

GUIDANCE

Risk assessment considerations for RNAi-based genetically modified plants

European Food Safety Authority (EFSA) | Francisco Barro | Albert Braeuning |
Tilemachos Goumperis | Aleksandra Lewandowska | Simon Moxon |
Nikoletta Papadopoulou | Elena Sánchez-Brunete

Correspondence: nif@efsa.europa.eu

The declarations of interest of all scientific experts active in EFSA's work are available at <https://open.efsa.europa.eu/experts>

Abstract

The risk assessment (RA) requirements for genetically modified plants (GMPs) are defined in Regulation (EU) No 503/2013 and the EFSA guidance on the RA of food and feed from GM plants (EFSA GMO Panel, 2011). When a GMP is developed to silence transcripts by RNA interference (RNAi), some specific additional analysis needs to be provided by the applicant. This guidance describes the requirements and recommendations for the GMP applications submitted to EFSA. It covers the molecular characterisation, focusing on bioinformatic analysis and confirmation of the trait, as well as the food and feed safety and dietary exposure assessment of RNAi-based GMPs. This document replaces the GMO panel strategy for the risk assessment of RNAi off targets in plants, described in Annex II to the minutes of the 118th Plenary meeting of the Scientific Panel on GMO and takes into account the current knowledge on the mechanisms of RNAi in plants.

KEYWORDS

genetically modified organisms, molecular characterisation, non-coding RNA, off-target, risk assessment, RNA interference

CONTENTS

Abstract.....	1
1. Introduction	3
2. Data and Methodologies.....	3
3. Requirements.....	3
3.1. Bioinformatic analyses	3
3.1.1. Parameters for in silico search for plant endogenous off-target transcripts	4
3.1.2. Considerations on how the results should be discussed	4
3.2. Confirmation of the trait	4
3.3. ncRNA stability and effects on humans/animals.....	5
3.4. Dietary exposure to short ncRNAs	5
4. Supporting information.....	5
Abbreviations	5
Acknowledgements	6
Requestor	6
Question number	6
Copyright for non-EFSA content.....	6
References.....	6
Annexes	8

1 | INTRODUCTION

Genetically modified plants (GMPs) can be designed to silence specific genes in the plant itself or other organisms by RNA interference (RNAi). This is achieved by inserting in the plant genome either a double-stranded RNA (dsRNA) or an artificial microRNA (miRNA) precursor. These molecules are cleaved into small RNAs that are 20–30 nucleotides long (small interfering RNAs [siRNAs] or miRNAs), which act as a template for an RNA-induced silencing complex (RISC) to specifically bind the target/messenger RNA (mRNA) with perfect or near-perfect complementarity, leading to cleavage of the target RNA (Burand & Hunter, 2013; Cagliari et al., 2019; Koch & Kogel, 2014). In addition to RNAi-based GMPs that are designed to reduce mRNA levels of specific endogenous plant gene(s) (e.g. EFSA GMO Panel, 2012, 2024a), RNAi-based GM plants may also induce silencing of mRNA transcripts in pests that potentially infect the plant (e.g. EFSA GMO Panel, 2018, 2024b). This last strategy is termed environmental RNAi.

GMPs and/or derived food/feed (FF) products are subject to a risk assessment (RA) and regulatory approval before entering the market in the European Union (EU), according to relevant legislation (Directive 2001/18/EC,¹ Regulation (EC) No 1829/2003² and Regulation (EU) No 503/2013³) and the EFSA guidance documents on the RA of food and feed from GMPs (EFSA GMO Panel, 2011) and on the environmental RA of GMPs (EFSA GMO Panel, 2010).

While these regulations and guidance are applicable to RNAi-based GMPs, some specificities have been identified for the RA of these plants (Casacuberta et al., 2015; Papadopoulou et al., 2020; Ramon et al., 2014). This guidance describes the information required for RNAi-based GMPs in applications submitted to EFSA. It provides specific requirements and recommendations for applicants regarding the molecular characterisation and risk assessment of the food and feed safety of RNAi-based GMPs. This guidance document does not cover environmental RA considerations. For more information on the topic, please refer to previous EFSA output on RNAi-based GMPs (Christiaens et al., 2018; Papadopoulou et al., 2020).

This document replaces the GMO panel strategy for the risk assessment of RNAi off-target studies in plants, described in Annex II to the minutes of the 118th Plenary meeting of the Scientific Panel on GMO⁴ and takes into account the current knowledge on the mechanisms of RNAi in plants.

2 | DATA AND METHODOLOGIES

In order to develop this guidance, EFSA consulted experts from the GMO Panel Cross-cutting working group, with expertise in Molecular Characterisation and Food and Feed risk assessment of RNAi-based GMPs. The EFSA staff and the experts took into account published scientific literature relevant for safety of RNAi-based GMPs (including Dávalos et al., 2019; Pačes et al., 2016; Papadopoulou et al., 2020), RA considerations from other regulatory authorities (e.g. relevant sections of OECD, 2023), experience from the assessment of RNAi-based GMO applications and preparatory work performed under the framework contract on literature reviews [OC/EFSA/MESE/2022/03] and specific contracts (OC/EFSA/MESE/2022/03-CT02 – SC08 and OC/EFSA/MESE/2022/03-CT03 – SC12). The search strings used and the retrieved papers from the updated scientific literature review are provided in Annexes A and B.

3 | REQUIREMENTS

3.1 | Bioinformatic analyses

For RNA interference, specificity is based on the sequence identity between the small silencing RNAs and their mRNA targets. Other transcripts that are not intended to be silenced but have sufficient sequence identity to the small silencing RNAs can also be targeted for degradation, which is referred to as off-target effects (Casacuberta et al., 2015; EFSA, 2014; Federal Insecticide, Fungicide and Rodenticide Act [FIFRA], Scientific Advisory Panel [SAP], 2014; Ramon et al., 2014). Off-target effects could occur in the GMP itself, or in other organisms that consume the GMP and derived products. Bioinformatic analyses for off-target transcripts are based on several criteria (e.g. the degree and position of base-pairing between the small RNA and the transcripts) that determine the efficiency of silencing (reviewed by Pačes et al., 2016). In silico target prediction algorithms are designed based on criteria related to the biochemical and thermodynamical properties of base-pairing (Pasquinelli, 2012; Rhoades et al., 2002). Depending on whether a dsRNA or artificial miRNA is used, a heterogeneous pool of siRNAs or a more homogeneous pool of miRNAs will be produced, impacting the silencing of the potential off-target transcript (Pačes et al., 2016). In case of siRNAs derived from dsRNAs, the number of different small RNAs targeting a particular off-target transcript will also influence the potential off-target silencing effect (Pačes et al., 2016).

¹Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 17.4.2001, pp. 1–38.

²Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, pp. 1–23.

³Commission Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. OJ L157, 8.6.2013, pp. 1–48.

⁴<https://www.efsa.europa.eu/sites/default/files/event/171025-m.pdf>.

According to the Implementing Regulation (EU) No 503/2013, Annex II, section 1.2.2.3, when silencing approaches by RNAi are used in GM plant applications, a bioinformatic analysis to identify potential off-target gene transcripts is required. In plants, a set of parameters allows for a reasonable prediction of RNAi off-target transcripts because exact alignments can be strict with respect to mismatches and hence are fast to compute. In contrast, in humans and other animals, the extent of complementarity between the small RNA and its target is more limited. Additionally, the complexity of microRNA gene targeting, the high level of noise and the absence of universally accepted methodology for off-target prediction in humans and animals does not allow for accurate predictions of off-target effects (Fridrich et al., 2019; Kern et al., 2020; Pinzón et al., 2017). The likelihood of a biological effect caused by such an off-target transcript is described in Section 3.3. Taking these into account, the applicant should only search for small RNAi off-target transcripts in the GMP.

3.1.1 | Parameters for in silico search for plant endogenous off-target transcripts

Based on the above, the EFSA developed a bioinformatics-based strategy for the RA of plant endogenous RNAi off-target transcripts. The parameters for identifying off-target transcripts in plants are applicable to both siRNAs and miRNAs, and are based on a conservative approach, relying primarily on knowledge from miRNA-target specificity that accounts for complementarity mismatches between the small RNA and target gene (Liu et al., 2014).

The applicants will be requested to report on possible off-target plant transcripts complying with all the following rules for all 21nt small RNAs potentially produced:

- No more than four mismatches (complementarity mismatches) with no gap or three mismatches and one gap;
- A G: U pair is considered as a mismatch, however it counts as half a mismatch;
- Only one single gap can be present either within the target sequence or the small RNA itself;
- Gaps cannot be of more than one nucleotide;
- No mismatches/gap at position 10 or 11 of the small RNA sequence;
- No more than two mismatches (or 1 mismatch and a gap) in the first 12 nt at the 5' of the small RNA;
- The minimum free energy of the duplex divided by the minimum free energy of the perfect complement should be greater than 0.75 (Allen et al., 2005).

The transcriptome of the GM plant species (the most up-to-date version available) should be used for off-target searches. Moreover, the search should also be performed on all newly expressed transcripts in the event (either single or stack) under assessment. If it is not feasible to perform an off-target search on all newly expressed transcripts, the applicant should provide a supplementary theoretical analysis, considering the likelihood of novel transcript formation and its potential impact on off-target silencing based on known mechanisms of RNAi.

3.1.2 | Considerations on how the results should be discussed

The RA of the potential off-target gene silencing in the plant should be based on:

- The number of different small RNAs showing significant similarity to the same potential off-target transcript. The potential for repression of a gene by a small RNA is correlated with the number of different small RNAs that can bind the same transcript (Hannus et al., 2014).
- Off-target transcripts with multiple hits should be the prime focus of the assessment. However, in GMP applications where a single small RNA is produced at high abundance (e.g. production of artificial miRNA), a single off-target sequence within a transcript may also be of relevance.
- The established or predicted function of the potential off-target genes and their potential impact on the safety of the GM plant and/or derived products as food/feed and for the environment.

The outcome of plant off-target transcript analyses will be assessed in light of the agronomic/phenotypic and compositional field-trial data gathered as part of GMP market applications, as they are designed to identify intended and unintended changes in GMPs. On a case-by-case basis, if a potential plant off-target transcript is identified, additional experimental data may be needed to investigate the predicted silencing effect.

3.2 | Confirmation of the trait

According to the implementing regulation (EU) No 503/2013, Annex II, section 1.2.2.3, the applicant shall provide information on the expression of the insert. More specifically, the applicant is requested to 'demonstrate whether the inserted/modified sequence results in intended changes at the protein, RNA and/or metabolite levels' and 'When justified by the nature of the insert (such as silencing approaches or where biochemical pathways have been intentionally modified), specific RNA(s) or metabolite(s) shall be analysed'. To comply with the requirement of the Implementing Regulation 503/2013,

the applicants are required to provide information confirming that the trait is observed. This will be assessed case-by-case, depending on the trait introduced (e.g. downregulation of an endogenous transcript, change in metabolite levels – for an example, see EFSA GMO Panel, 2024a) and the type of short RNA (siRNA or miRNA). Moreover, as described in Section 3.1.2, if there are any potential off-target transcripts identified, additional information might be requested.

In some RNAi-based GMP applications (EFSA GMO Panel, 2018, 2019, 2024b), the applicants have provided dsRNA levels measured in different GM plant tissues (Urquhart et al., 2015). However, since it is likely that plant Dicer proteins will process some of the dsRNA into siRNAs, the GMO Panel considered that ‘the levels of dsRNA are not a good proxy for the levels of the active siRNAs present in plants’ (EFSA GMO Panel, 2018, 2019, 2024b; Pačes et al., 2016). While there are techniques that allow for measurement of short non-coding RNAs (ncRNAs) in plant tissues (e.g. short RNA sequencing, QuantiGene assay, stem-loop RT-qPCR for miRNAs), the optimal experimental design for these analyses remains unclear, i.e. which tissues and developmental stages should be analysed. Considering that RNA pools are highly variable and there are no clear thresholds of biologically relevant miRNA and siRNA concentrations, such data would be difficult to analyse. For these reasons, and taking into account the considerations further discussed below (see Sections 3.3 and 3.4), the exact levels of short ncRNAs are of limited use for risk assessment.

3.3 | ncRNA stability and effects on humans/animals

ncRNAs, including silencing RNAs, are ubiquitous constituents of human and animal diets. In general, dietary ncRNAs are rapidly denatured, depurinated and degraded shortly after ingestion due to enzymatic activities (e.g. salivary and pancreatic RNases, digestive enzymes) and physico-chemical conditions (e.g. pH) in the gastrointestinal (GI) tract lumen. Furthermore, the presence of barriers (e.g. mucus, cellular membranes) limits the cellular uptake of ncRNAs by gastrointestinal cells and rapid intracellular degradation of potentially internalised ncRNAs occurs (Dávalos et al., 2019). Due to the above, the amount of RNAs taken up and absorbed after oral ingestion is considered negligible in humans and animals (mammals, birds and fish). Therefore, in cases where the ncRNA does not contain any structural modification aimed to increase stability or cellular uptake in the GI tract following oral administration, it is deemed highly unlikely that the short ncRNAs might exert biological effects once ingested by humans, mammals, birds and fish. In this case, no data on stability or fate or toxicological studies of the ncRNA would be required. On the other hand, when the ncRNA under assessment is modified to increase its stability and/or cellular uptake in the GI tract and systemic absorption following oral administration, additional data might be requested on a case-by-case basis.

3.4 | Dietary exposure to short ncRNAs

As described above, since ncRNAs are generally rapidly denatured, depurinated and degraded shortly after ingestion, and given that the general scientific consensus is that the likelihood that ncRNAs exert any biological effects once ingested by humans and animals is very low, no dietary exposure assessment needs to be conducted. However, if the ncRNA has been modified to increase its stability, additional data might be requested on a case-by-case basis to assess the impact of such modification on dietary exposure to the ncRNA.

4 | SUPPORTING INFORMATION

In order to perform the *in silico* analyses of plant RNAi off-target transcripts, it is recommended that the applicants use the bioinformatic script available on EFSA repository together with its documentation:

https://dev.azure.com/efsa-devops/EFSA/_git/efsa.bioinfo.RNAi.

The script can be run by users on an environment of their choice, and is not linked to any EFSA database, repository or system of any kind. Required inputs (the query sequence, the transcriptomes and/or other target sequences) are inserted by users at every new run. The script runs the analysis off-line, without sending information over the internet, for confidentiality reasons. The outcome of the analysis (a structured file with a sorted list of potential off-target transcripts and their alignments to the query sequence) is owned by the applicant. In case the corresponding dossier is submitted to EFSA, the outcome of the analysis performed using this script shall be included in the dossier, together with the information required in Section 3.

ABBREVIATIONS

dsRNA	double-stranded RNA
FF	food and feed
GI	gastrointestinal
GM	genetically modified
GMO Panel	EFSA Panel on Genetically Modified Organisms

GMO	genetically modified organism
GMP	genetically modified plant
miRNA	microRNA
ncRNA	non-coding RNA
OECD	Organisation for Economic Co-operation and Development
RA	risk assessment
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
siRNA	small interfering RNA

ACKNOWLEDGEMENTS

EFSA wishes to thank the members of the Working Groups on Molecular characterisation and Food/feed for reviewing this scientific output, hearing expert Alberto Dávalos for participation in the discussions of the Cross-cutting working group.

REQUESTOR

EFSA

QUESTION NUMBER

EFSA-Q-2024-00613

COPYRIGHT FOR NON-EFSA CONTENT

EFSA may include images or other content for which it does not hold copyright. In such cases, EFSA indicates the copyright holder and users should seek permission to reproduce the content from the original source.

REFERENCES

- Allen, E., Xie, Z., Gustafson, A. M., & Carrington, J. C. (2005). microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell*, 121(2), 207–221.
- Burand, J. P., & Hunter, W. B. (2013). RNAi: Future in insect management. *Journal of Invertebrate Pathology*, 112, S68–S74. <https://doi.org/10.1016/j.jip.2012.07.012>
- Cagliari, D., Dias, N. P., Galdeano, D. M., dos Santos, E. A., Smagghe, G., & Zotti, M. J. (2019). Management of pest insects and plant diseases by nontrans-formative RNAi. *Frontiers in Plant Science*, 10, 1319. <https://doi.org/10.3389/fpls.2019.01319>
- Casacuberta, J. M., Devos, Y., du Jardin, P., Ramon, M., Vaucheret, H., & Nogué, F. (2015). Biotechnological uses of RNAi in plants: Risk assessment consid-erations. *Trends in Biotechnology*, 33(3), 145–147.
- Christiaens, O., Dzhambazova, T., Kostov, K., Arpaia, S., Joga, M. R., Urru, I., Sweet, J., & Smagghe, G. (2018). Literature review of baseline information on RNAi to support the environmental risk assessment of RNAi-based GM plants. *EFSA supporting publications*, 15(5), EN-1424. <https://doi.org/10.2903/sp.efsa.2018.EN-1424>
- Dávalos, A., Henriques, R., Latasa, M. J., Laparra, M., & Coca, M. (2019). Literature review of baseline information on non-coding RNA (ncRNA) to support the risk assessment of ncRNA-based genetically modified plants for food and feed. *EFSA supporting publications*, 16(8), EN-1688. <https://doi.org/10.2903/sp.efsa.2019.EN-1688>
- EFSA (European Food Safety Authority). (2014). International scientific workshop ‘risk assessment considerations for RNAi-based GM plants’ (4–5 June 2014, Brussels, Belgium). *EFSA supporting publications*, 11(12), EN-705. <https://doi.org/10.2903/sp.efsa.2014.EN-705>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms). (2010). Guidance on the environmental risk assessment of genetically modified plants. *EFSA Journal*, 8(11), 1879. <https://doi.org/10.2903/j.efsa.2010.1879>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms). (2011). Guidance for risk assessment of food and feed from genetically modified plants. *EFSA Journal*, 9(5), 2150. <https://doi.org/10.2903/j.efsa.2011.2150>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms). (2012). Scientific Opinion on application (EFSA-GMO-NL-2010-78) for the placing on the market of herbicide tolerant genetically modified soybean MON 87705 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Monsanto. *EFSA Journal*, 10(10), 2909. <https://doi.org/10.2903/j.efsa.2012.2909>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Mullins, E., Bresson, J.-L., Dalmay, T., Dewhurst, I. C., Epstein, M. M., Firbank, L. G., Guerche, P., Hejatko, J., Moreno, F. J., Naegeli, H., Nogué, F., Rostoks, N., Sánchez Serrano, J. J., Savoini, G., Veromann, E., Veronesi, F., Ardizzone, M., De Sanctis, G., ... Xiftou, K. (2024a). Assessment of genetically modified maize MON 94804 (application GMFF-2022-10651). *EFSA Journal*, 22(4), 8714. <https://doi.org/10.2903/j.efsa.2024.8714>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Mullins, E., Bresson, J.-L., Dalmay, T., Dewhurst, I. C., Epstein, M. M., Firbank, L. G., Guerche, P., Hejatko, J., Moreno, F. J., Naegeli, H., Nogué, F., Rostoks, N., Sánchez Serrano, J. J., Savoini, G., Veromann, E., Veronesi, F., Ardizzone, M., Camargo, A. M., ... Raffaello, T. (2024b). Assessment of genetically modified maize DP23211 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2019-163). *EFSA Journal*, 22(1), 8483. <https://doi.org/10.2903/j.efsa.2024.8483>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Naegeli, H., Birch, A. N., Casacuberta, J., De Schrijver, A., Gralak, A. M., Guerche, P., Jones, H., Manachini, B., Messéan, A., Nielsen, E. E., Nogué, F., Robaglia, C., Rostoks, N., Sweet, J., Tebbe, C., Visioli, F., Wal, J.-M., Ardizzone, M., ... Ramon, M. (2018). Scientific Opinion on the assessment of genetically modified maize MON 87411 for food and feed uses, import and processing, under regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2015-124). *EFSA Journal*, 16(6), 5310. <https://doi.org/10.2903/j.efsa.2018.5310>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Naegeli, H., Bresson, J.-L., Dalmay, T., Dewhurst, I. C., Epstein, M. M., Firbank, L. G., Guerche, P., Hejatko, J., Moreno, F. J., Mullins, E., Nogué, F., Rostoks, N., Serrano Sánchez, J. J., Savoini, G., Veromann, E., Veronesi, F., Álvarez, F., Ardizzone, M., ... Paraskevopoulos, K. (2019). Scientific opinion on the assessment of genetically modified maize MON 87427 × MON 89034 × MIR162 × MON 87411 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2017-144). *EFSA Journal*, 17(11), 5848. <https://doi.org/10.2903/j.efsa.2019.5848>
- Federal Insecticide, Fungicide and Rodenticide Act [FIFRA], Scientific Advisory Panel [SAP]. (2014). *Transmittal of the meeting, minutes of the FIFRA SAP meeting held January 28, 2014 on the scientific issues associated with the use of “RNAi technology as a pesticide: Problem formulation for human health*

- and ecological risk assessment." SAPanel minutes no. 2014-02. <https://www.epa.gov/sap/meeting-materials-january-28-2014-scientific-advisory-panel>
- Fridrich, A., Hazan, Y., & Moran, Y. (2019). Too many false targets for MicroRNAs: Challenges and pitfalls in prediction of miRNA targets and their gene ontology in model and non-model organisms. *BioEssays*, 41(4), 1800169.
- Hannus, M., Beitzinger, M., Engelmann, J. C., Weickert, M. T., Spang, R., Hannus, S., & Meister, G. (2014). siPools: Highly complex but accurately defined siRNA pools eliminate off-target effects. *Nucleic Acids Research*, 42(12), 8049–8061.
- Kern, F., Backes, C., Hirsch, P., Fehlmann, T., Hart, M., Meese, E., & Keller, A. (2020). 2020. What's the target: Understanding two decades of in silico microRNA-target prediction. *Briefings in Bioinformatics*, 21(6), 1999–2010. <https://doi.org/10.1093/bib/bbz111>
- Koch, A., & Kogel, K. H. (2014). New wind in the sails: Improving the agronomic value of crop plants through RNAi-mediated gene silencing. *Plant Biotechnology Journal*, 12, 821–831. <https://doi.org/10.1111/pbi.12226>
- Liu, Q., Wang, F., & Axtell, M. J. (2014). Analysis of complementarity requirements for plant MicroRNA targeting using a *Nicotiana benthamiana* quantitative transient assay. *The Plant Cell*, 26, 741–753.
- OECD. (2023). *Considerations for the human health risk assessment of externally applied dsRNA-based pesticides*. <https://doi.org/10.1787/54852048-en>
- Pačes, J., Nič, M., Novotný, T., & Svoboda, P. (2016). Literature review of baseline information to support the risk assessment of RNAi-based GM plants. *EFSA supporting publications*, 14(6), EN-1246. <https://doi.org/10.2903/sp.efsa.2017.EN-1246>
- Papadopoulou, N., Devos, Y., Álvarez-Alfageme, F., Lanzoni, A., & Waigmann, E. (2020). Risk assessment considerations for genetically modified RNAi plants: EFSA's activities and perspective. *Frontiers in Plant Science*, 11, 445. <https://doi.org/10.3389/fpls.2020.00445>
- Pasquinelli, A. E. (2012). MicroRNAs and their targets: Recognition, regulation and an emerging reciprocal relationship. *Nature Reviews Genetics*, 13, 271–282.
- Pinzón, N., Li, B., Martinez, L., Sergeeva, A., Presumey, J., Apparailly, F., & Seitz, H. (2017). microRNA target prediction programs predict many false positives. *Genome Research*, 27(2), 234–245.
- Ramon, M., Devos, Y., Lanzoni, A., Liu, Y., Gomes, A., Gennaro, A., & Waigmann, E. (2014). RNAi-based GM plants: Food for thought for risk assessors. *Plant Biotechnology Journal*, 12(9), 1271–1273.
- Rhoades, M. W., Reinhart, B. J., Lim, L. P., Burge, C. B., Bartel, B., & Bartel, D. P. (2002). Prediction of plant micro RNA targets. *Cell*, 110(4), 513–520.
- Urquhart, W., Mueller, G. M., Carleton, S., Song, Z., Perez, T., Uffman, J. P., Jensen, P. D., Levine, S. L., & Ward, J. (2015). A novel method of demonstrating the molecular and functional equivalence between in vitro and plant-produced double-stranded RNA. *Regulatory Toxicology and Pharmacology*, 73(2), 607–612. <https://doi.org/10.1016/j.yrtph.2015.09.004>

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: EFSA (European Food Safety Authority), Barro, F., Braeuning, A., Goumperis, T., Lewandowska, A., Moxon, S., Papadopoulou, N., & Sánchez-Brunete, E. (2025). Risk assessment considerations for RNAi-based genetically modified plants. *EFSA Journal*, 23(3), e9321. <https://doi.org/10.2903/j.efsa.2025.9321>

ANNEXES

ANNEX A

Methodology for the literature studies

Annex A is available under the Supporting Information section on the online version of the scientific output.

ANNEX B

Outcome of the scientific literature search

Annex B is available under the Supporting Information section on the online version of the scientific output.