


NGT plants of the future: EFSA overlooked most powerful and risky applications

Different views on the risks of NGT plants

Commission and the Parliament, based on EFSA opinions, are proposing **formal criteria** to take decisions on safety, by counting number of mutations and classifying the type of mutations.

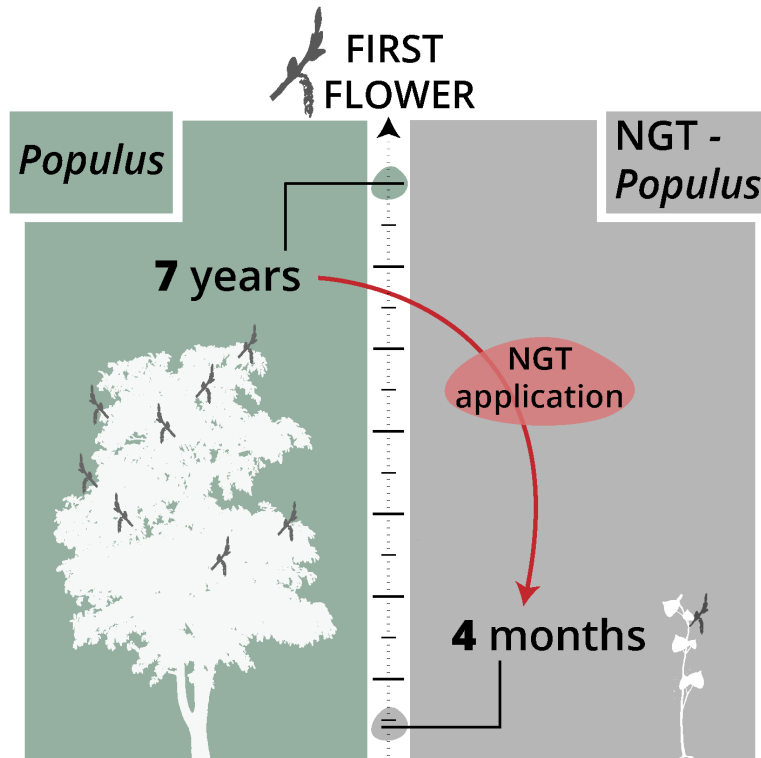
 ANSES and other experts are looking at the site of mutations in the genome (specific genotype) and are taking into account the observed effects (resulting phenotype) and therefore see the need for a **case by case** risk assessment.

Formal criteria are insufficient to ensure safety

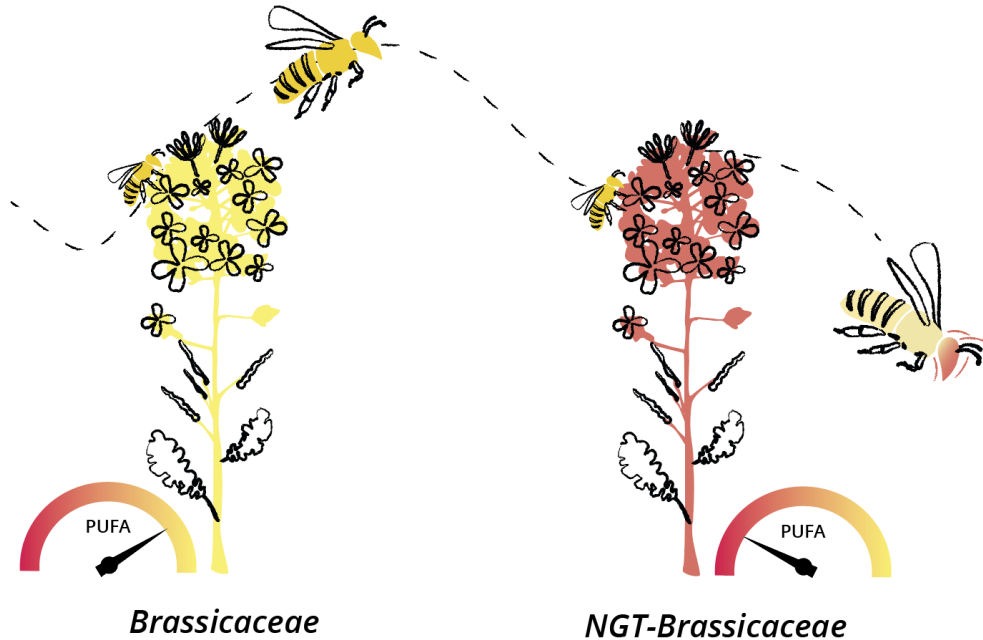
Case studies show genotypic and phenotypic differences between conventionally bred and NGT plants that definitely require risk assessment. But according to the Commission and Parliament, these NGT plants nevertheless would be included in 'Category 1' and equated to conventional breeding.

Consequently, the formal criteria being proposed by Commission and the Parliament do not ensure the safe use of NGT plants.

Case study: Early first flowering in NGT poplar



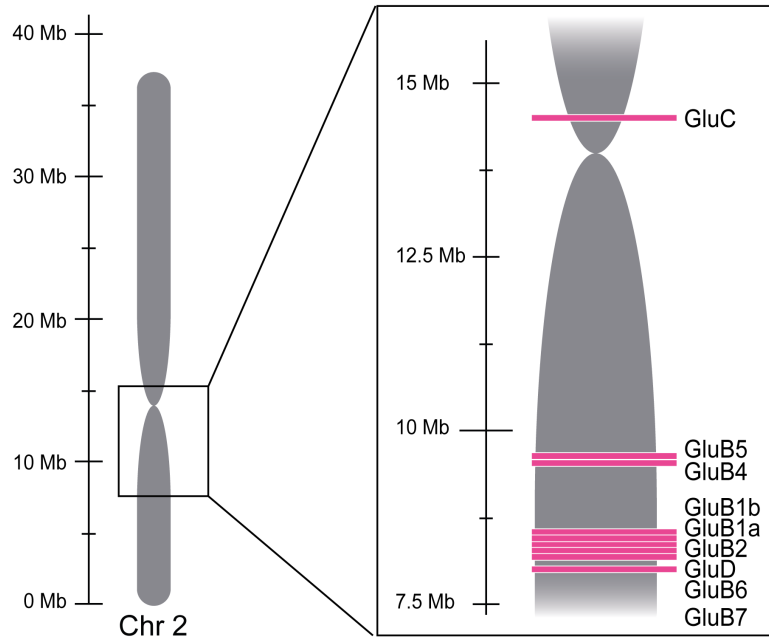
Case study: NGT plants with changes in oil composition



reducing or increasing polyunsaturated fatty acids (PUFA)

<https://www.preprints.org/manuscript/202402.0255/v1>

Case study: NGT rice with reduced glutelin content



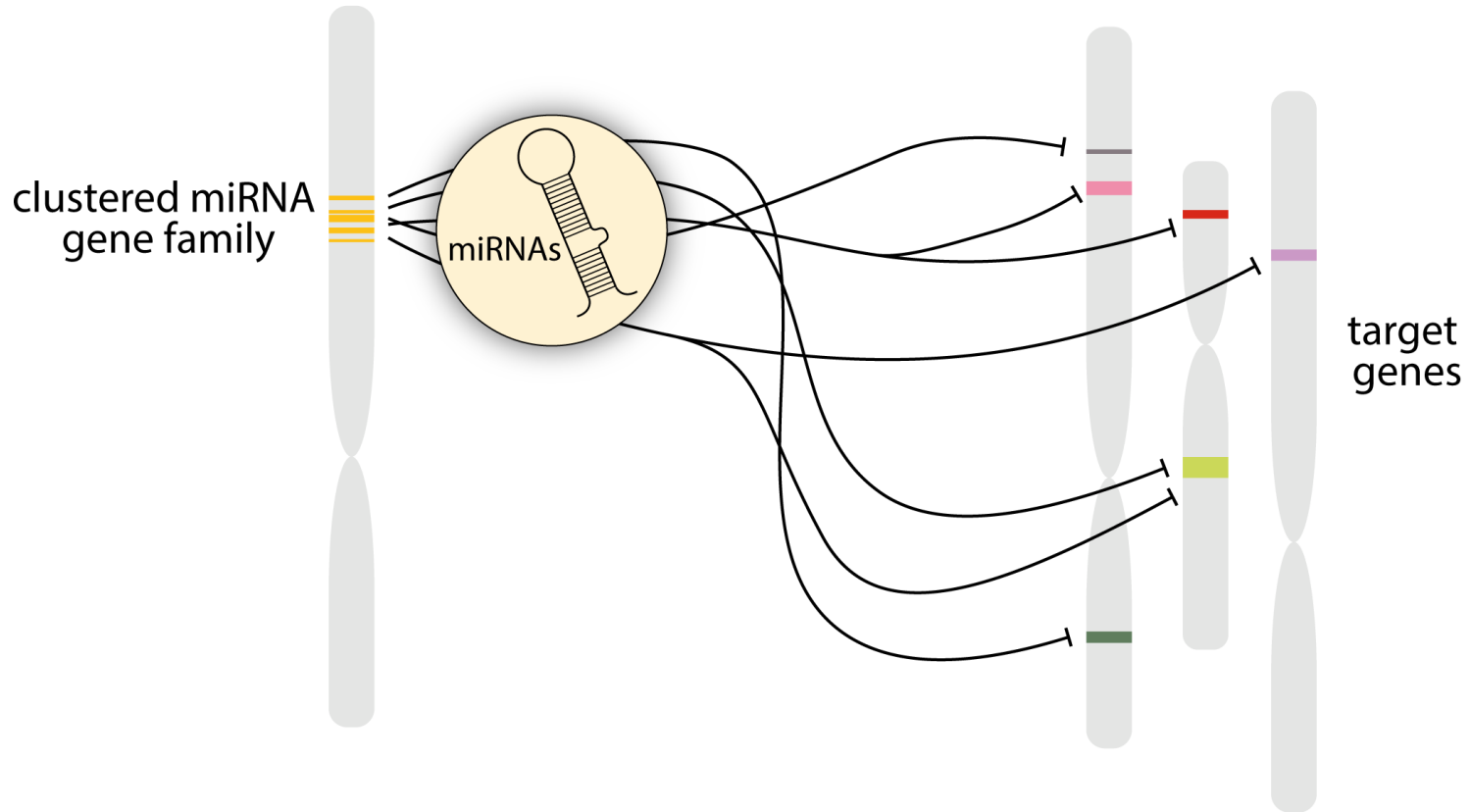
New case studies: NGT applications targeting miRNA

MicroRNAs (miRNAs) **regulate various complex functions** related to growth, development, and stress response in plants and animals.

Hundreds of genes coding for miRNAs are already known in plants (e. g. for more than 600 miRNAs in rice) which are often organised in **clustered gene families** <http://www.mirbase.org>.

miRNA is impacting its target gene(s) 'simply' by downregulating. Depending on the **regulatory networks**, further genes (often involving several hundred genes) may be downregulated as well or also show higher expression.

miRNAs downregulate plant genes



miRNAs can escape knock-out via random mutation

Most experts agree, that it is **practically impossible** to effectively knock-out specific miRNA genes with non-targeted methods for purposeful use in plant breeding. Some reasons:

- miRNA genes are often **clustered** in several **copies** in close proximity within a genomic site and therefore can hardly be recombined by crossings.
- After random mutations of **single nucleotids, the function of the gene is often maintained**. Therefore, several mutations may be needed within the short miRNA gene sequences.
- Other genes of the same family of miRNA genes may **compensate the knock-out** of single copies.
- Very often, there are minor (but biological relevant) **differences between the members of a miRNA family** that only can be addressed by targeted methods.

NGTs are most powerful tool to knock-out miRNA genes

Knock-out of specific miRNA genes, typically, was not possible by random mutagenesis, but their **knock-down in transgenic plants**.

CRISPR/Cas has been proven to **effectively knock-out whole miRNA families** as well as **only some of its members in a targeted way**. It can also be used to knock-out several miRNA families simultaneously.

The NGT effects can be reached by **minor genetic changes** such as 3 base pairs within a gene sequence **or gene deletions**.

Examples: Knockdown of miRNA genes in rice

Zhao et al. (2017) successfully targeted two miRNA families, each with three members, thereby changing the expression of **dozens other miRNAs**.

Zhang et al. (2020) successfully knocked-out eight members of one miRNA family; one knock-out alone caused **more than 300 genes to be up-regulated and nearly 250 down-regulated**.

Zhou et al. (2022) knocked-out two members of a miRNA family. They observed **changes in expression in 119 miRNAs and further 763 genes coding for proteins**.

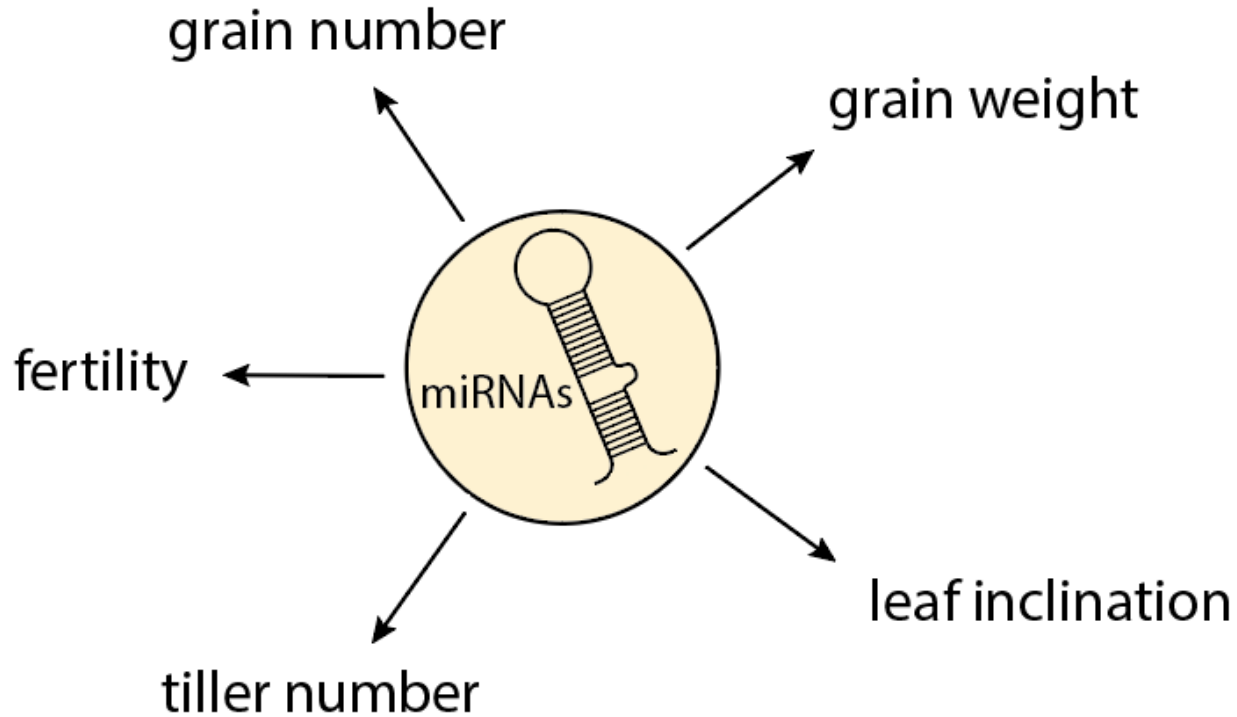
Examples: Knockdown of miRNA genes in tomatoes

Hong et al. (2021) knocked-out **two miRNAs that together target 22 genes**, with seven of them being targeted by both miRNAs.

Lin et al. (2022), knocked-out two miRNAs of one family and observed changes of gene activity in **several thousand genes** that then could be categorized to six clusters.

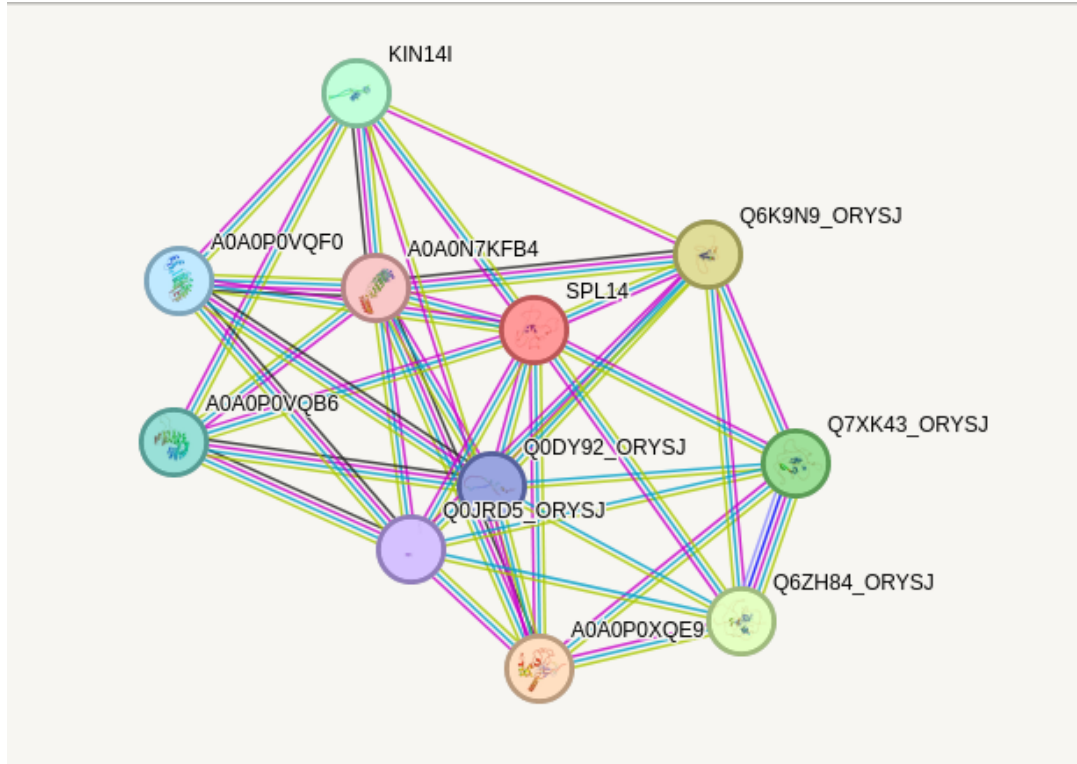
There are further examples for soybeans and maize.

Examples for miRNA applications in rice



(derived from: Peng et al., 2019)

Example: Targeting miRNA156 gene in rice

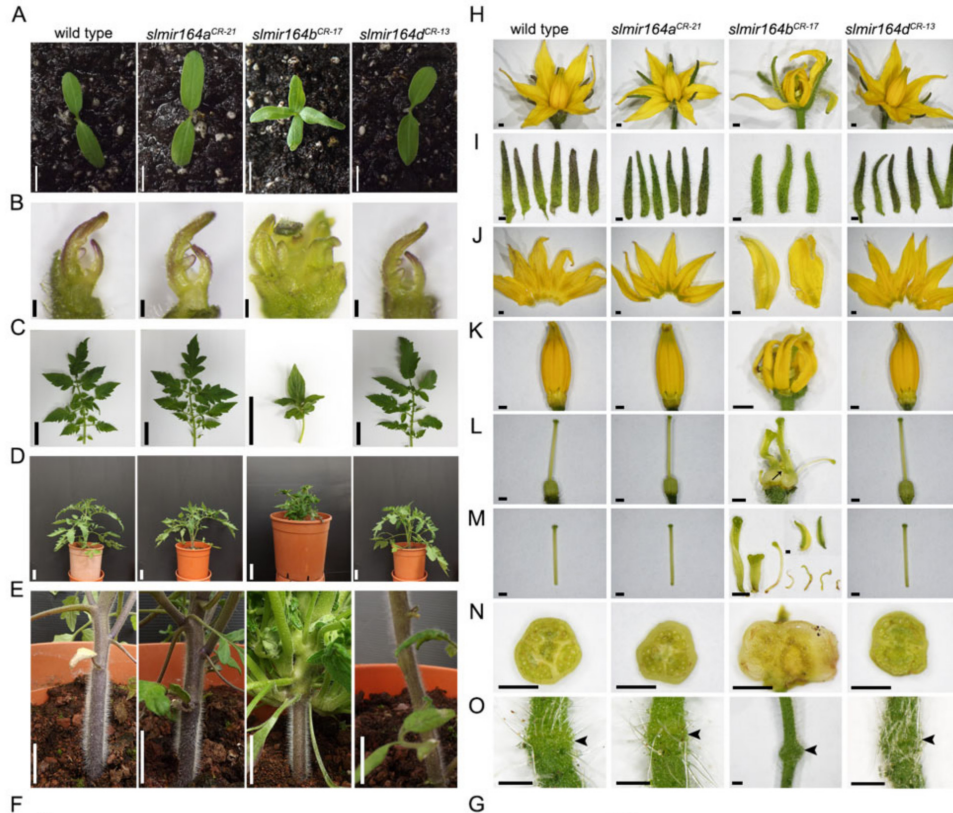


Network of proteins influenced by **SPL14** in rice which is a target gene of **miRNA156** and in the focus of several experiments.

miRNA156 via SPL14 is supposed to regulate **panicle branching and grain numbers in rice** (Peng et al., 2019).

<https://string-db.org/cgi/network?taskId=bTDW9KzCxSZp&sessionId=bfFl4u293E49>

Example: Targeting miRNA163 gene in tomatoes



Source:

Gupta et. al., (2021), CRISPR/Cas9 mutants of tomato MICRORNA164 genes uncover their functional specialization in development, *Plant Phys.*, 187; 1636–165

Between basic research and commercial applications

“Our transgene-free [...] knockout lines can be directly translated into the breeding practice that **may face less regulatory burden** in many countries.” (Zhou et al., 2022)

(!) Currently, mandatory risk assessment as set out in Directive 2001/18 is required if miRNAs are knocked-down via transgenesis with the same or similar results as those obtained from NGTs.

Perfect match with proposed regulatory loopholes

- miRNA are produced by regulatory genes, that **do not code for proteins**. Changes in regulatory genes are explicitly exempt from risk assessment as proposed by EU Parliament.
- **Knock-out** of miRNA genes can be achieved **by less than 20 changes in nucleotides or through deletions**. According to proposals made by Commission and Parliament, such genetic alterations are exempt from risk assessment.
- miRNAs, after processing in the cells, only have a length of around 20 nucleotides. The proposed regulation would not require risk assessment if **new functions** could be achieved by **changing less than 20 nucleotides** (see also Bohle et al. 2024).
- Knock-out of miRNA family can typically be achieved by making **less than 20 genetic changes (overall)**. Thus according to the Commission proposal, the plants could be equated to conventionally-bred plants.

Conclusions

The formal criteria as proposed by Commission and Parliament do not ensure the safe use of NGT plants:

- NGT plants that are substantially different to conventionally-bred plants would be included in the same category as conventionally-bred plants.
- Most powerful NGT applications would be exempt from regulation.

References

- Bohle et al. 2024, Where does the EU-path on new genomic techniques lead us?, *Frontiers in Genome Editing*, 6, <https://www.frontiersin.org/articles/10.3389/fgeed.2024.1377117>
- Hong et al. (2021) Editing miR482b and miR482c simultaneously by CRISPR/Cas9 enhanced tomato resistance to *Phytophthora infestans*. *Phytopathology* 111:1008–1016
- Lin et al. (2022) SIMIR164A regulates fruit ripening and quality by controlling SINAM2 and SINAM3 in tomato. *Plant Biotechnol J.* 20(8):1456-1469. doi: 10.1111/pbi.13824.
- Peng et al. (2019) (2019) MicroRNAs meet with quantitative trait loci: small powerful players in regulating quantitative yield traits in rice. *Wiley Interdisciplin Rev: RNA* 10(6):e1556
- Zhang et al. (2020) Disruption of MIR396e and MIR396f improves rice yield under nitrogen-deficient conditions. *Natl Sci Rev.* 7(1):102-112. doi: 10.1093/nsr/nwz142
- Zhao et al. (2017) Suppression of microRNA159 impacts multiple agronomic traits in rice (*Oryza sativa* L.). *BMC Plant Biol* 17:215
- Zhou et al. (2022) CRISPR-Cas9 mediated OsMIR168a knockout reveals its pleiotropy in rice. *Plant Biotechnol J* 20(2):310–322