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Summary:	This document provides guidance on preparing proposals to exempt plants with additional modifications from regulation, as described in 7 CFR § 340.1(b)(4). APHIS protects and enhances U.S. agricultural and natural resources using a science-based and risk-based regulatory framework to ensure the safe movement – including importation, interstate movement, and confined environmental release – of organisms developed using genetic engineering. APHIS receives its regulatory authority from the Plant Protection Act of 2000, and oversees organisms developed using genetic engineering in accordance with its regulations under 7 CFR part 340 (*Movement of Organisms Modified or Produced Through Genetic Engineering*).
Disclaimer:	The contents of this guidance document do not have the force and effect of law and are not meant to bind the public in any way. This document is intended only to provide clarity to the public regarding existing requirements under the law or agency regulations.

USDA-APHIS Biotechnology Regulatory Services

Guidance for Preparing Proposals to Exempt Plants with Additional Modifications from Regulation Pursuant to 7 CFR § 340.1(b)(4)

v. 01/07/2021

Biotechnology Regulatory Services
Animal and Plant Health Inspection Service
United States Department of Agriculture
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Pursuant to the Congressional Review Act (5 U.S.C. § 801 et seq.), the Office of Information and Regulatory Affairs designated this document as a non-major rule, as defined by 5 U.S.C. § 804(2).

The information contained in this document is intended solely as guidance. Except where noted, persons may choose to follow APHIS guidance or follow different procedures, practices, or protocols that meet applicable statutes and regulations.

Language implying that guidance is mandatory (e.g., "shall," "must," "required," or "requirement") should not be construed as binding unless the terms are used to refer to a statutory or regulatory requirement.

Following the guidance contained in this document should not be construed as a guarantee of compliance with applicable statutes and regulations.

Guidance for Preparing Proposals to Exempt Plants With Additional Modifications from Regulation Pursuant to 7 CFR § 340.1(b)(4)

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Introduction to Preparing Proposals to Exempt Plants with Additional Modifications Plants from Regulation Pursuant to 7 CFR § 340.1(b)(4)

APHIS regulations at 7 CFR part 340 govern the movement of certain organisms that are modified or produced through genetic engineering. The regulations specify certain plants that are exempt from the regulations (7 CFR § 340.1(b), (c)). Among plants containing genetic modifications (i.e., changes to the plant genome), that could be achieved through conventional breeding, only plants containing a single genetic modification of one of the types listed in 7 CFR § 340.1(b)(1-3) are initially exempt from the regulations. Over time, APHIS expects new plant breeding innovations to evolve with advancements in science and technology, along with further development of scientific information related to conventional plant breeding. To ensure the regulations keep pace with advancements in science and technology, § 340.1(b)(4) of the regulations establishes a process by which the Administrator can identify additional modifications that plants can contain and be exempt from the regulations, based on what could be achieved through conventional breeding. Through this process the Administrator could, for example, exempt plants that contain multiple genetic modifications on a species-specific basis, or the Administrator could list a new type of genetic modification that any plant could contain and be exempt from regulation.

Proposals to exempt plants with additional modifications from the regulations may be APHIS-initiated or may be initiated by another party accompanied by adequate supporting information. In either case, assuming APHIS finds the proposal by the outside party to be scientifically credible, APHIS will publish a notice in the *Federal Register* of the proposal, along with the supporting information and will request public comments. After reviewing the comments, APHIS will publish a subsequent notice in the *Federal Register* announcing its action on the proposal. A list specifying modifications that plants can contain and be exempt pursuant to § 340.1(b) including § 340.1(b)(4) will be available on the APHIS website at: https://www.aphis.usda.gov/aphis/ourfocus/biotechnology.

APHIS is providing the following guidance to help those who wish to prepare and submit proposals for exempting plants with additional modifications from the regulations pursuant to 7 CFR § 340.1(b)(4). We recommend discussing your proposal with APHIS prior to your first submission.

Submitting Proposals Under 7 CFR § 340.1(b)(4)

You must electronically submit your proposal via email (ExemptionProposals@usda.gov) to:

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services

The proposal must include the following information:

- Proposer's name and contact information, including email address.
- A description that clearly describes the additional modification(s) that a plant or plants can contain and qualify for exemption (e.g., a number of changes achieved in all plants generally; a number of changes in a particular plant species; a type of modification other than those already listed 7 CFR § 340.1(b)(1-3)).
- The factual grounds demonstrating that the proposed genetic modification(s) could be achieved through conventional plant breeding[1].
- Copies of scientific literature or publicly available information that support the proposal and demonstrate the stated modification(s) can be achieved in conventional breeding.
- Any information known to the proposer that would be unfavorable to the proposal.

Examples of how to prepare a proposal for additional modifications plants can contain and qualify for exemption, based on existing exemptions (7 CFR § 340.1(b)(1) and (2)), are shown in Appendix 1.

After APHIS receives all the information required for a proposal, APHIS will complete its review and make a final determination within 12 months, except in circumstances that could not reasonably have been anticipated.

- If, after review of the proposal, APHIS determines there is insufficient publicly available information and/or data or disagrees with the conclusion that plants containing the modification(s) could be achieved through conventional breeding methods, APHIS will return the proposal to the submitter and note the reasons for the return in writing.
- If, after review of the proposal, APHIS initially determines that plants containing the modification(s) could be achieved through conventional breeding methods, APHIS will publish a notice in the *Federal Register* proposing to exempt plants containing the additional modification(s) from the regulations, including publicly available information and/or data demonstrating that the modification(s) could be achieved through conventional breeding methods, in accordance with the process set forth in § 340.1(b)(4)(i), requesting public comment. After reviewing the comments, APHIS will publish a subsequent notice in the *Federal Register* announcing its final determination.

A list specifying additional modifications that plants can contain and be exempt from regulation pursuant to 7 CFR § 340.1(b)(4) will be available on the APHIS website at:
https://www.aphis.usda.gov/aphis/ourfocus/biotechnology

[1] As described in the Preamble to the revised 7 CFR part 340, our standard for "could be achieved through conventional breeding" is that the genetic modification could practically be expected to be pursued and achieved in a conventional breeding program. For example, evidence that multiple desired traits or genetic modifications can be introduced in a plant in a single step on a practical basis is needed to meet this standard. We are unlikely to adopt an exemption for plants containing statistically improbable modifications.

Appendix 1

Example 1 based on § 340.1(b)(1)

Name: Dr. Professor Scientist, 111 Plant Breeding Road, Anycity, Anystate, 00000
drprofessorscientist@any.edu

Description of the modification.
The genetic modification is a single change in any plant species resulting from cellular repair of a targeted DNA break in the absence of an externally provided repair template.

The factual grounds demonstrating that the proposed modification(s) could be achieved through conventional plant breeding.
Spontaneous mutation rates in higher plants are low, ranging from 10^{-5} to 10^{-8} (1). During the 1920s and 1930s, scientists discovered that mutation rates could be increased using radiation and chemical treatments (2). Breeding programs based on efficient mutation techniques have been widely used by plant breeders and many of our food crops are derived either directly or indirectly from such programs (2, 3). The molecular basis of spontaneous mutation, chemical mutagenesis, and radiation mutagenesis is known to result from damage to DNA(2-4). Plants have evolved a number of DNA repair pathways to repair damaged DNA. One of the common types of DNA damage is the double strand break (3, 4). When double strand breaks are repaired, the frequent outcome is a deletion or insertion (also known as an indel) (5). Genome editing, just like conventional breeding, can be used to generate indels. Whole genome sequencing of individuals within a breeding population reveals the wide extent of polymorphisms created from DNA repair events (6, 7). As cellular repair of a targeted DNA break in the absence of an externally provided repair template is a common occurrence in conventional breeding, a plant containing a modification involving DNA breaks induced through genome editing should be eligible for exemption.

Copies of scientific literature, unpublished studies, or other data that support the proposal demonstrating that the stated modification(s) can routinely be achieved in conventional breeding.

1. S.-Y. Jiang, S. Ramachandran, Assigning biological functions to rice genes by genome annotation, expression analysis and mutagenesis. *Biotechnology Letters* **32**, 1753-1763 (2010).
2. M. C. Kharkwal, "A brief history of plant mutagenesis" in Plant Mutation Breeding and Biotechnology, Q. Y. Shu, B.P. Forster, and H. Nakagawa, Ed. (CABI, 2012), 10.1079/9781780640853.0000 chap. 2, pp. 21-30.
3. Y. Oladosu *et al.*, Principle and application of plant mutagenesis in crop improvement: a review. *Biotechnology & Biotechnological Equipment* **30**, 1-16 (2016).
4. Q. Que *et al.*, Plant DNA Repair Pathways and Their Applications in Genome Engineering. *Methods Mol Biol* **1917**, 3-24 (2019).
5. H. Puchta, The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *Journal of Experimental Botany* **56**, 1-14 (2004).
6. S. Li *et al.*, Frequency and type of inheritable mutations induced by γ rays in rice as revealed by whole genome sequencing. *J Zhejiang Univ Sci B* **17**, 905-915 (2016).
7. S. Sun *et al.*, Extensive intraspecific gene order and gene structural variations between Mo17 and other maize genomes. *Nat Genet* **50**, 1289-1295 (2018).

Information unfavorable to the proposal
None

Example 2 based on § 340.1(b)(2)

Name: Dr. Professor Scientist, 111 Plant Breeding Road, Anycity, Anystate, 00000
drprofessorscientist@anycompany.com

Description of the modification.
The genetic modification is a single targeted single base pair substitution in the genome of any plant species.

The factual grounds demonstrating that the proposed modification(s) could be achieved through conventional plant breeding.
Chemical and radiation mutagenesis has been widely used in plant breeding (1-4). The Food and Agriculture Organization of the United Nations/International Atomic Energy Agency – Mutant Variety Database (FAO/IAEA-MVD) data (2019) reports on the developed and officially released mutants, a total of 3,275 accessions from 225 species (4). The types of chemical mutagens most widely used in plants include ethyl-methane sulfonate (EMS) (1, 3, 5, 6), methyl-methansulfonate (MMS) (3), sodium azide (7), N-methyl-N-nitrosourea (MNU) (8) and hydroxylamine (4, 9).

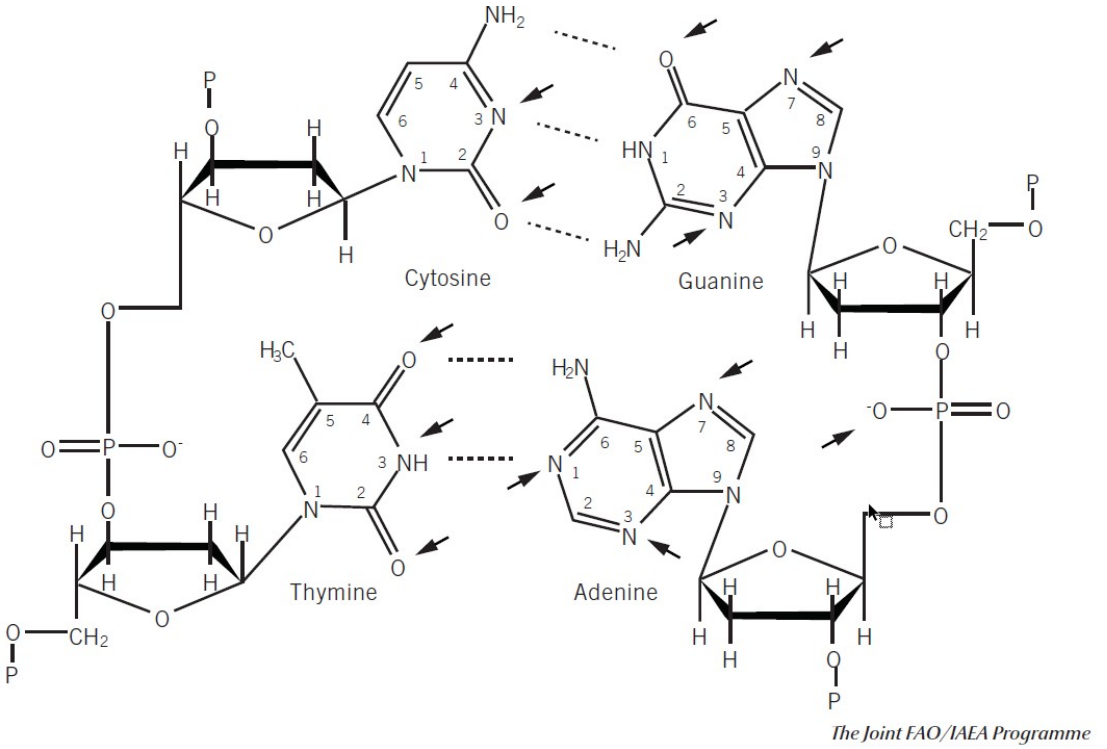

Figure 12.4 Most frequently alkylated sites in DNA.

From (2).

EMS, MMS, and MNU are strong alkylating agents (2). These chemicals modify nucleotides by attaching an ethyl (EMS) or methyl (MMS and MNU) to the N or O moieties of the DNA bases.(10). The most frequently alkylated sites are shown in Figure 12.4 from reference (2). Modifications that occur on Guanine-O6, cytosine-

N3, cytosine O2, Adenine-N1, Adenine-N3, Thymine-O4, Thymine-N3, and Thymine-O2 can influence base pairing (2, 11-14). In some cases, the modification causes mispairing that leads to mutation. The most well documented case of mispairing results from ethylation of Guanine-O6, after treatment with ethyl methane sulfonate (EMS), diethyl sulfate, or ethyl nitrosourea (ENU) (9). When Guanine-O6 is ethylated, guanine can pair with thymine instead of its usual cytosine (2, 9). During DNA replication, a DNA repair process removes the substituted guanine and replaces it with an adenine, the usual base pair for thymine (2, 9). This mutation results in a G to A transition (2, 9). Transitions are substitutions for purines with purines (A for G and G for A) and pyrimidines with pyrimidines (C for T and T for C). Transversions are substitutions between purines and pyrimidines (A or G for C or T and vice versa). The predominant mutation resulting from EMS mutagenesis is the G to A transition (1, 2). At a low frequency, EMS generates G to C or G to T transversions by 7-ethylguanine hydrolysis or A to G transition by 3-ethyladenine pairing errors (1, 13). Minoia *et al*. (2010) also observed A to C transversions in EMS treated tomato (15).

Some of the alkylation sites on DNA bases do not influence base pairing. The most heavily alkylated site by methyl methanesulfonate (MMS) is Guanine-N7 representing 83% of the alkylation sites (2, 11) and this modification does not influence base pairing (14). It is thought that when alkylation of the base occurs on a moiety not involved in base pairing, mutagenesis can still occur. The modified base is likely to be removed from the DNA strand (11-14) and when depurination occurs, faulty excision repair may lead to mutagenesis (16). MMS frequently leads to the formation of T to G transversions (2) and G to A and A to G transitions (1, 2).

MNU alkylates Guanine-O6 to a much greater extent than MMS showing a bias for G to A transitions (2). It can also alkylate Guanine-N7 and Adenine at N1, N3, and N7 (2). Suzuki *et al*. (2008) sequenced mutants isolated after MNU mutagenesis and also detected C to T transitions at high frequency, as well as T to A and A to T transversions (8).

Non-alkylating mutagenesis.
Hydroxylamine and sodium azide are non-alkylating mutagens frequently used in plant mutagenesis (2, 3). Hydroxylamine reacts with N substituents that are not alkylated by alkylating agents forming either N4 hydroxycytosine or N6 hydroxyadenosine (9, 17). As depicted in Figure 12.4, both target sites play a role in base pairing. Brown (1968) speculated that the Cytosine derivative can base pair with T leading to a C to A transition (17).

Sodium azide is the principle chemical mutagen used in barley breeding (7). Sodium azide reacts with the endogenous metabolite O-acetylserine to form a reactive intermediate, beta-azidoalanine; presumably the beta azidoalanine reacts directly with DNA or through another intermediate to modify the DNA(7). It is not clear how this DNA modification results in base pairing substitutions (7). Olsen et al. (7) isolated several mutant lines from a screen for pigment mutants and sequenced candidate genes. They identified a number of single base pair substitutions in azide mutagenized barley including A to G; G to A, and T to C transitions and A to T and T to A transversions (7).

Summary of reviewed mutagenesis outcomes.
Chemical mutagenesis results in single base pair mutations as a result of DNA modification and repair. The agents used in mutagenesis modify DNA in various ways. In some cases, the modification leads to mispairing and repair. In other cases, excision of the modified base followed by faulty repair is thought to lead to the mutation. In most cases, the mutation is a G to A transition. However, all 12 possible combinations of single base pair substitution have been reported in the course of breeding experiments. These data are summarized in the table below:

	Original base	SBS		
1	A	G	Transition	MMS (1); NA (7); EMS ((1)
2	A	C	Transversion	EMS (15)
3	A	T	Transversion	MMS (1); MNU (8); NA (13)
4	G	A	Transition	EMS (1); MNU (2, 8); MMS (2); NA (7)
5	G	C	Transversion	EMS (1)
6	G	T	Transversion	EMS (1)
7	C	G	Transversion	MNU (2); MMS(2)
8	C	T	Transition	MNU (8); EMS (6))
9	C	A	Transversion	HA (17)
10	T	A	Transversion	MNU (8); NA (7); EMS (6)
11	T	C	Transition	NA (7)
12	T	G	Transversion	MMS (1); EMS (6)

MMS-methyl methane sulfonate
NA-sodium azide
EMS-ethane methyl sulfonate
MNU-methyl nitrosourea
HA-hydroxylamine
SBS-single base pair substitution
Numbers in parentheses refer to references.

Conclusion

As chemical mutagenesis is widely used in plant breeding, as single base pair substitutions are a frequent outcome of chemical mutagenesis, and all possible single nucleotide substitutions have been observed by sequencing plant DNA derived from plants subjected to chemical mutagenesis, a modification that results in a single nucleotide substitution should be exempt from regulation.

Copies of scientific literature, unpublished studies, or other data that support the proposal demonstrating that the stated modification(s) can routinely be achieved in conventional breeding.

1. Y. Kim, K. S. Schumaker, J. K. Zhu, EMS mutagenesis of Arabidopsis. *Methods Mol Biol* **323**, 101-103 (2006).
2. J. M. Leitao, "Chemical Mutagenesis" in Plant Mutation Breeding and Biotechnology, Q. Y. Shu, Forster, B. P., Nakagawa, H., Ed. (CABI, 2012), http://doi.org/10.1079/9781780640853.0135 chap. 12, pp. 135-158.
3. M. A. J. Parry *et al.*, Mutation discovery for crop improvement. *Journal of Experimental Botany* **60**, 2817-2825 (2009).
4. V. E. Viana, C. Pegoraro, C. Busanello, A. Costa de Oliveira, Mutagenesis in Rice: The Basis for Breeding a New Super Plant. *Frontiers in Plant Science* **10** (2019).
5. J. Maple, S. G. Møller, Mutagenesis in Arabidopsis. *Methods Mol Biol* **362**, 197-206 (2007).
6. X. Serrat *et al.*, EMS mutagenesis in mature seed-derived rice calli as a new method for rapidly obtaining TILLING mutant populations. *Plant Methods* **10**, 5 (2014).
7. O. Olsen, X. Wang, D. von Wettstein, Sodium azide mutagenesis: preferential generation of A.T-->G.C transitions in the barley Ant18 gene. *Proc Natl Acad Sci U S A* **90**, 8043-8047 (1993).
8. T. Suzuki *et al.*, MNU-induced mutant pools and high performance TILLING enable finding of any gene

mutation in rice. *Molecular Genetics and Genomics* **279**, 213-223 (2008).
9. B. Singer, J. T. Kusmierek, Chemical Mutagenesis. *Annual Review of Biochemistry* **51**, 655-691 (1982).
10. G. R. Hoffmann, Genetic effects of dimethyl sulfate, diethyl sulfate, and related compounds. *Mutation Research/Reviews in Genetic Toxicology* **75**, 63-129 (1980).
11. D. T. Beranek, Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutation Research* **231**, 11-30 (1990).
12. J. G. Jansen *et al.*, Molecular analysis of hprt gene mutations in skin fibroblasts of rats exposed in vivo to N-methyl-N-nitrosourea or N-ethyl-N-nitrosourea. *Cancer Res* **54**, 2478-2485 (1994).
13. D. R. Krieg, Ethyl Methanesulfonate-induced reversion of bacteriophage T4rII mutants. *Genetics* **48** (1963).
14. R. Saffhill, G. P. Margison, P. J. O'Connor, Mechanisms of carcinogenesis induced by alkylating agents. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* **823**, 111-145 (1985).
15. S. Minoia *et al.*, A new mutant genetic resource for tomato crop improvement by TILLING technology. *BMC Res Notes* **3**, 69-69 (2010).
16. M. Ensminger *et al.*, DNA breaks and chromosomal aberrations arise when replication meets base excision repair. *Journal of Cell Biology* **206**, 29-43 (2014).
17. D. M. Brown, M. J. E. Hewlins, P. Schell, The tautomeric state of N(4)-hydroxy- and of N(4)-amino-cytosine derivatives. *Journal of the Chemical Society C: Organic* 10.1039/J39680001925, 1925-1929 (1968).

Information Unfavorable to the Proposal
None