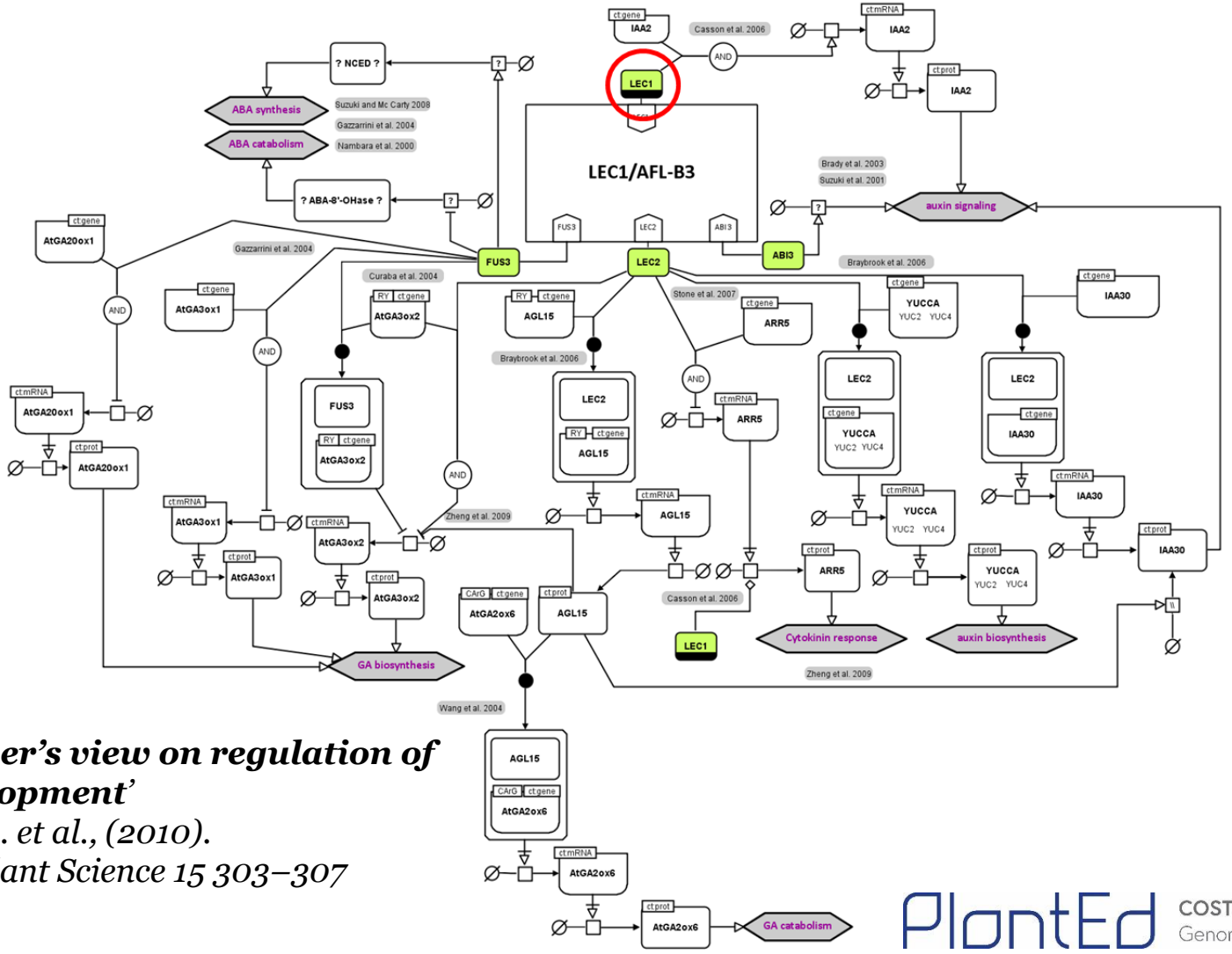
The target gene is **a master transcription factor**
A 'Systems Biology' approach-finding the connections among the parts



'An engineer's view on regulation of seed development'
by Junker A. et al., (2010).
Trends in Plant Science 15 303–307

From Genome Editing to Phenotyping

What is the probability for a DNA disruption to yield a phenotype?

Probability<1:2000x1:2000=1:4.000.000

Phyllotaxis: In the position of the first compound leaf, two compound leaves are found from the same node in slightly spiral arrangement. The wild-type had alternate leaves

From Genome Editing to Phenotyping during reproduction

WT

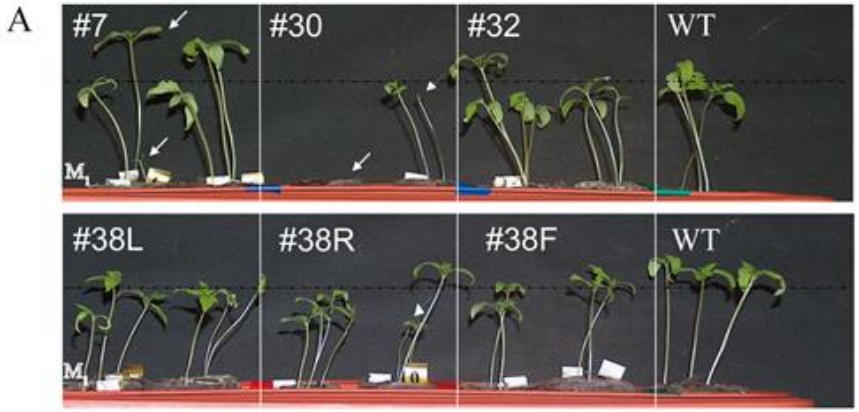
ZFN-*L1L4*

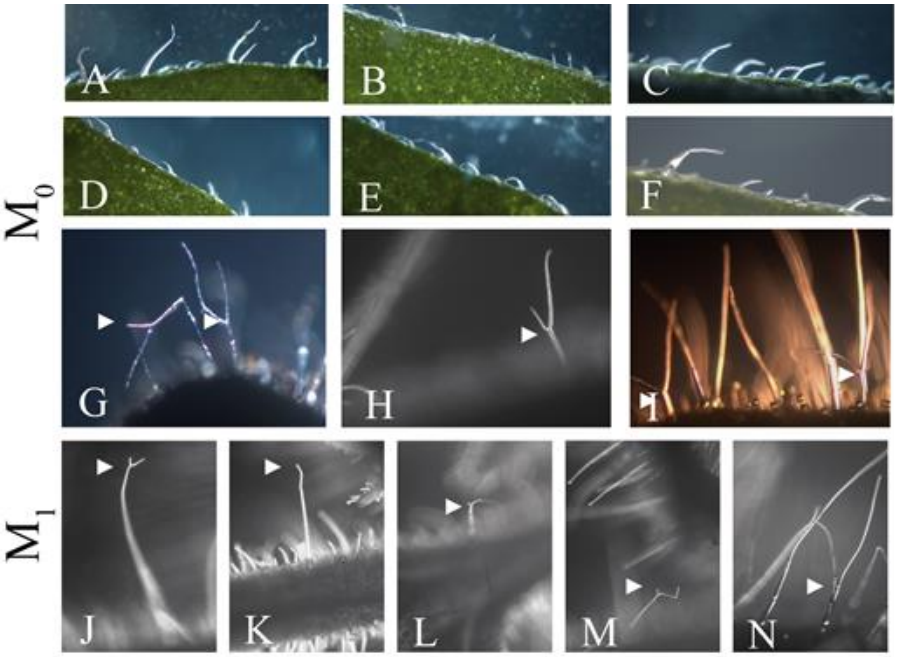
ZFN-*L1L4*

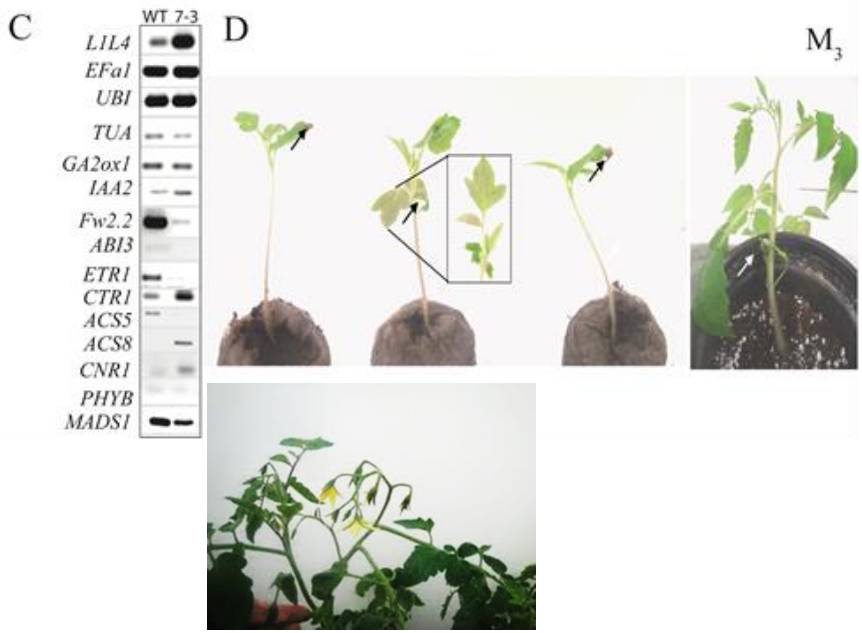
Pedicel length and its correlation to fertility

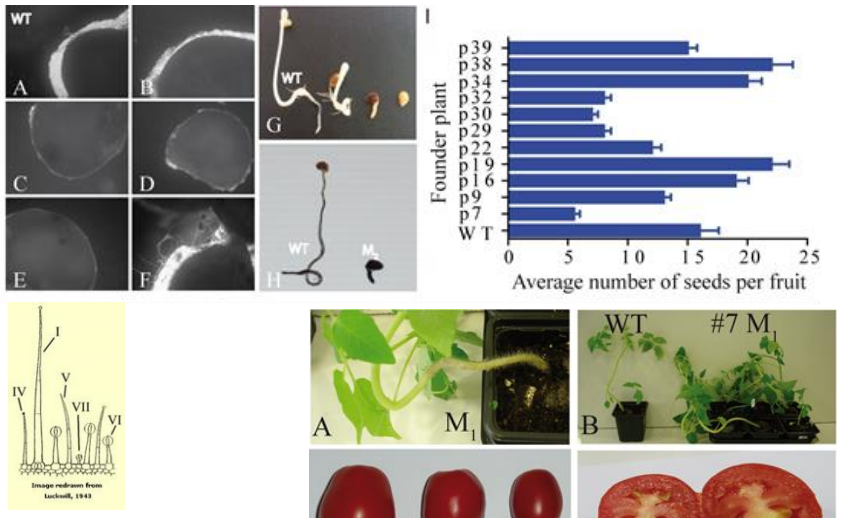
- The most prominent phenotype was observed at this stage: 45% (18 out of 40) of the founders had short pedicel and they were infertile

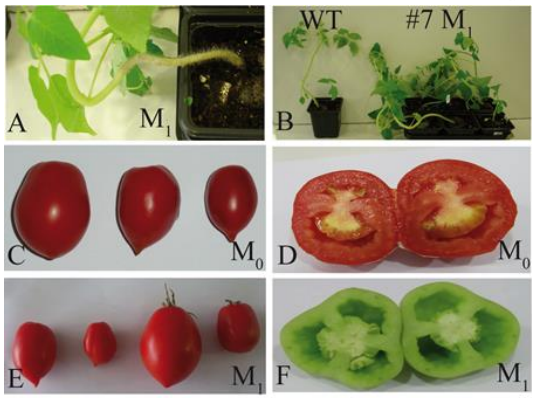
- From the fertile mutants (~50%), 23 % were created by the wild-type FokI construct while 27 % by the heterodimer FokI. This difference in effectiveness was considered to be not statistically significant (Chi square test)











Natural selection on heritable mutations and a population carrying mutations advantageous to the organism's reproductive success (fitness)

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From Genome Editing to Fruit Quality parameters

Fruit quality parameters

lines	Diameter (mm)	Length (mm)	Weight (g)	Lightness	a*	b*	a*/b*	Firmness (N)	Soluble solid content (%)	Titratable acidity	Taste index
WT	34.32±0.44**a**	51.35±0.4**a**	32.66±1.52**ab**	46.08±2.48**a**	22.03±2.98**a**	19.65±2.57**abc**	1.12±0.02**ab**	5.37±0.56**a**	3.6±0.17**d**	0.68±0.04**a**	0.95±0.03**abc**
2	34.06±1.38**a**	53.17±1.08**a**	35.77±5.25**ab**	44.56±1.92**a**	22.57±1.68**a**	22.33±3.09**ab**	1.04±0.10**ab**	4.46±0.36**a**	3.85±0.06**cd**	0.65±0.03**ab**	0.95±0.01**abc**
3	32.7±0.38**a**	51.55±1.00**a**	29.35±0.55**b**	46.13±2.66**a**	21.67±1.67**a**	24.76±1.88**a**	0.94±0.02**b**	4.92±0.9**a**	3.9±0.4**cd**	0.49±0.01**d**	0.89±0.02**c**
4	35.79±0.29**a**	56.31±1.23**a**	40.51±1.86**a**	42.15±1.69**a**	22.17±2.07**a**	20.35±1.83**abc**	1.09±0.00**ab**	4.84±0.34**a**	4.55±0.1**ab**	0.56±0.01**bcd**	0.97±0.01**ab**
5	34.34±1.6**a**	55.18±3.29**a**	37.01±5.51**ab**	47.73±0.19**a**	17.37±0.11**a**	14.77±0.99**c**	1.19±0.09**a**	5.21±0.36**a**	3.93±0.03**cd**	0.56±0.03**cd**	0.91±0.01**bc**
6	36.02±1.11**a**	53.57±1.53**a**	38.18±2.9**ab**	45.82±1.77**a**	19.48±1.97**a**	16.24±2.21**bc**	1.21±0.04**a**	5.28±0.84**a**	4.2±0.1**bc**	0.62±0.04**abc**	0.96±0.02**ab**
7	36.69±0.35**a**	52.11±1.48**a**	38.61±1.02**ab**	43.25±1.87**a**	19.57±1.20**a**	17.35±2.34**bc**	1.17±0.15**a**	4.65±0.35**a**	4.58±0.2**ab**	0.65±0.01**ab**	1.00±0.00**a**
8	35.6±2.57**a**	53.73±1.62**a**	31.77±2.4**ab**	44.42±2.02**a**	22.28±2.55**a**	21.01±2.13**abc**	1.06±0.07**ab**	5.4±0.74**a**	3.81±0.51**cd**	0.55±0.04**cd**	0.90±0.04**c**
9	35.66±1.08**a**	55.12±2.01**a**	38.98±3.44**ab**	44.09±1.47**a**	21.72±1.51**a**	17.65±2.13**abc**	1.25±0.08**a**	4.55±0.4**a**	4.68±0.46**a**	0.55±0.02**cd**	0.98±0.02**ab**

Values are mean±SE. The values followed by the same letter, in the same column/ parameter are not significantly different (Duncan's new multiple range test at P<0.05)

Gago, C., Drosou, V., Paschalidis, K., Guerreiro, A., Miguel, G., Antunes, D., Hilioti, Z, 2017. ***Targeted gene disruption coupled with metabolic screen approach to uncover the LEAFY COTYLEDON1-LIKE4 (L1L4) function in tomato fruit metabolism****. Plant Cell Reports 36: 1065-1082.*

From Genome Editing to Fruit Quality parameters

Fruit composition

	Moisture (%)	Fiber (%)	Protein (%)	Ash (%)	Fructose (g/kg DW)	Glucose (g/kg DW)	Ascorbic acid (g/kg DW)	Citric acid (g/kg DW)	Oxalic acid (g/kg DW)	Quinic acid (g/kg DW)	Shikimic acid (g/kg DW)	Succinic acid (g/kg DW)
WT	95.34±0.21**a**	1.77±0.08**b**	0.81±0.11**a**	0.43±0.02**a**	43.58±1.28**b**	34.17±5.12**a**	1.03±0.01**b**	21.72±3.46**a**	20.67±3.9**a**	6.67±0.12**bc**	0.22±0.004**c**	n.d.
2	94.88±0.11**ab**	1.99±0.18**ab**	0.48±0.03**e**	0.43±0.04**a**	53.2±0.82**a**	32.92±0.32**a**	1.00±0.07**b**	7.66±1.77**d**	5.84±1.11**de**	n.d.	0.22±0.007**c**	0.26±0.08**d**
3	94.25±0.22**bc**	2.08±0.18**ab**	0.57±0.01**de**	0.48±0.05**a**	54.16±1.15**a**	34.17±0.56**a**	1.21±0.01**b**	15.93±1.08**bc**	9.26±0.37**cd**	5.03±1.16**bc**	0.26±0.011**a**	0.36±0.08**d**
4	93.97±0.1**cd**	2.56±0.18**a**	0.63±0.04**bcde**	0.56±0.02**a**	52.32±1.67**a**	33.71±0.52**a**	2.80±0.95**a**	10.96±0.41**cd**	9.03±0.53**cd**	2.66±0.84**d**	0.25±0.009**ab**	1.16±0.03**a**
5	94.18±0.44**bc**	2.39±0.33**a**	0.58±0.04**de**	0.59±0.06**a**	53.65±1.5**a**	33.95±0.35**a**	n.d.	15.73±1.67**bc**	3.74±0.29**e**	3.64±0.25**cd**	0.25±0.006**ab**	0.26±0.01**d**
6	93.56±0.32**cd**	2.10±0.16**ab**	0.71±0.04**abcd**	0.58±0.02**a**	54.1±0.87**a**	34.58±0.31**a**	0.92±0.05**b**	16.63±0.93**abc**	12.71±1.04**bc**	11.51±1.26**a**	0.24±0.001**abc**	0.70±0.03**c**
7	93.4±0.09**cd**	2.12±0.07**ab**	0.75±0.02**abc**	0.51±0.04**a**	52.69±0.51**a**	33.57±0.32**a**	0.98±0.04**b**	20.29±2.63**ab**	17.17±1.49**ab**	3.73±0.22**cd**	0.23±0.005**bc**	0.95±0.06**b**
8	94.14±0.41**bc**	2.36±0.14**a**	0.78±0.06**ab**	0.52±0.07**a**	46.89±1.25**b**	31.51±0.6**a**	0.98±0.09**b**	21.88±0.43**a**	19.19±0.68**a**	4.23±0.11**cd**	0.25±0.004**ab**	1.25±0.09**a**
9	93.2±0.31**d**	2.31±0.14**ab**	0.60±0.02**cde**	0.55±0.07**a**	54.21±0.49**a**	34.26±0.06**a**	1.04±0.11**b**	13.87±0.86**c**	12.78±0.96**bc**	3.23±0.19**cd**	0.25±0.007**ab**	0.16±0.08**d**

Values are mean±SE. The values followed by the same letter, in the same column/ parameter are not significantly different (Duncan's new multiple range test at P<0.05). n.d = not detected.

Targeted metabolic analysis

Gago, C., Drosou, V., Paschalidis, K., Guerreiro, A., Miguel, G., Antunes, D., Hilioti, Z, 2017. ***Targeted gene disruption coupled with metabolic screen approach to uncover the LEAFY COTYLEDON1-LIKE4 (L1L4) function in tomato fruit metabolism****. Plant Cell Reports 36: 1065-1082.*

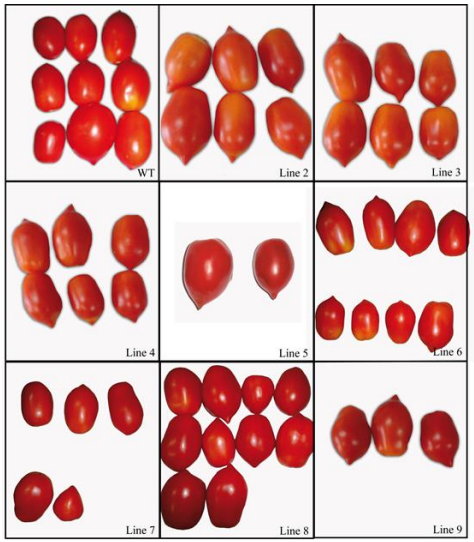
From Genome Editing to Fruit Quality parameters

- The current data establish **a relationship between high-order regulators of gene expression** and **metabolic phenotypes** of tomato fruit.

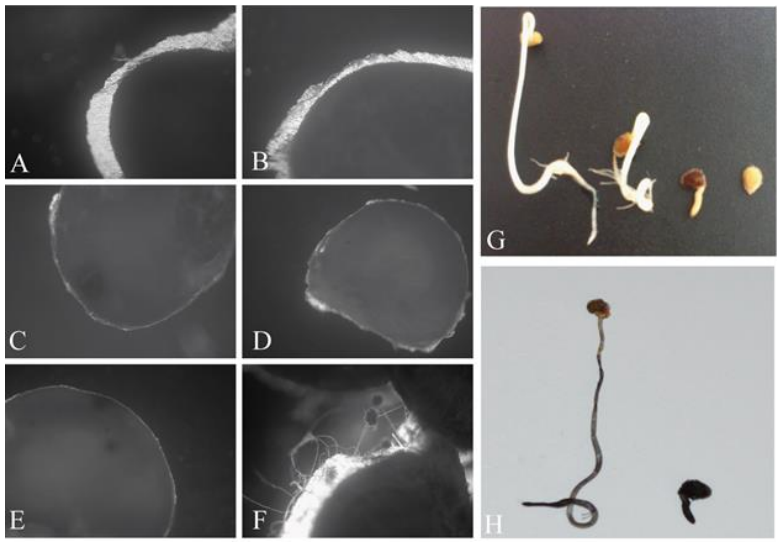
- A **high-fructose-to-glucose-ratio** phenotype was prominent in mutant fruits, while **low oxalic** and **citric acid** contents were also detected. Line 4 had high **vitamin C** content.

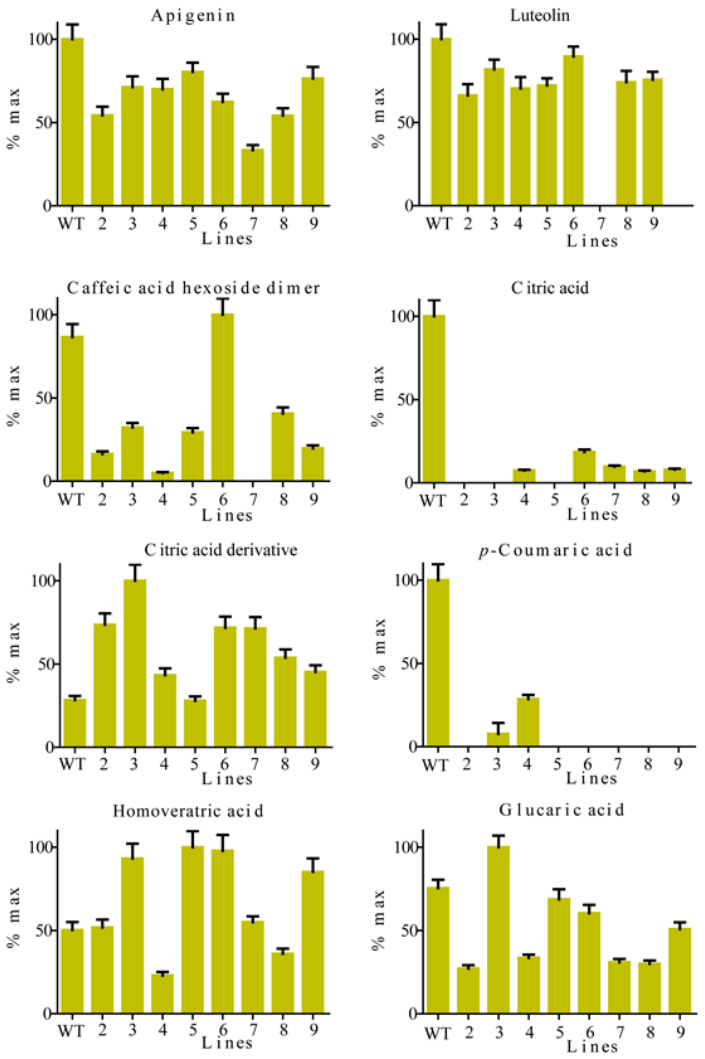
- The identification of identical phenotypes (e.g., metabolic and morphologic) in several mutant fruits shows the robustness of the ZFN technology.

- L1L4 TF appears to be **a negative regulator of fiber, fructose, succinic and shikimic acid** biosynthesis and **a positive regulator of citric and oxalic acid**.



From Genome Editing to seed metabolites

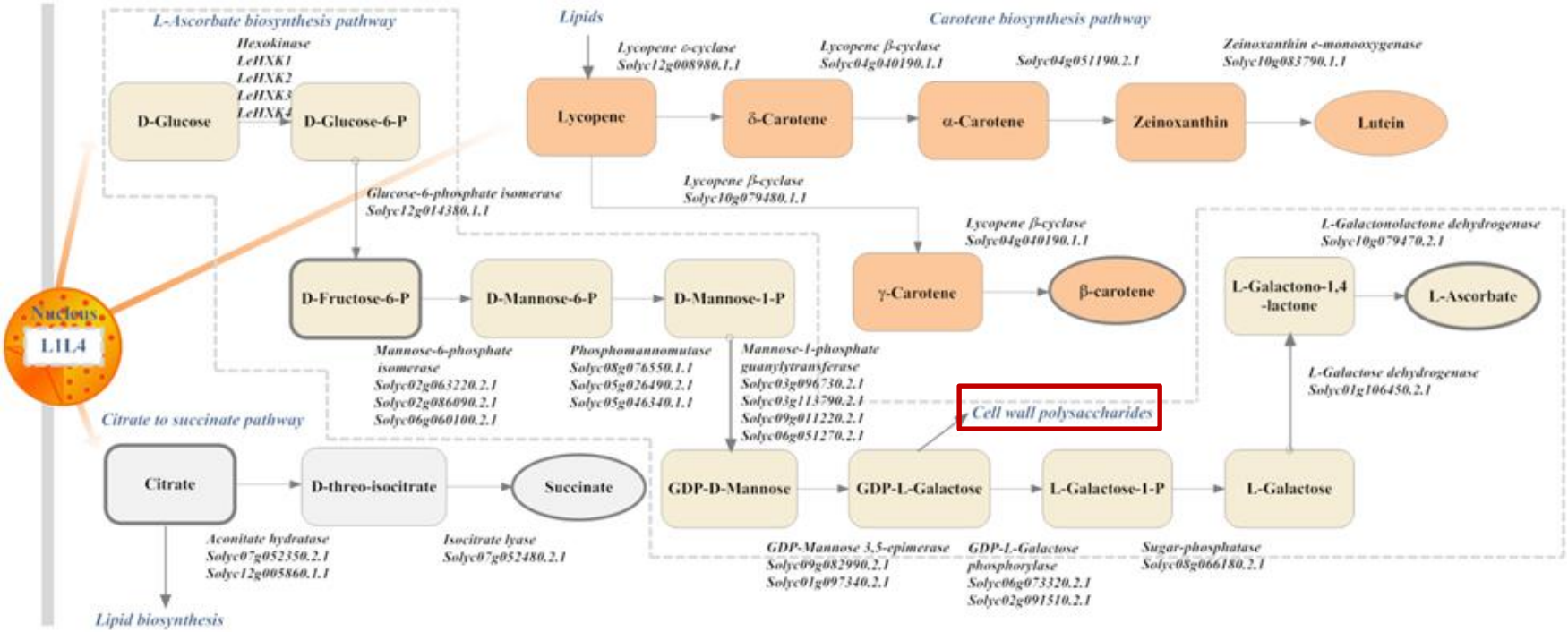




Gago, C., Drosou, V., Paschalidis, K., Guerreiro, A., Miguel, G., Antunes, D., Hilioti, Z, 2017.
Targeted gene disruption coupled with metabolic screen approach to uncover the LEAFY COTYLEDON1-LIKE4 (L1L4) function in tomato fruit metabolism. *Plant Cell Reports 36: 1065-1082.*

Untargeted LC-MS/MS metabolic analysis

From Genome Editing to reconstruction of metabolic biosynthetic pathways

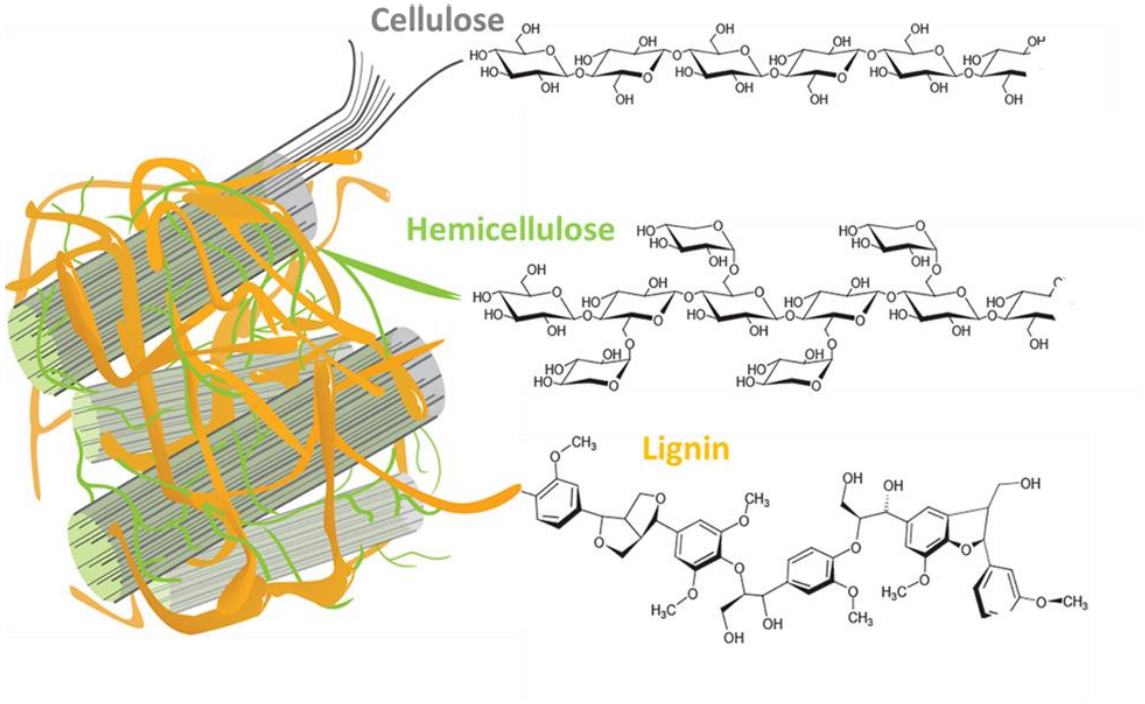


Inference of putative metabolic pathways affected by L1L4 coding sequence disruption in lycopene, ascorbate and citrate biosynthesis. The predicted identities of genes encoding key enzymes in the metabolic pathways are shown. Key metabolites in each pathway are shown with squares and end products with circles. Significantly affected metabolites are shown with thick border.

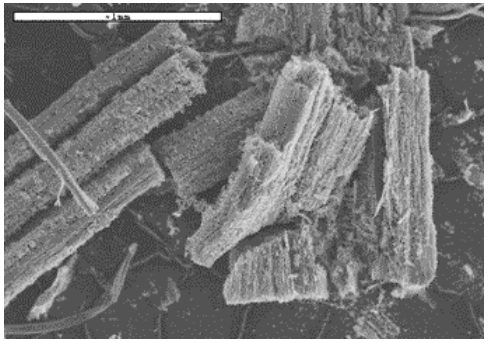
Gago, C., Drosou, V., Paschalidis, K., Guerreiro, A., Miguel, G., Antunes, D., Hilioti, Z, 2017. Plant Cell Reports 36: 1065-1082.

Lignocellulosic biomass

engineering plant feedstocks with tailored cell walls via genome editing?



The last decade plant feedstocks became a major industry lignocellulosic material



From Genome Editing to modifying plant cell walls

Genetic engineering can alter the **content** and **composition** of plant cell wall polymers, particularly lignin

The recalcitrance of cell walls to hydrolysis to yield the component sugars of the major cellulosic and hemicellulosic polysaccharides affects **biofuels** production and the **digestibility of forage crops** for animal feed

High-value products from isolated **lignin** are **carbon fibers**, **commodity chemicals**, **engineering plastics**, **thermoplastic elastomers**, **polymeric foams** and **membranes**, a variety of **fuels** and **chemicals** (all currently sourced from petroleum).

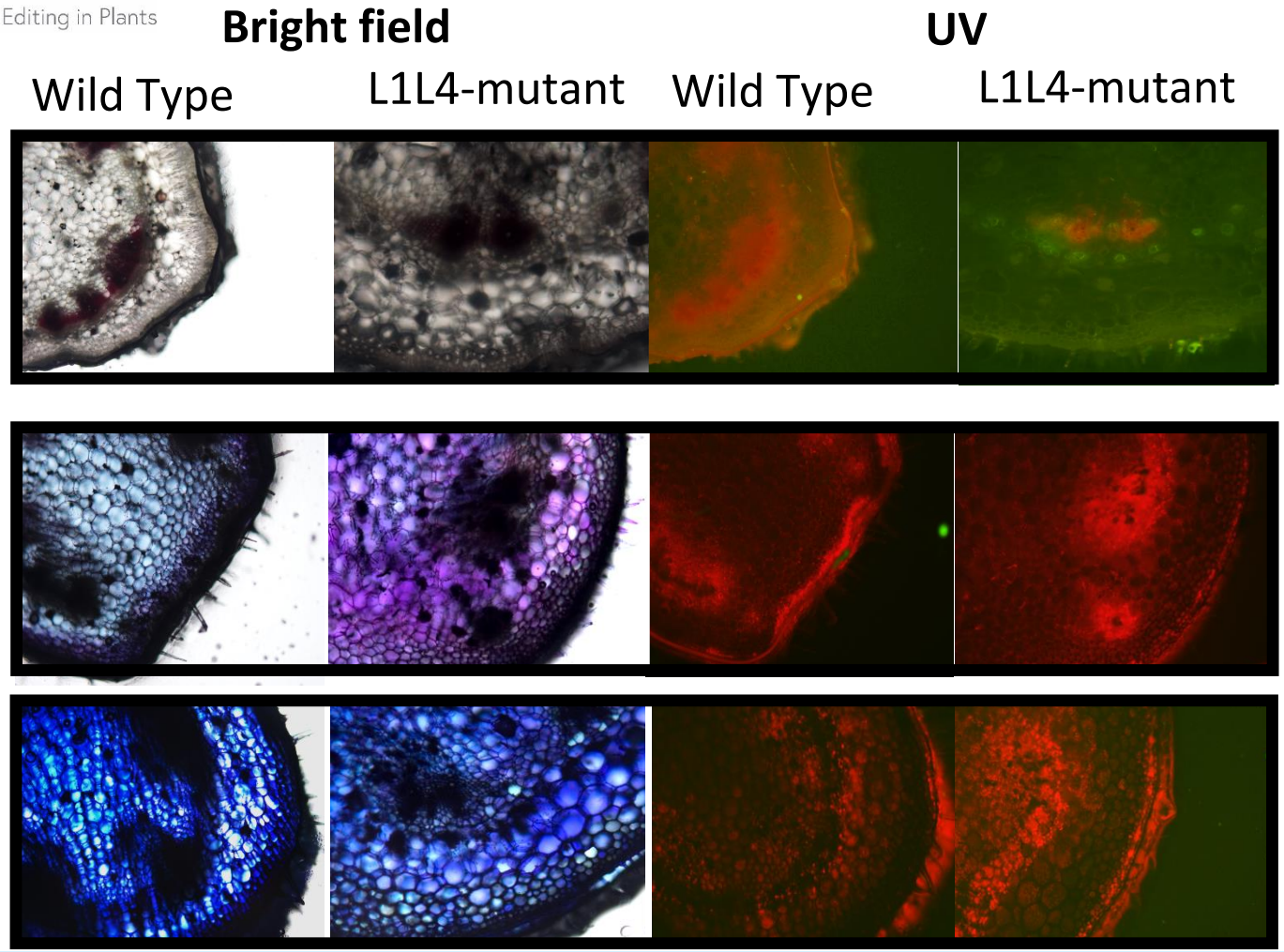
In years 2003-2012 more than **25.000 patents** have been filled on lignin valorization

There is demand for engineering plant feedstocks for greater structural homogeneity and **tailored functionality**.

Lignin has been associated to **pest/disease resistance**

Histochemical Staining of tomato Cell Wall Elements (stem cross-sections)

PlantEd COST Action CA18111 Genome Editing in Plants



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THANK YOU