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SPPS

New Breeding Technologies in the Plant Sciences – Applications and Implications

07 July 2017, Gothenburg

Dr Attila Molnar
University of Edinburgh, UK

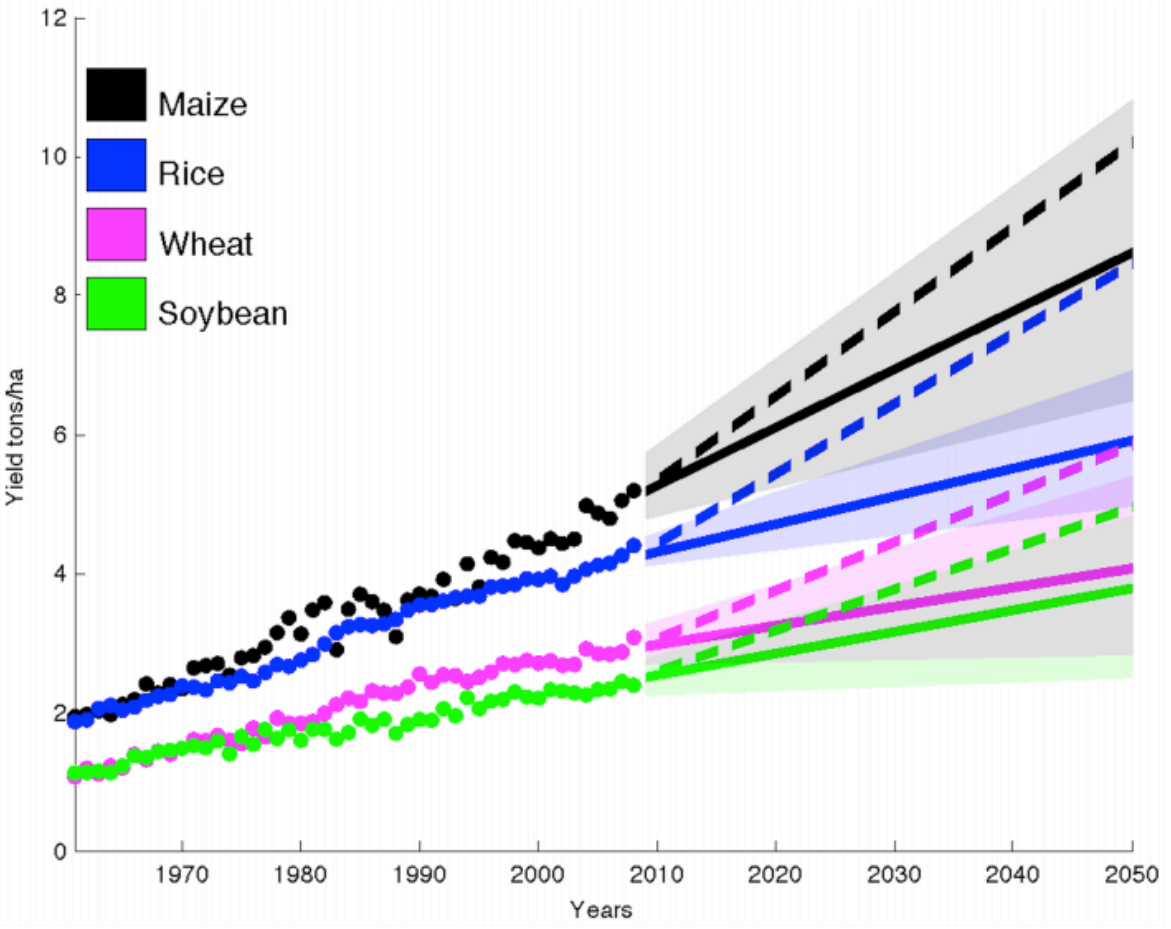
Using CRISPR to develop *Arabidopsis* viral resistance

Viral infections in crops threaten global food security

Global food production will have to at least double by 2050 to support our expanding population. (Tilman *et al*., 2011)

Viruses claim 10-15% of our annual harvest, globally (Regenmortel & Mahy, 2009)

Therefore, mitigating crop losses to viruses is a feasible way to close the gap between food supply and demand.



(Ray et al., 2013)

Potyviruses are an important focus for virology research

Potyviruses are the largest taxonomic grouping of all plant viruses (~30% of all plant viruses)

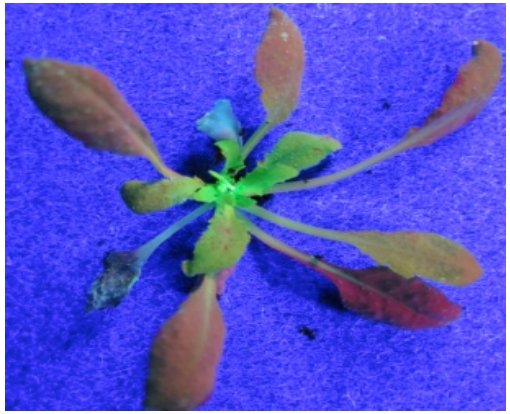
Certain *Potyvirus* species cause significant damage to economically important crops

eg: **P**otato **V**irus **Y** (PVY)
Turnip **M**osaic **V**irus (TuMV)

PVY infecting potato

(Karasev *et al*., 2013)

TuMV-GFP infecting Arabidopsis

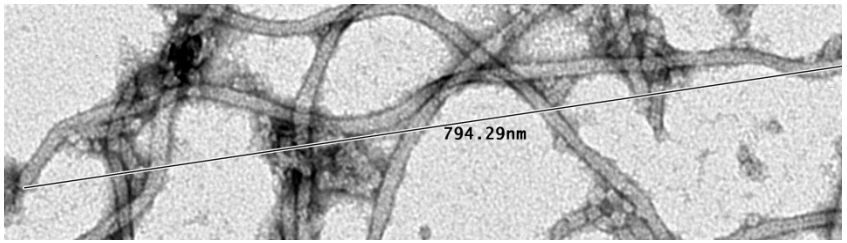


TuMV infecting cabbage

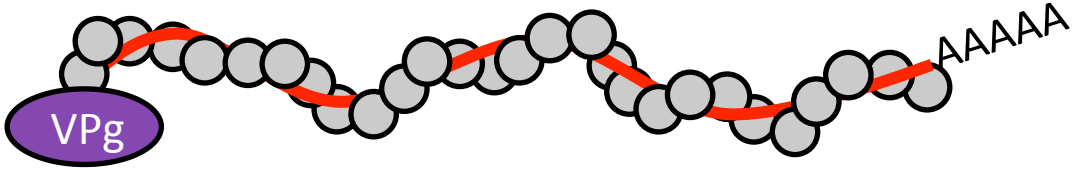
(Walsh, 2010)

A brief introduction to *Potyviruses*

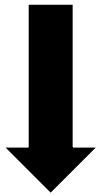
Potyviruses exist as flexuous, filamentous virions (approximately 650-900nm long)



Potyviruses have a +ssRNA genome (approximately 10kb long)

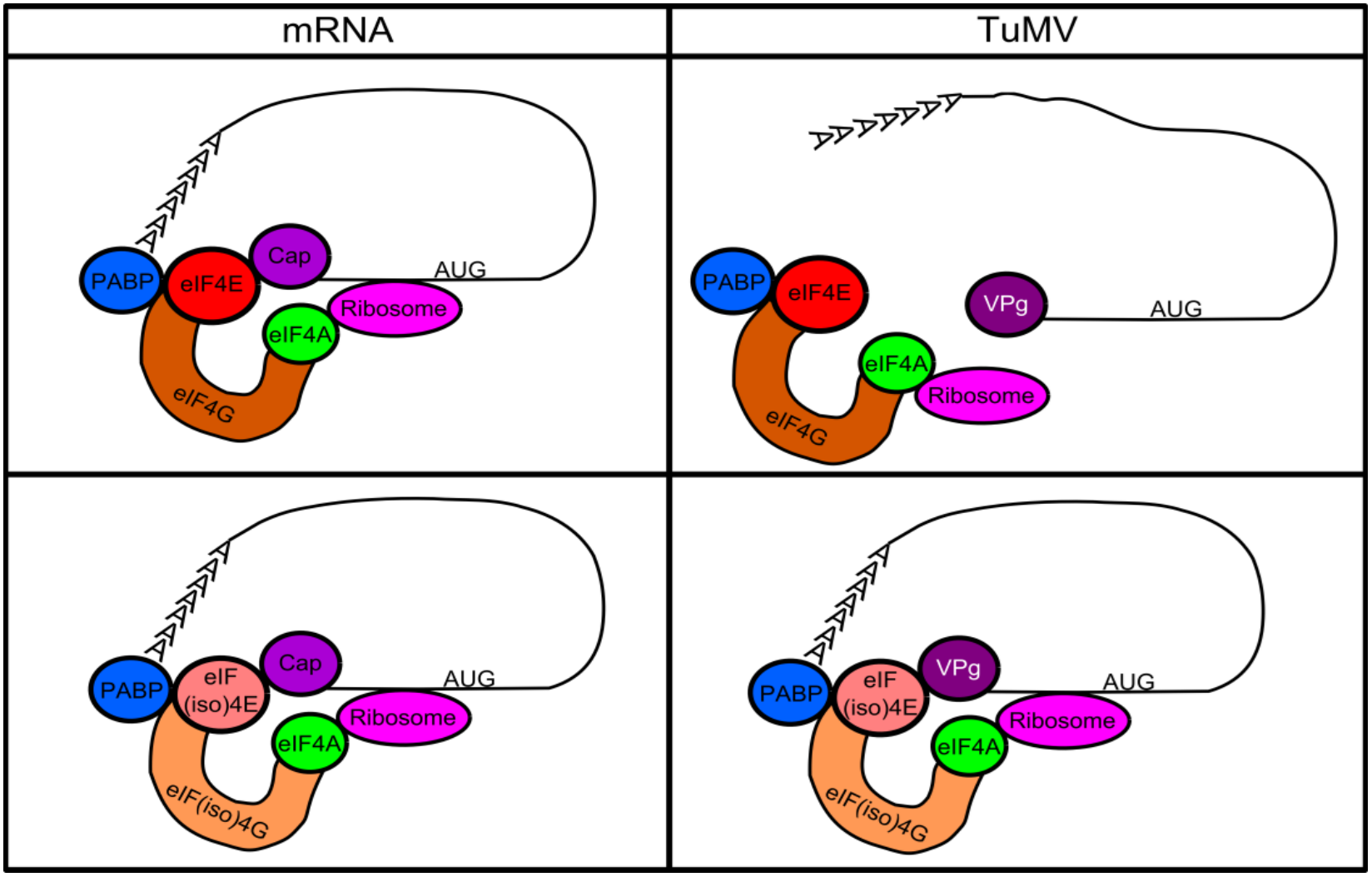


The mRNA-like genome is translated using the plant's translation apparatus



The *potyviral* protein VPg (**V**iral **P**rotein **g**enome-linked) acts as an mRNA cap analogue to aid 'mRNA mimicry'

Viral specificity for host translation factors – an Achilles heel?



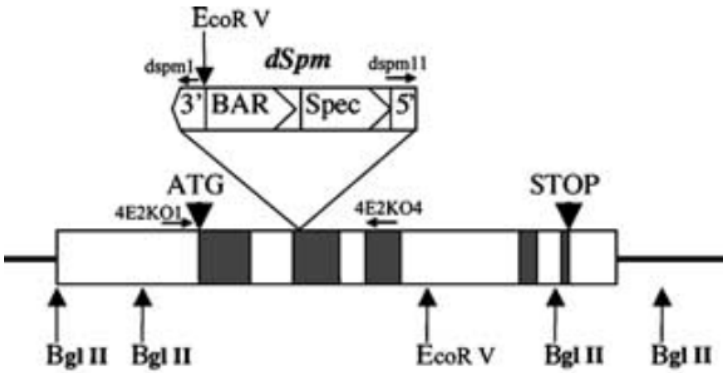
Cellular mRNA can utilise both eIF4E and eIF(iso)4E isoforms

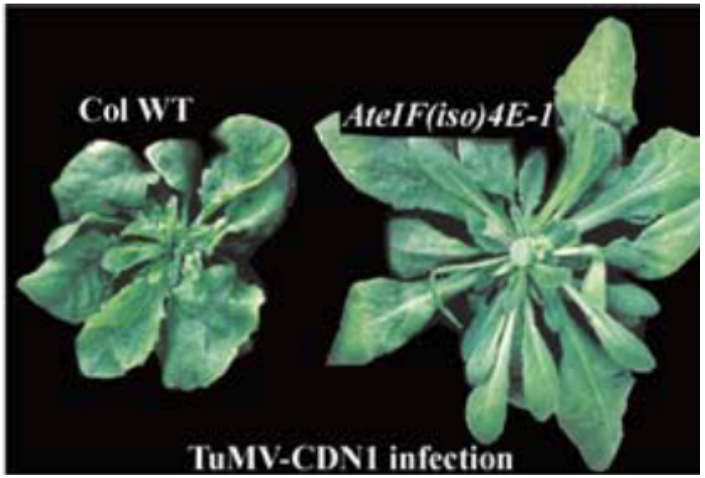
In contrast, TuMV has evolved strict specificity for the eIF(iso)4E isoform

The Plant Journal (2002) **32**, 927–934

The *Arabidopsis* eukaryotic initiation factor (iso)4E is dispensable for plant growth but required for susceptibility to potyviruses

Anne Duprat[1], Carole Caranta[2], Frédéric Revers[3], Benoît Menand[1], Karen S. Browning[4] and Christophe Robaglia[1,*]





Transposon mutagenesis of *eIF(iso)4E* results in resistance to TuMV

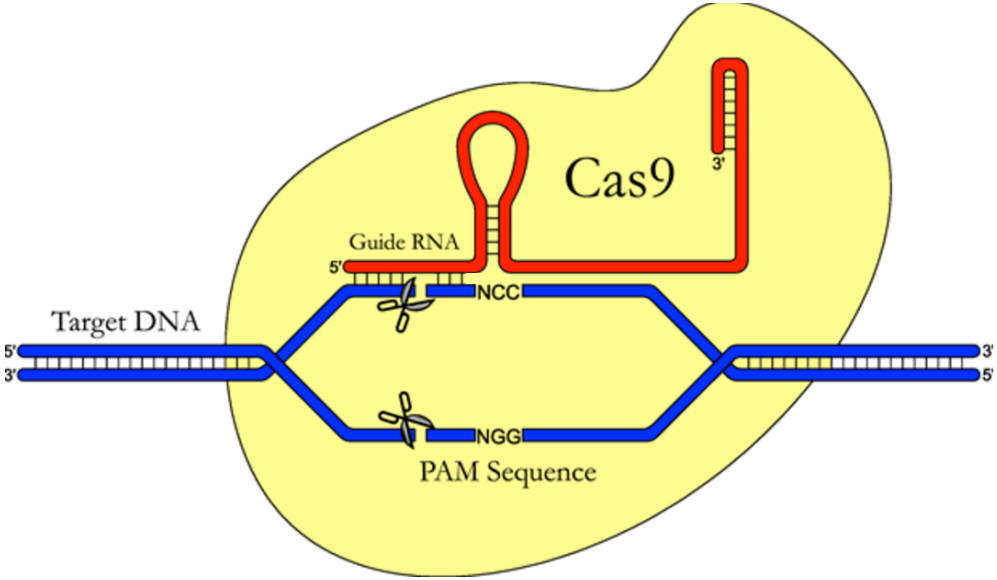
This resistance is **recessive** – ie both alleles must be mutated to render plants resistant

CRISPR/Cas9 induced genome editing

CRISPR/Cas9 is a new genome editing technology.

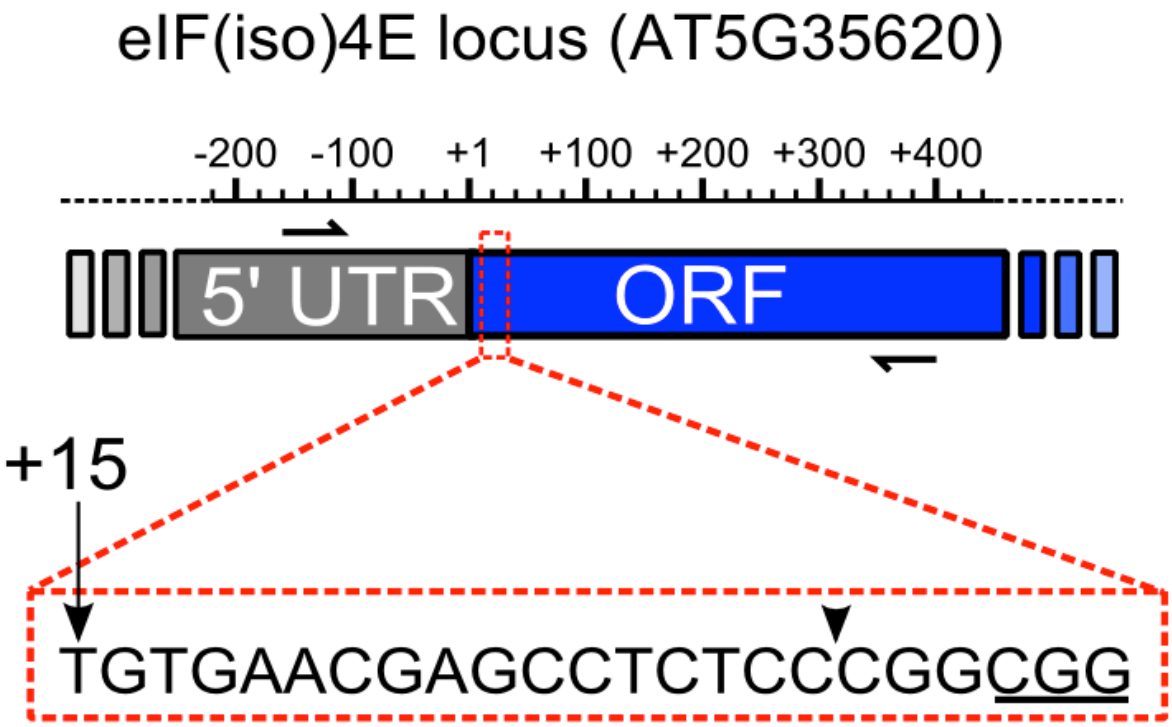
The Cas9 nuclease can be guided by a synthetic sgRNA (single-guide RNA) to induce double-stranded DNA breaks (DSBs) at almost any genomic site.

DSBs repaired by the cell's non-homologous end joining (NHEJ) pathway can result in point mutations at the target locus.



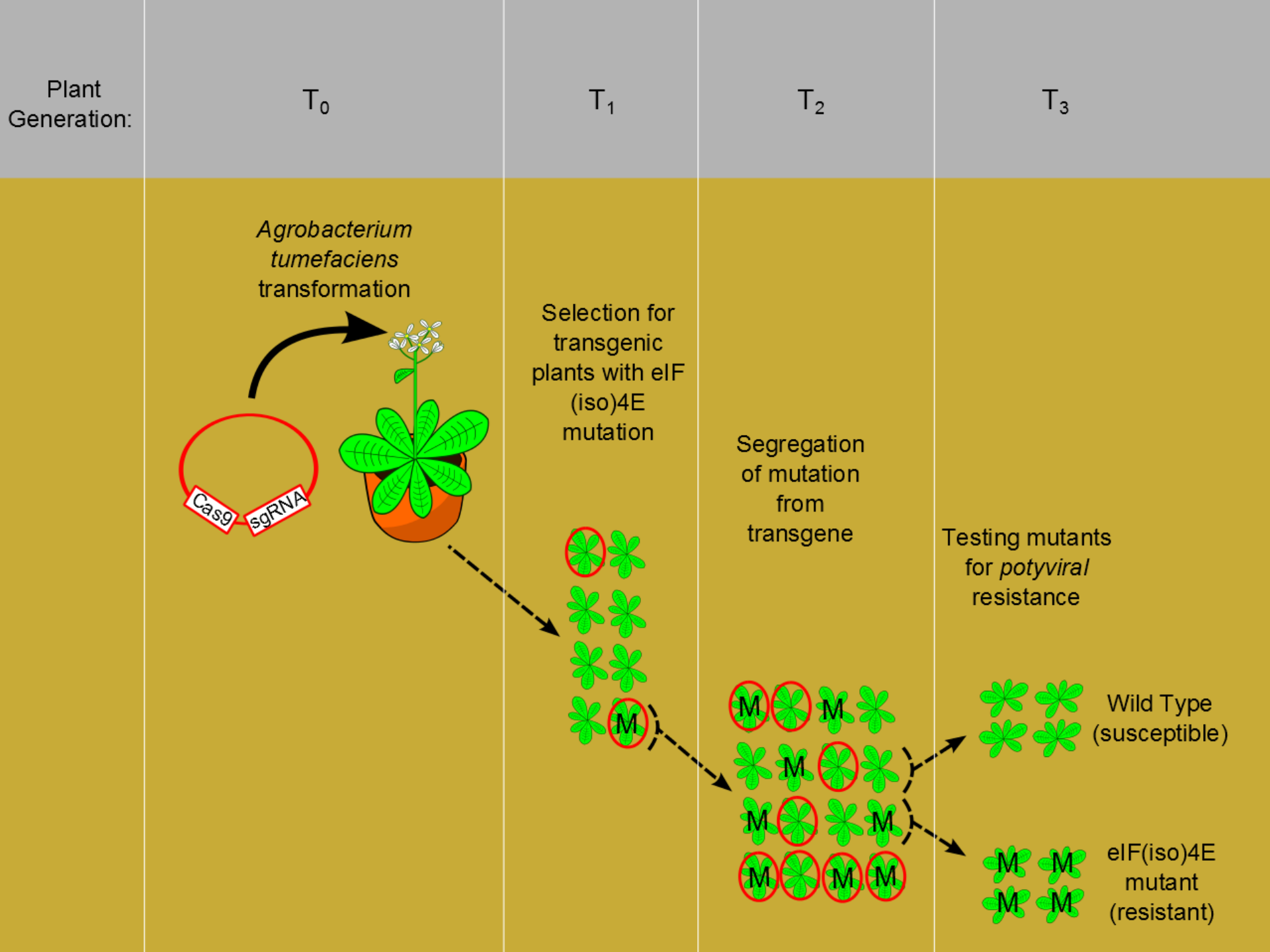
Our aim was to use CRISPR/Cas9 technology to knock out *eIF(iso)4E* to generate a novel resistance allele to TuMV.

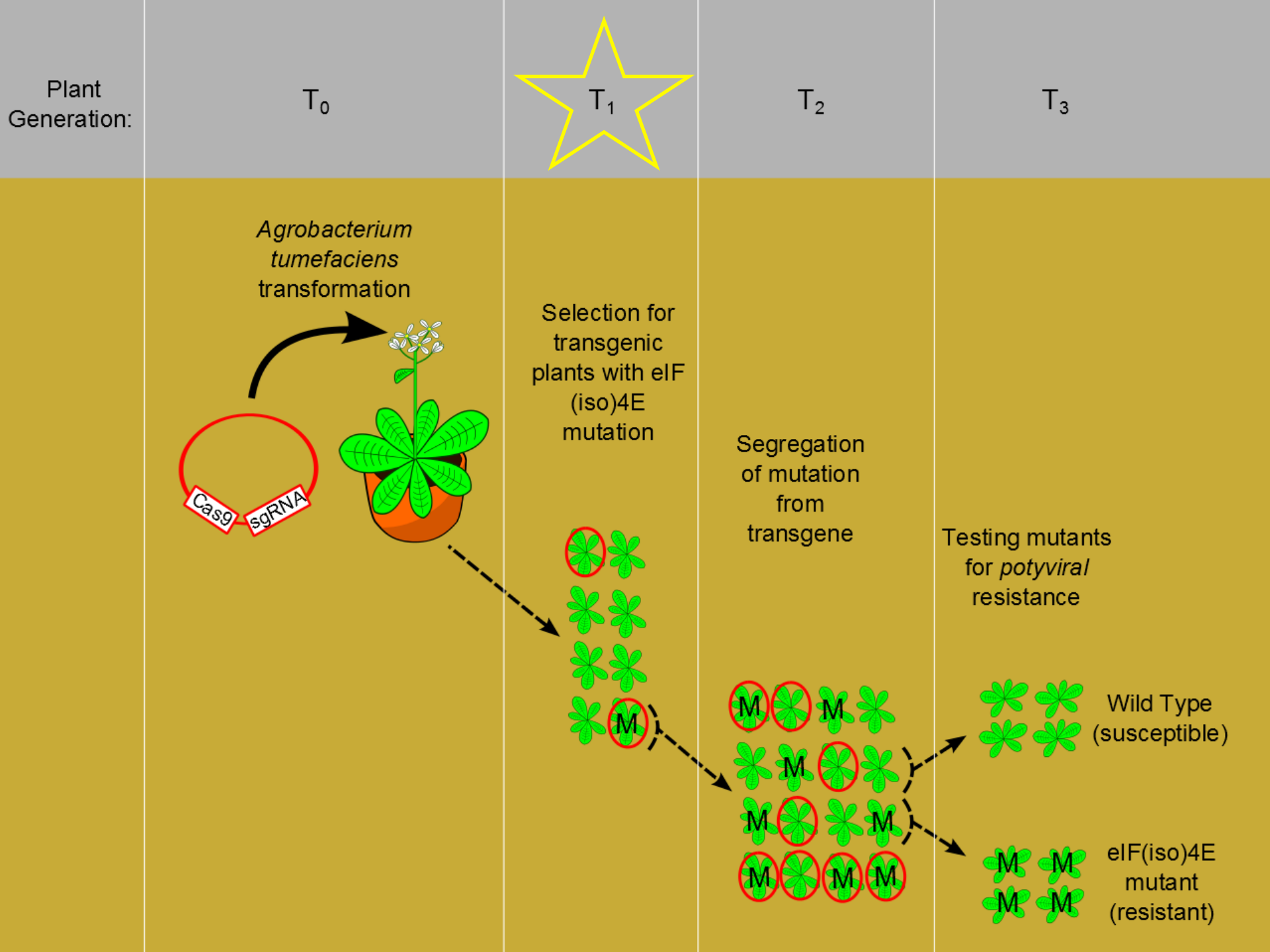
sgRNA design for targeting *eIF(iso)4E*



An sgRNA was designed to target the 5’ of the ORF to disrupt the entire protein by point mutation

A region with a GG dinucleotide immediately upstream of the PAM was selected to increase the editing efficacy.


Plant Generation:
T0
T1
T2
T3
Agrobacterium tumefaciens transformation
Cas9
sgRNA
Selection for transgenic plants with eIF (iso)4E mutation
Segregation of mutation from transgene
Testing mutants for potyviral resistance
M
Wild Type (susceptible)
eIF(iso)4E mutant (resistant)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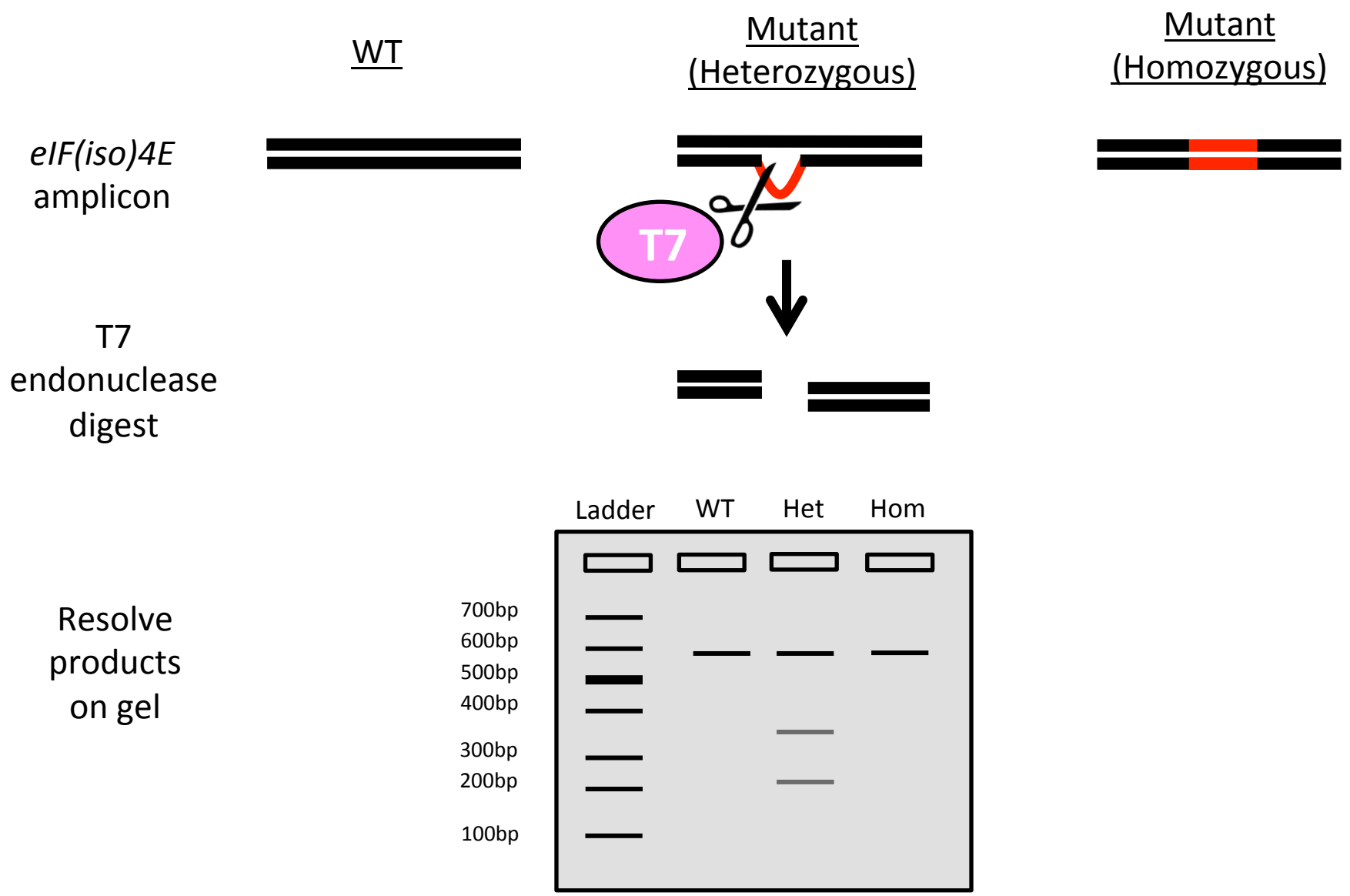
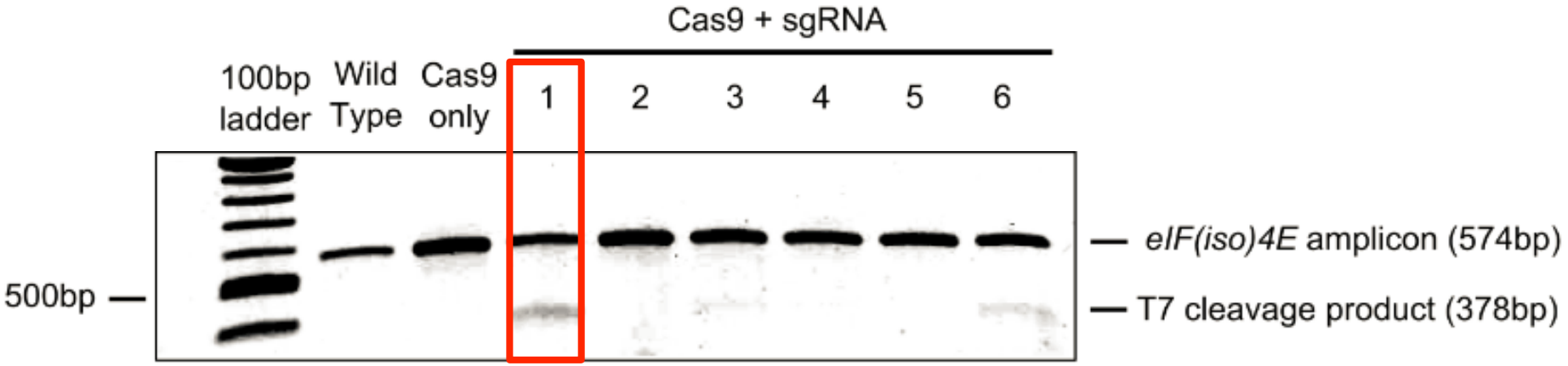
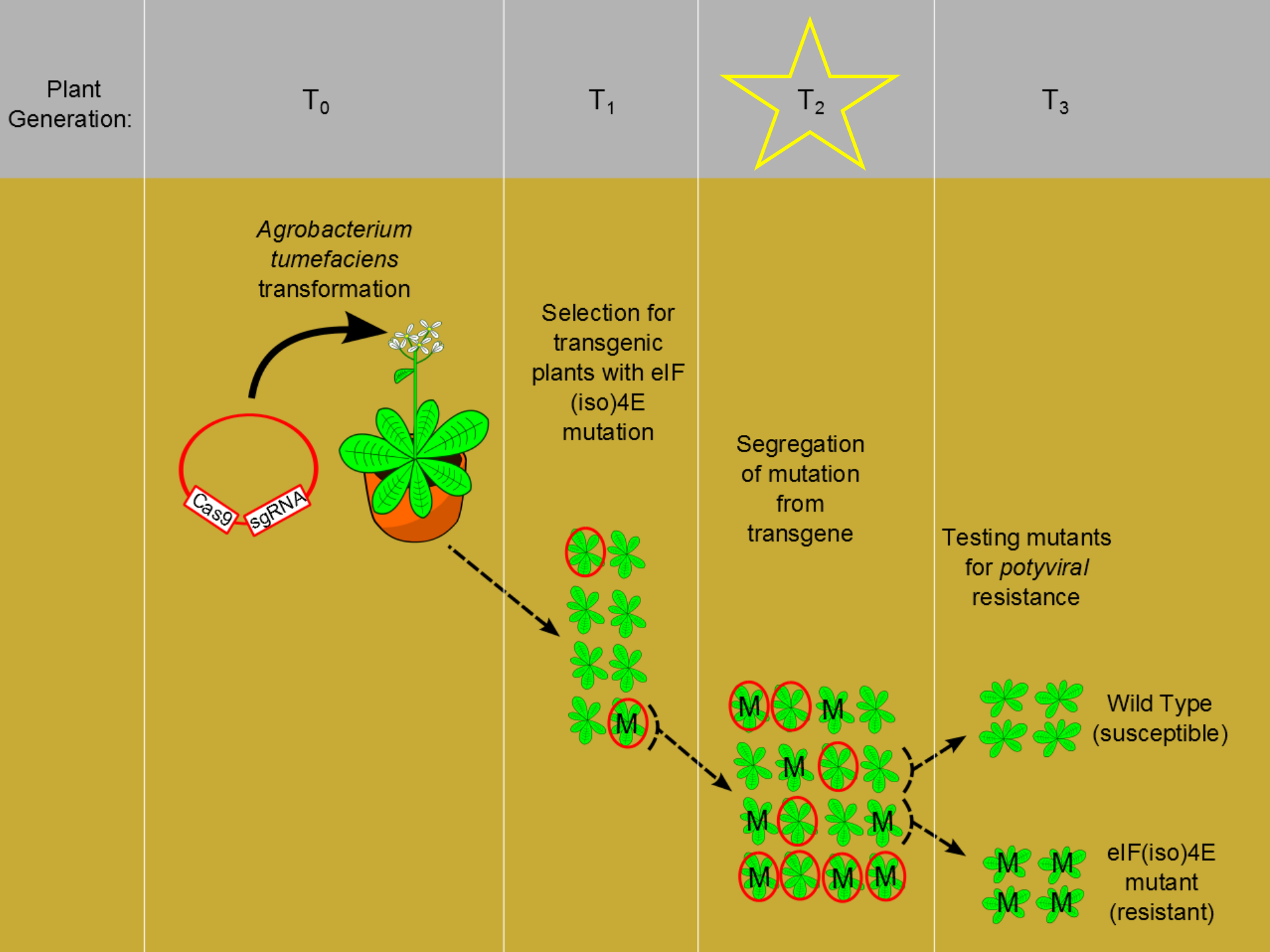
Testing for CRISPR/Cas editing by T7 endonuclease assay



eIF(iso)4E editing detected in the T_1 generation



T_1 transformant number 1 was selected to produce T_2 seed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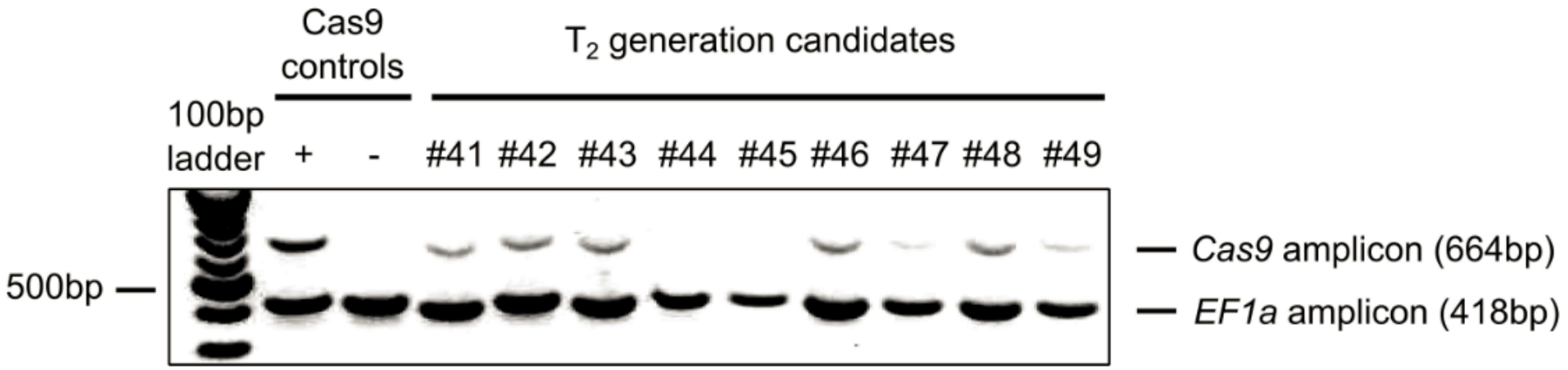
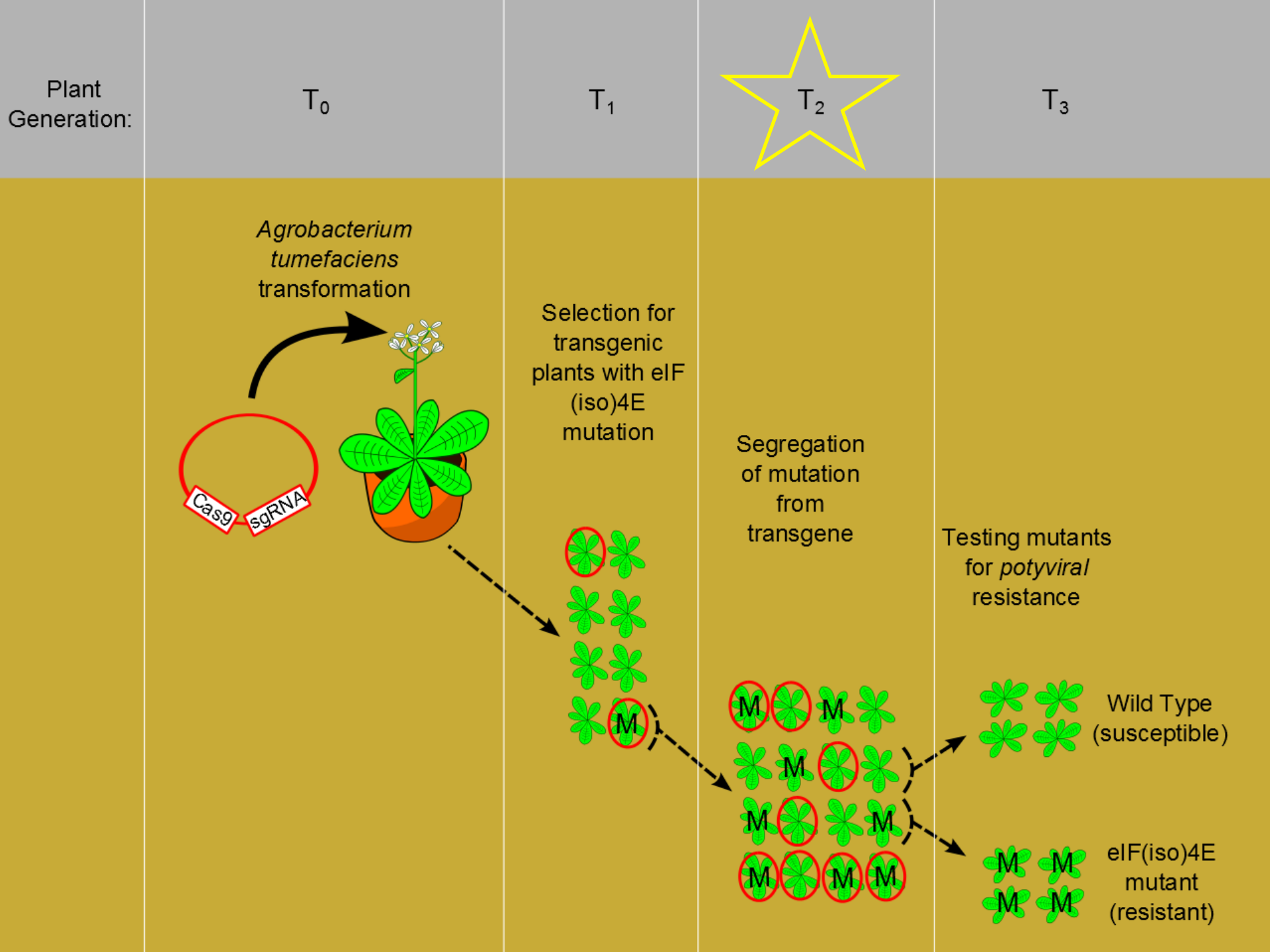
‘Weeding out’ the transgene in the T_2 generation



55 transgene-free plants were identified out of **144** T_2 candidates (**~38%**)

These **55** non-trangenic plant were then screened for CRISPR/Cas9-induced mutations in *eIF(iso)4E*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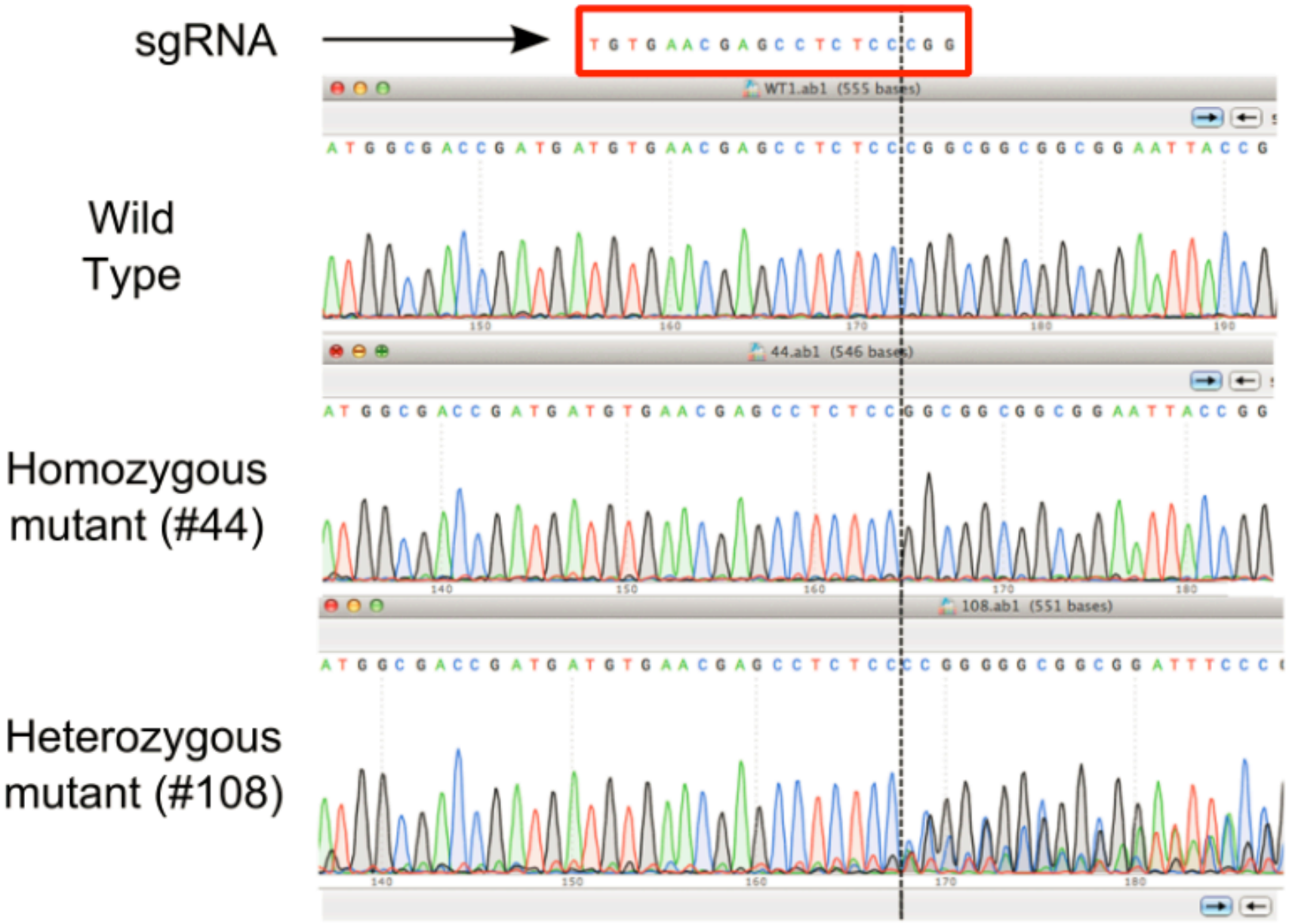
Identification of *eIF(iso)4E* mutations by Sanger sequencing



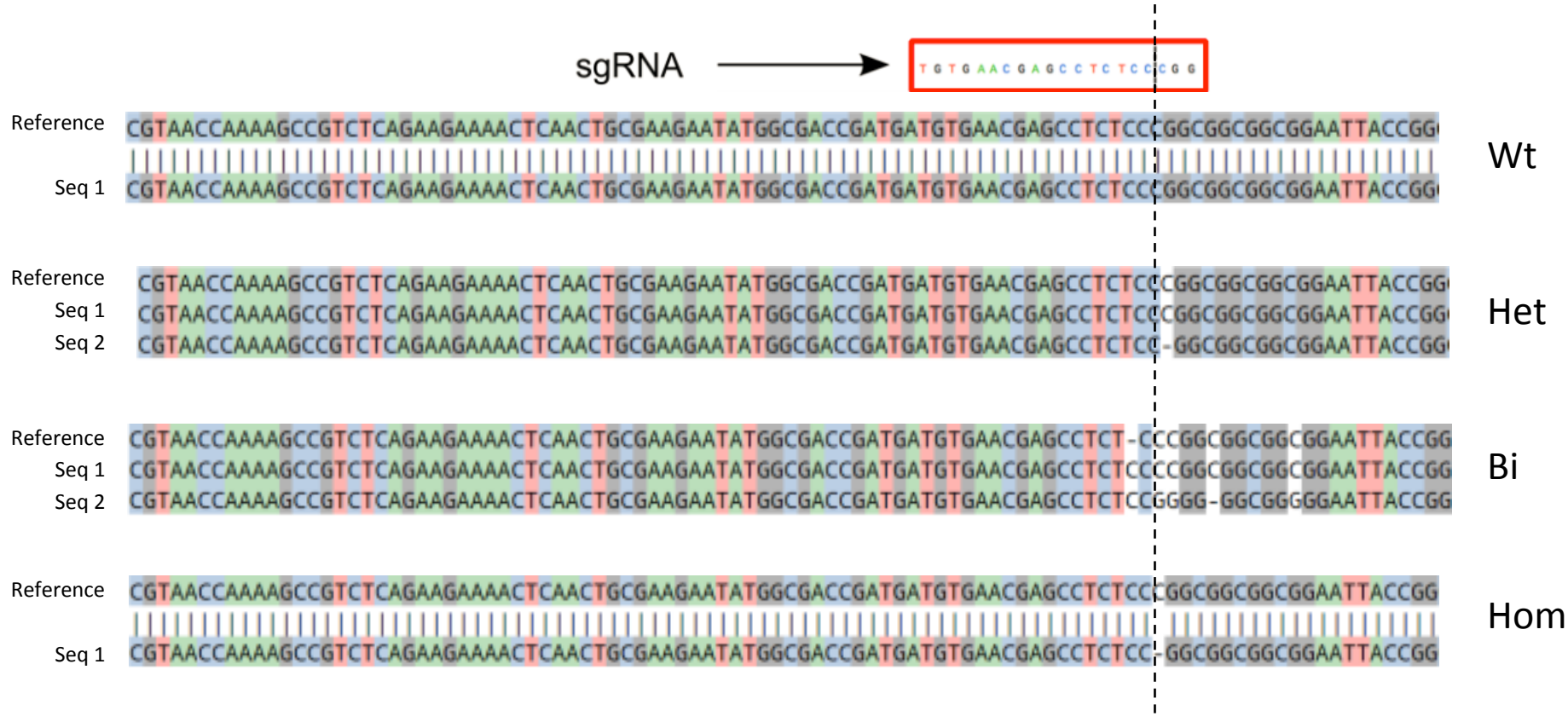
Decoding indels

SCIENTIFIC REPORTS

Article | OPEN

CRISP-ID: decoding CRISPR mediated indels by Sanger sequencing

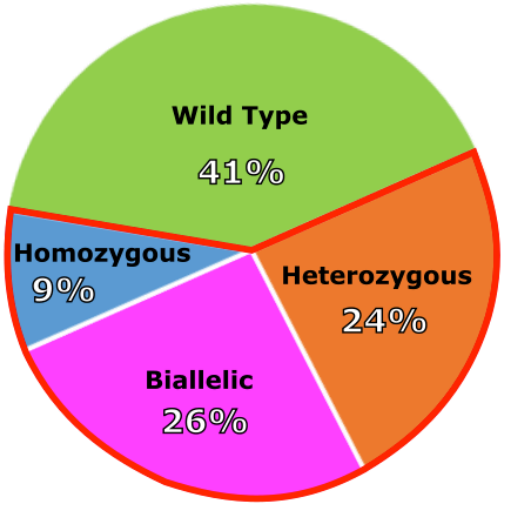
Jonas Dehairs, Ali Talebi, Yacine Cherifi & Johannes V. Swinnen



Wt, wild type; Het, heterozygous; Bi, bi-allelic; Hom, homozygous

Summary of non-transgenic T_2 plants with *eIF(iso)4E* mutations

55 non-transgenic T_2 plants were analysed by CRISP-ID

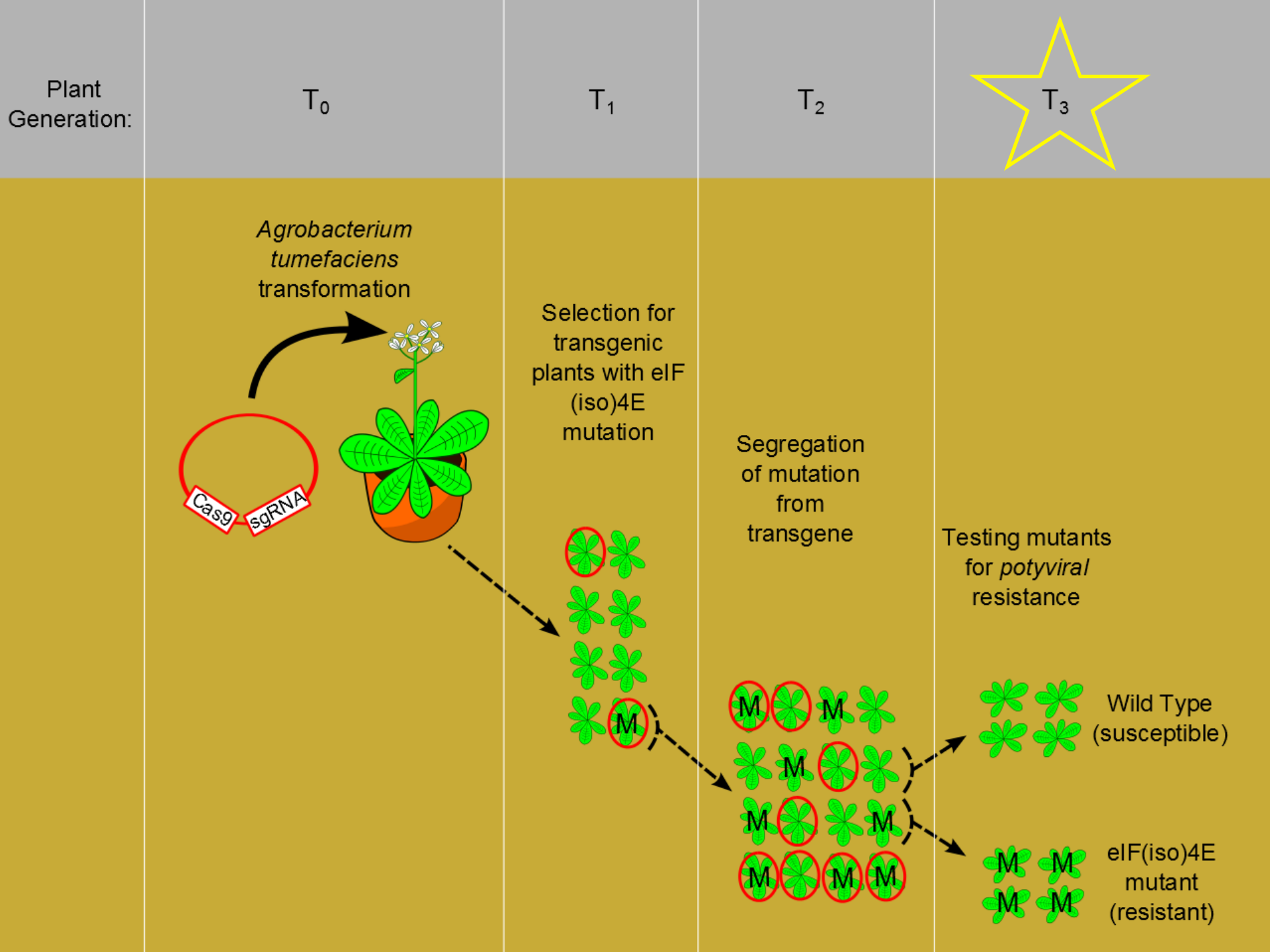


59% mutation frequency

Sample	Nucleotide Sequence	Mutation
Wild Type	TGTGAACGAGCCTCTCC-CGGCGG	
#44	TGTGAACGAGCCTCTCC--GGCGG	Deletion (-C)
#65	TGTGAACGAGCCTCTCCACGGCGG	Insertion (+A)
#68	TGTGAACGAGCCTCTCCTCGGCGG	Insertion (+T)
#98	TGTGAACGAGCCTCTCCCCGGCGG	Insertion (+C)

The point mutations in *eIF(iso)4E* induce protein truncation

Sample	Amino Acid Sequence
Wild Type	1 MATDDVNEPLPAAAELPATEAEKQPHK.LERKWSFWFDNQSKKGAAWGASLRKAYTFDTV
#44	1 MATDDVNEPLRRRRNYRRQRRRNNHTSSKESGVSGSITNQRKA..PPGELLFVKPILSTP
#65	1 MATDDVNEPLHGGGGITGDRGGETTTQ.ARKKVEFLVR*~~~~~~~~~~~~~~~~~~~~
#68	1 MATDDVNEPLLGGGGITGDRGGETTTQ.ARKKVEFLVR*~~~~~~~~~~~~~~~~~~~~
#98	1 MATDDVNEPLPGGGGITGDRGGETTTQ.ARKKVEFLVR*~~~~~~~~~~~~~~~~~~~~
Wild Type	60 EDFWGLHETIFQTSKLTANAEIHLFKAGVEPKWEDPECANGGKWTWVVTANRKEALDKGW
#44	59 SKIFGDCTRLY.....FRLAN*~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
#65	38 ~~~~~~
#68	38 ~~~~~~
#98	38 ~~~~~~
Wild Type	120 LETLMALIGEQFDEADEICGVVASVRPQSKQDKLSLWTRTKSNEAVLMGIGKKWKEILDV
#44	75 ~~~~~~
#65	38 ~~~~~~
#68	38 ~~~~~~
#98	38 ~~~~~~
Wild Type	180 TDKITFNNHDDSRRSRFTV*
#44	75 ~~~~~~~~~~~~~~~~~~~
#65	38 ~~~~~~~~~~~~~~~~~~~
#68	38 ~~~~~~~~~~~~~~~~~~~
#98	38 ~~~~~~~~~~~~~~~~~~~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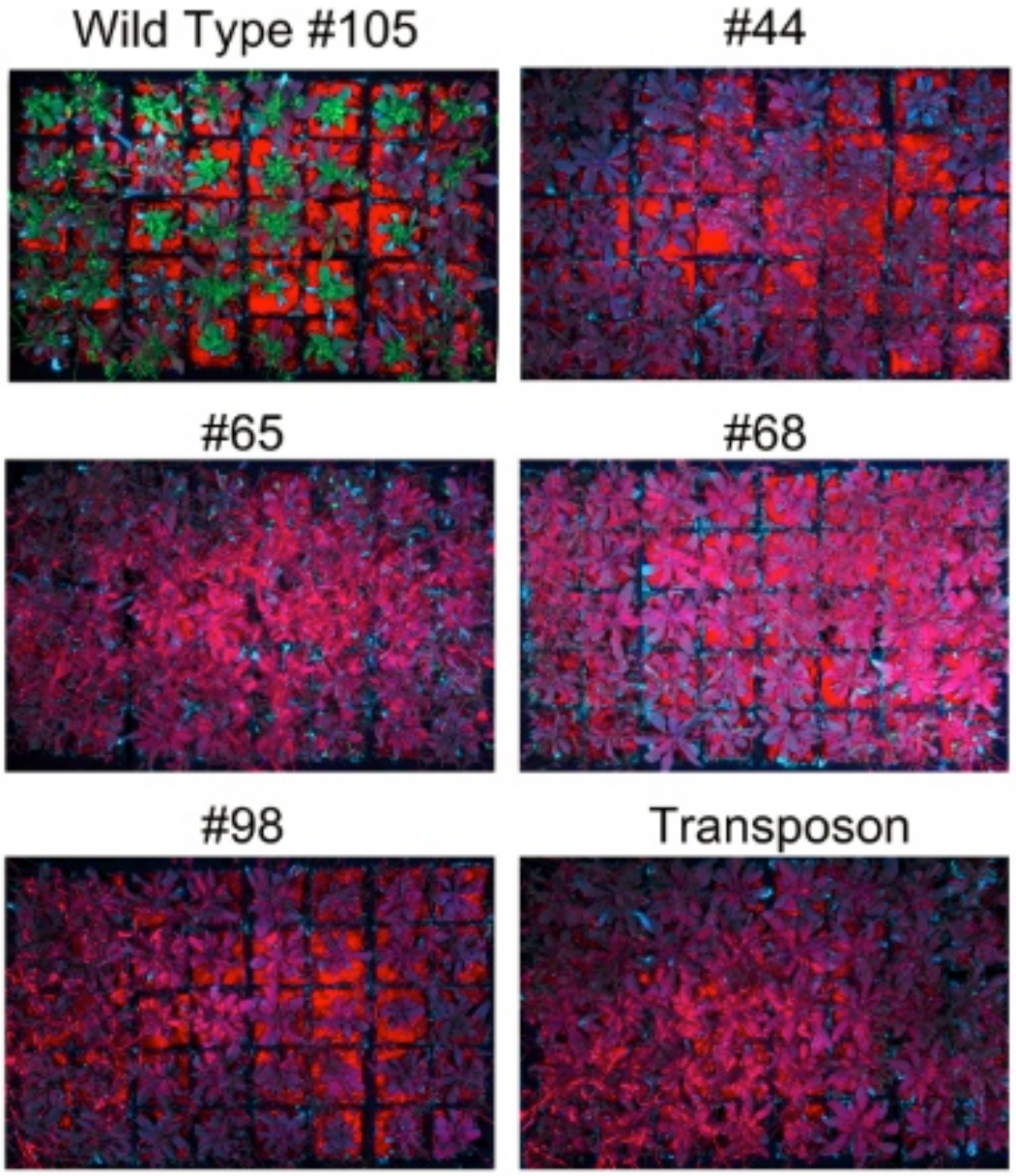
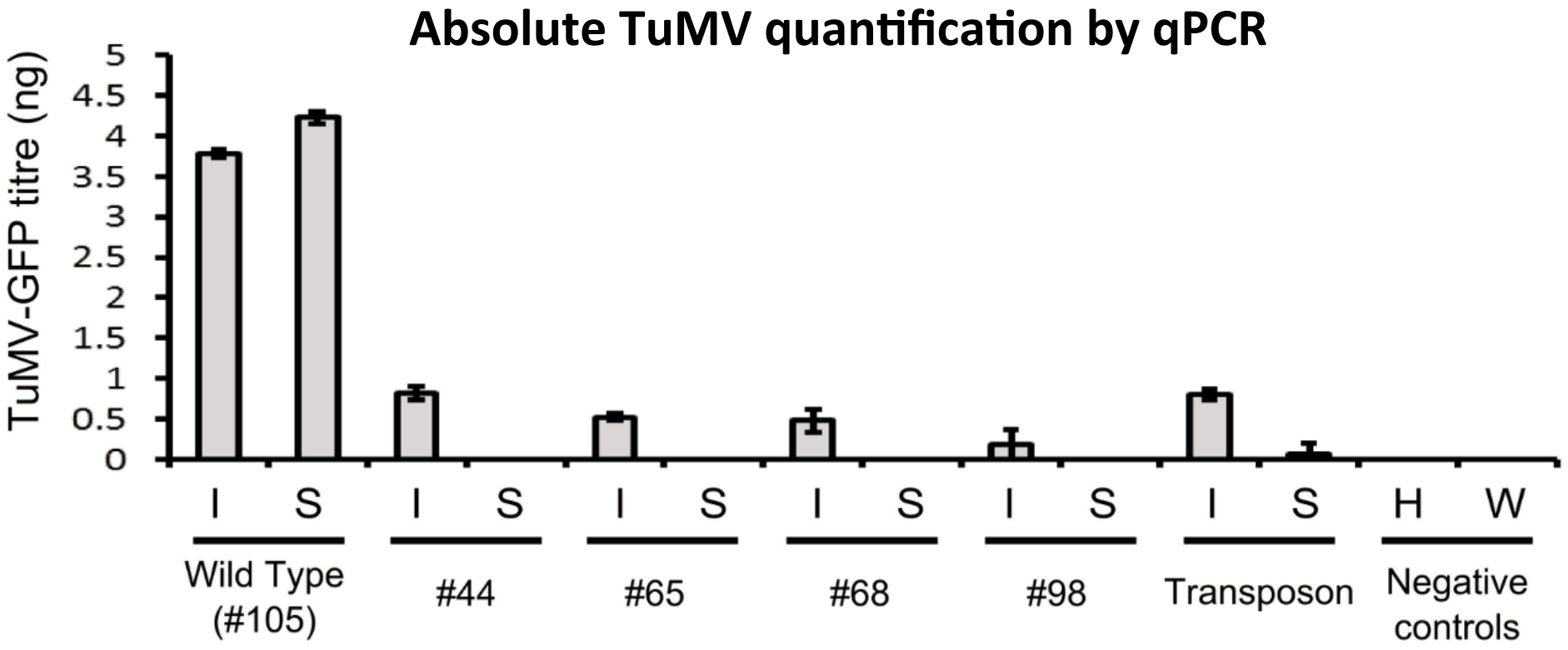
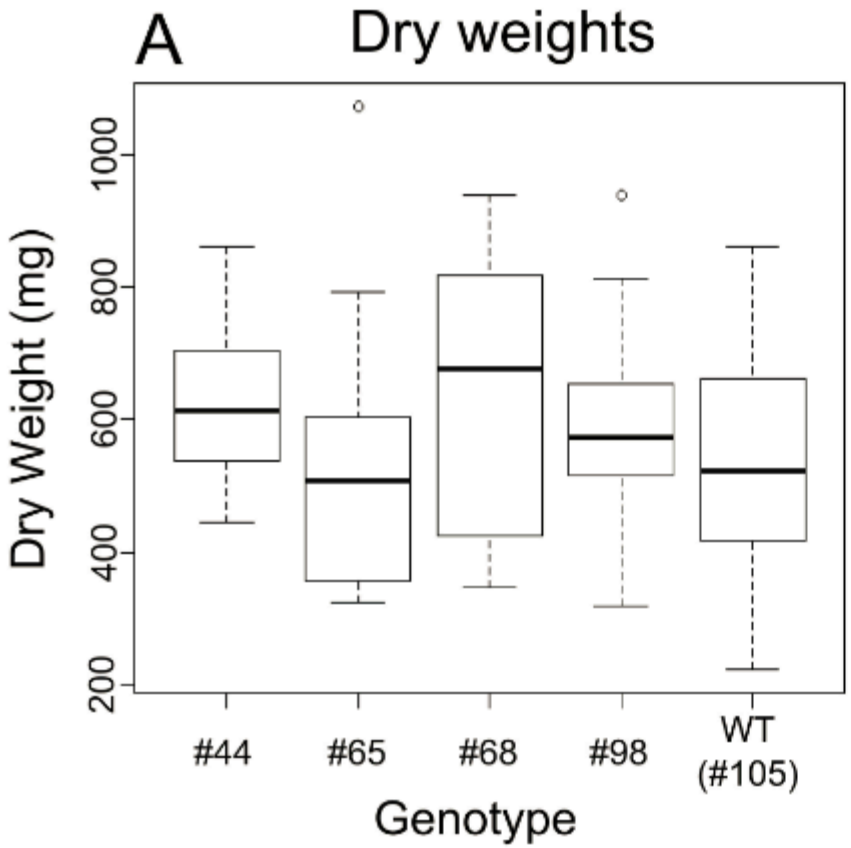
Whole tray infections

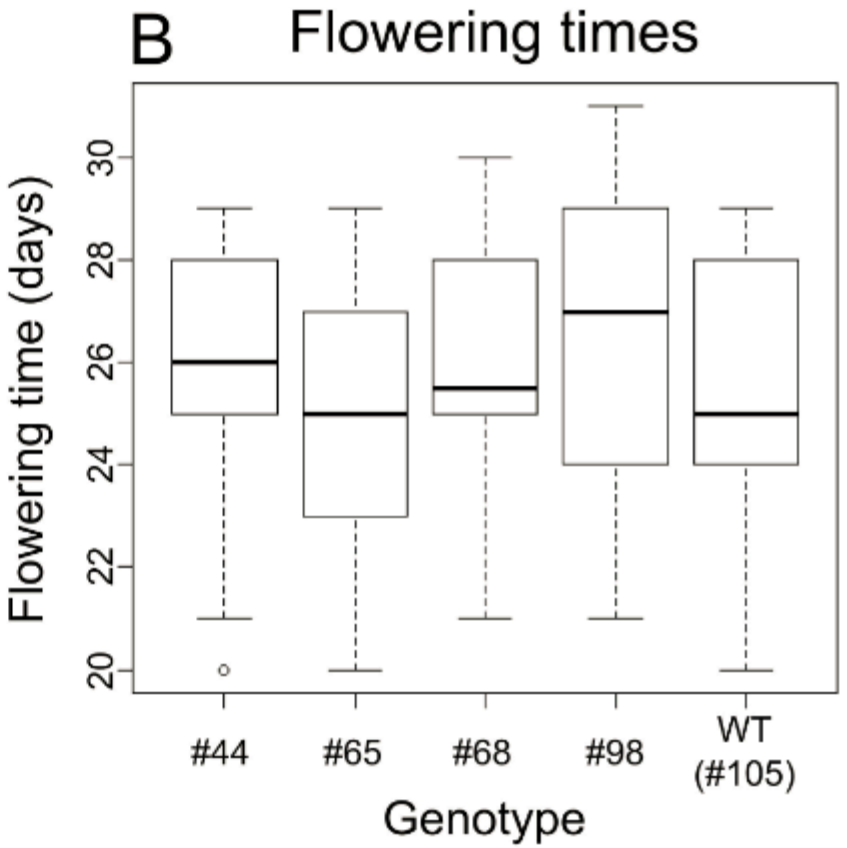


Homozygous *eIF(iso)4E* mutants are completely resistant to TuMV



Plant growth is not compromised by *eIF(iso)4E* knock-out





$F_{4,70} = 1.372$, $p=0.252$

$F_{4,119} = 1.597$, $p=0.180$

Conclusions/Future directions

- Homozygous point mutations at *eIF(iso)4E* were generated by CRISPR/Cas9 genome editing, creating complete resistance to TuMV
- We show that these mutations do not affect the dry mass of mature plants or their flowering time, under 'normal' growth conditions.
- We hope test in greater detail whether the *eIF(iso)4E* mutation would be detrimental to plant growth under certain environments (eg. stress).
- We plan to test how broad and stable the engineered resistance is by infecting with other viruses/strains.
- Our study provides a proof of concept for generating non-transgenic, virus resistant crops which can later be applied to important crops.

Molecular Plant Pathology

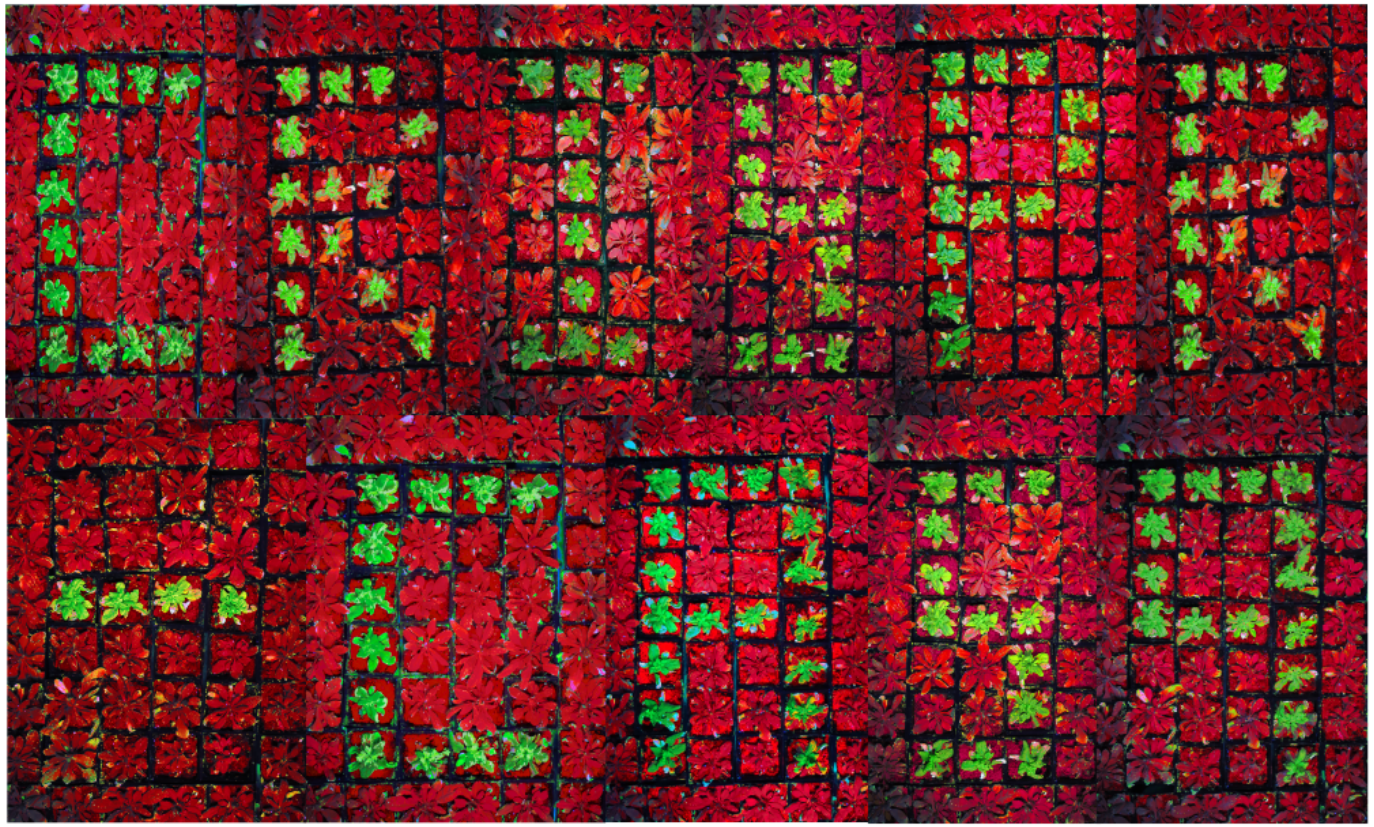
MOLECULAR PLANT PATHOLOGY

DOI: 10.1111/mpp.12417

Engineering of CRISPR/Cas9-mediated potyvirus resistance in transgene-free *Arabidopsis* plants

DOUGLAS E. PYOTT, EMMA SHEEHAN AND ATTILA MOLNAR*

Institute of Molecular Plant Sciences, University of Edinburgh, Edinburgh EH9 3JR, UK



What did we learn about the CRISPR/Cas9 technology throughout our work with eIF(iso)4E?

1. The ubiquitin promoter confers strong expression of Cas9 in the germline, which results in high number of heritable mutations in progenies.

2. Like in animals, guide RNAs ending with two Gs are very efficient inducers of sequence-specific mutations.

3. T7 endonuclease assay is superior over restriction-enzyme based methods to select for the lines with the highest level of genome editing in T_1 population.

4. Direct sequencing of target gene in the T_2 population is the fastest and cheapest way to detect homozygous mutations.

5. Growing plants at slightly higher temperature (25-27^{o}C) can promote flowering and subsequently can reduce generation time. Homozygous, transgene-free mutants can be recovered within 4 months from dipping the *Arabidopsis* flowers in *Agrobacterium* suspension.

We believe that CRISPR-engineered crops will be acceptable for commercial applications, and should not be restricted by current legislation for genetically modified organisms for the following reasons:

1) While transgenes may be used to initially deliver the CRISPR nuclease/guide RNA complex, they are not needed once the genome has been edited and, because they are located elsewhere in the genome, can be inherited independently of the edited gene. Hence **the final engineered product can be made completely free of transgenes** by simple breeding.

2) The strategy of knocking out eIF genes mimics natural mutations which have occurred multiple times to give rise to the majority of known Potyvirus resistance alleles. Hence, **natural and artificial selection of mutated eIF genes provide testament to the success of this approach**. It also reveals that mutated plants pose no additional risks to health or the environment.

3) The **mutations induced by CRISPR nuclease-mediated DNA cleavage arise by the cell's natural process for repairing DNA, which occurs under natural growth conditions** (e.g., when DNA is broken by sunlight).

contacted

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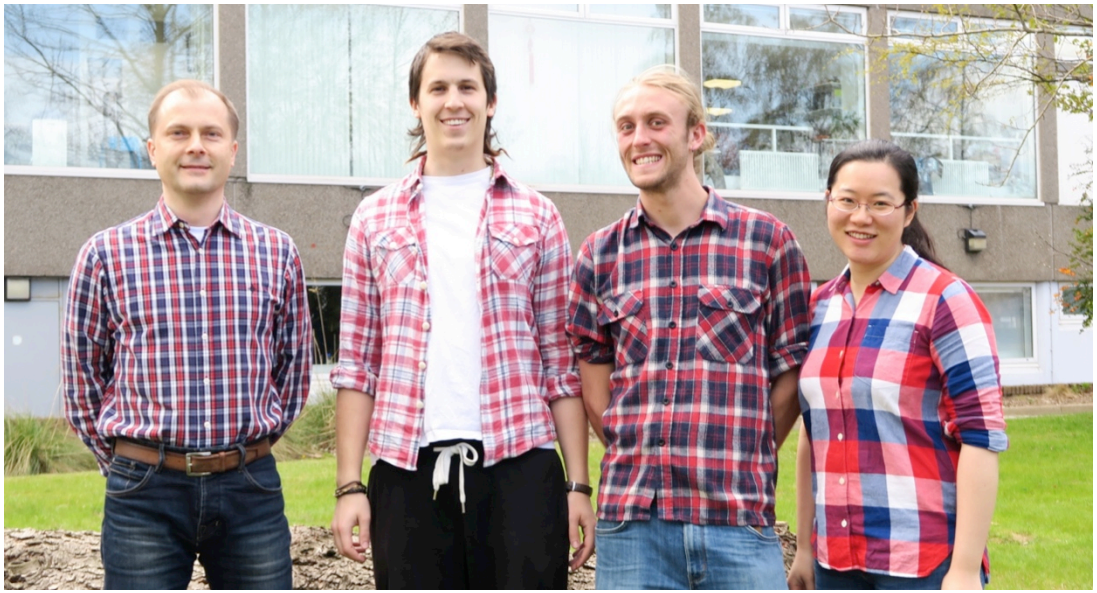
Aknowledgements



Douglas
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