Haut Conseil des Biotechnologies

SCIENTIFIC COMMITTEE

SCIENTIFIC OPINION
ON
NEW PLANT BREEDING TECHNIQUES

Paris, 2 November 2017

(adopted by the Scientific Committee on 26 April 2017)

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Summary

The abbreviation NPBT[1] refers to any of a heterogeneous set of techniques for plant breeding. A key issue currently being debated in the European Union is the regulatory framework for these techniques, which are the focus of considerable interest in the agricultural sector. This raises multiple questions about environmental and health assessment of plants obtained by NPBTs as well as how they are to be detected, traced and, where applicable, labelled.

The HCB Scientific Committee has drafted this opinion on the basis of a report by a working group[2] and the discussions at the Scientific Committee's four plenary sessions.[3] Its remit[4] was to provide answers to the questions in a referral from the Minister of the Environment and the Minister of Agriculture relating to:

- Assessment and **traceability** methods for plants and products obtained by NPBTs;
- Implications for supply chain coexistence (in connection with the above point);
- **Direct** risks to health and the environment associated with **novel characteristics** of the plants and products obtained;[5]
- **Management measures** required to prevent or limit risks to health and the environment associated with use of plants and products obtained by NPBTs if such risks are identified;
- Proposals for **intermediate options** between the provisions of the EU catalogue and those of Directive 2001/18/EC that would seem advisable for regulating use of NPBTs on EU territory.

Scope of the opinion

In its opinion the HCB Scientific Committee has taken account of all the NPBTs currently being discussed at EU level.[6] It has also addressed related techniques such as TALENs[7] and CRISPR/Cas9[8] and extended its discussion to all the uses of negative segregants as well as to RNA interference and the opportunity to modify several targets in one round allowed by some of these techniques. The opinion concerns use of NPBTs for plants of agricultural interest.

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[1] New plant breeding technique.

[2] See Appendix III.

[3] For convenience, the Scientific Committee has defined some of its terms in the glossary in Appendix VI.

[4] Following a decision by the HCB Board, see Appendix II.

[5] Thus the referral confined itself to asking HCB about *direct* and *specific* risks associated with NPBTs and the traits that they produce. Risks associated with characteristics shared with plants obtained by transgenesis, random mutagenesis or traditional breeding methods are described in Appendix V to this opinion.

[6] Oligonucleotide-directed mutagenesis (ODM), zinc finger nucleases (ZFNs), cisgenesis, intragenesis, grafting of GM and non-GM plants, agro-infiltration, epigenetic modifications using RNA-dependent DNA methylation (RdDM), and reverse breeding (list of techniques drawn up by the Netherlands in 2006: see fact sheets).

[7] Transcription activator-like effector nucleases.

[8] Clustered regularly interspaced palindromic repeats/CRISPR-associated protein 9.

Introduction to the opinion

In its introduction to the opinion the Scientific Committee addresses the question of natural genetic variation in crop plant species. The Scientific Committee also describes the type of mutations that might be obtained, in particular using site-directed nuclease (SDN) techniques. This point is essential in order to characterise the mutations that can be obtained by some NPBTs, particularly SDNs, for the purpose of comparing them with natural mutations and considering questions of detection, traceability, assessment and risk management for the varieties thus produced.

The Scientific Committee notes that mutations obtained by SDN techniques are defined by their genome targeting. It observes in its opinion that some targeted mutagenesis techniques may be associated with unwanted (off-target) mutations just like those associated with conventional mutagenesis techniques or occurring naturally – mutations that could modify genome sequences that are still poorly characterised with regard to function.

The opinion then considers the questions contained in the referral.

Assessment and traceability methods for plant and products obtained by NPBTs

The first question seeks to determine whether products obtained by NPBTs can be distinguished from products obtained using conventional crop breeding approaches.

If public authorities consider establishing coexistence rules and molecular traceability for products obtained by NPBTs,[9] this would very much depend on the ability to distinguish those obtained by NPBTs. However, for some of these techniques, the modifications obtained are technically very difficult to identify.

The Scientific Committee has determined three points for consideration concerning traceability of plants obtained from various NPBTs:

- Detectability of a molecular characteristic in DNA (mutations: insertion, deletion, substitution, etc.),
- Identification of the technique underlying the molecular characteristic: if a molecular modification of DNA is found, can it be traced back to the technique used?
- Routine practical screening of food processing and production: given current screening tools, what would be the limitations in terms of detection and identification?

Moreover, the existence of natural variation and random mutations occurring naturally (see "Introduction to the opinion" above) may complicate the question of whether an NPBT has actually been used to breed the crop plant.

The following ideas informed the discussion in the Scientific Committee:

- Without information about a modification, the Scientific Committee notes that, unlike the case of transgenic plants, for which molecular tools are used to detect certain transgenes,

[9] Along the same lines as those for genetically modified plants (GMPs).

it will be impossible to detect modifications in certain products obtained by NPBTs, such as those obtained by SDN-1, SDN-2, ODM, a few SDN-3 techniques,[10] RdDM, grafting a scion on GM plants, and negative segregants.

- Moreover, as in the case of transgenic plants, when detection of plants obtained by NPBTs is possible, whether in end products or at the various stages of production, the Scientific Committee notes that, depending on the complexity of mixtures, sampling bias combined with the technical limits of detection will affect the ability to detect some modifications.
- If molecular modifications are detectable and possibly quantifiable if the modified gene or sequence is known, the original technique used to obtain the modification will nevertheless be very hard to identify solely by studying the product's DNA. Breeder notification of the technique, together with availability of methods to identify the modification, as is the rule for transgenic plants, would help to rectify this situation.
- However, even assuming this information to be available, detection could turn out to be impossible in the following cases: some types of grafting, negative segregants[11], and processing chains in which mixtures of products are used.
- Lastly, effectors[12] are used for some NPBTs in the plant generation phases in a contained environment. Persistence of effector DNA fragments may generate plants that would then be transgenic. The Scientific Committee notes that these transgenic plants, which are easily identifiable and traceable owing to their transgenes, will be subject to GMO requirements for contained environments and will subsequently come under Directive 2001/18/EC if these effectors remain in the plants or products of these plants placed on the market. The Scientific Committee has therefore chosen to focus on effector-free plants that will be placed on the market.

Thus:

- For techniques generating a detectable genetic modification but one that cannot be clearly attributed to an NPBT, the Scientific Committee draws a distinction between a case in which precise information about the modification is available and a case in which there is no information about the modification:
    - If precise information about the modification is supplied by the breeder: in this case molecular (DNA) traceability is theoretically possible and the product, accompanied by information on the modification, can be identified molecularly[13] throughout the supply chain. However, for negative segregants and some cases of grafting it will still be technically difficult to identify modifications. Similarly, for modifications that can also be obtained by non-GM techniques, molecular assays alone cannot distinguish between the possible methods of obtaining the products. Only documentary traceability would allow this.

[10] Mainly plants obtained by SDN-3 through cisgenesis.

[11] Non-transgenic offspring of a transgenic plant (see fact sheets).

[12] Effectors: Molecules (proteins or nucleic acids (RNA or DNA)) used to obtain the desired modification in the plant (see glossary, Appendix VI).

[13] Within the limits of measurement and depending on the impact of industrial processing undergone by the plant.

- If there is no information about the modification: in this case, for SDN-1 and SDN-2, ODM and RdDM,[14] the HCB Scientific Committee has concluded that, without records, traceability will often be very complex or even sometimes impossible, since, in the absence of precise traceability data, molecular methods of characterising the end product cannot detect whether a product is obtained from a plant bred conventionally or by induced mutagenesis.
- For DNA insertion (or substitution) techniques (SDN-3, cis-/intragenesis), detection methods similar to those used for GMOs could be introduced but might sometimes be complicated to interpret in the event of combinations such as SDN-3 and cisgenesis, for example.

Implications for supply chain coexistence (from farm to fork)

In the event of a coexistence policy being introduced for one or more NPBT supply chains and other supply chains (conventional, "GMO-free", organic, etc.), the Scientific Committee asked whether it would be feasible to implement it. The scenarios set out by the Committee reflect the options available. The choice of one or more of these options is a matter for the supply chains and the public authorities.

The Scientific Committee has identified a number of scenarios, focusing on detection capabilities in commercial supply chains and their ability to coexist:

- Firstly, **for supply chains interested only in the end product**, regardless of breeding technique,[15] whether the supply chain was defined in terms of inclusion or non-inclusion of products obtained by NPBTs would not necessarily be an issue.
- **For supply chains interested in the plant breeding method**, although molecular detection of plants modified by some NPBTs is not always an option, documentary traceability could be introduced to help regulate crop coexistence. Specifications could be proposed for these supply chains.
- Secondly, the Scientific Committee has come to the conclusion that if Directive 2001/18/EC were to be interpreted as **excluding some NPBTs**, new supply chains could be established in which the products' molecular characteristics would be distinguished by using documentary traceability.
- If Directive 2001/18/EC were to be interpreted as **including some NPBTs**, the Scientific Committee believes that the question of detection would then become critical and would depend in large measure on technical constraints, especially for products imported from countries using NPBTs or for detection of adventitious presence of products obtained by NPBTs.[16]

[14] Since some forms of RdDM do not require expression of a transgene.

[15] Although some traits such as herbicide tolerance may necessitate specific management measures.

[16] For example, regulatory thresholds for presence of products from plants obtained by NPBTs will affect detection capability, and, as explained previously, in some cases it would not be possible to determine which methods had been used.

- For cases in which a coexistence policy is to be established, **and when genetic modifications can be detected**, the Scientific Committee notes that coexistence measures could be based on the analysis provided in its opinion on coexistence. Such measures would apply particularly in the field.

Direct risks to health and the environment associated with novel characteristics of products obtained

In response to the referral's question about risks, the Scientific Committee has focused on **direct risks associated with novel characteristics** of products obtained by NPBTs, whilst listing elsewhere (Appendix V of the opinion) indirect risks and risks shared with other breeding methods (GM and conventional).

The Scientific Committee has identified three categories of risk:

- Risks associated to technique's **unintended effects** on the end product (e.g. effector persistence and off-target modifications);
- Risks associated with **ease of use** of NPBTs, which could lead to a faster pace of production and faster cultivation of varieties obtained using these techniques;
- Risks associated with **desired traits** (novel traits, or new modifications of crop plants).

Among new direct risks, the Science Committee has come to the conclusion that the main risk is associated with the **technically avoidable** presence of effectors.[17] The Scientific Committee recommends verifying that effectors are absent, which is technically possible. The Scientific Committee notes that effector persistence in a plant's genome would make the plant transgenic.

Other risks are associated with the techniques' **efficiency, speed, and the option of making several genetic modifications at once** (multiplex genome editing) as well as the possibility of obtaining **novel traits**[18] :

- Acceleration of the breeding process for new varieties may be a factor in agronomic improvement but can also hold risks. It will have an effect on farming production and food-processing systems, whether economically, sociologically or ecologically. It is therefore possible that it will positively or negatively affect ecosystem functioning and dynamics, including ecosystem services that regulate the environment, since new balances may come into play.
- Moreover, while NPBTs would accelerate adoption of the new varieties obtained by these techniques, having an impact on agricultural ecosystems, this could lead to additional adjustment problems for biodiversity and associated ecosystem services. These problems

[17] Effectors are the molecules (proteins or nucleic acids (RNA or DNA)) used to obtain the desired modification in the plant.

[18] A definition of novel traits can be found in the glossary in Appendix VI.

would be compounded if these genetic modifications spread into wild species sexually compatible with the crop varieties.

- As for risks associated with desired **novel traits**, the Scientific Committee defines a *Novel Trait* as the introduction into a variety of an entirely new trait not already present in the species itself and/or a related species.[19] In this case, the Scientific Committee **cannot identify any specific risks, since, by definition, these traits have not yet been described**. The Scientific Committee therefore recommends **case-by-case assessment** taking account of the trait itself and the species into which it has been introduced (see below). In this connection, the Scientific Committee has discussed the likelihood of unanticipated ecological effects, which might be greater in the case of *Novel Traits* drastically modifying a plant's metabolism.
- Lastly, the Scientific Committee has addressed the issue of off-target genome modifications using directed approaches (SDNs and ODM). A mutation outside the target region could in some cases have an unintended effect on the plant's phenotype. The Scientific Committee notes that technical developments are leading to a substantial reduction in these off-target modifications. It further notes that off-target modifications are also found with other widely used unregulated techniques. The latter also induce mutations outside the sites selected. The Scientific Committee adds that off-target mutations[20] with unwanted phenotypic effects could be removed by backcrossing in the case of annuals. Nevertheless, the Scientific Committee notes that this might be difficult or even impossible to do for some perennials or plants that reproduce mainly through vegetative propagation. In this case, additional molecular data might be requested on a case-by-case basis.

Management measures required to prevent or limit risks to health and the environment associated with use of products obtained by NPBTs if such risks are identified

If risk management measures were to be required, the Scientific Committee suggests that they be introduced in the light of the results of an assessment conducted on the basis of the proposals set out below.[21]

For risks associated with presence of effectors

Plants in which effectors are still present are transgenic plants. Directive 2001/18/EC lays down that such plants shall be subject to assessment and appropriate management measures (including surveillance and post-market monitoring).

For risks associated with off-target modifications

[19] By way of example, a herbicide tolerance trait already obtained in the same species by other methods is not a novel trait. In this particular case there is room for cross-cutting discussion of the ways in which herbicides are used, regardless of the technique employed.

[20] This applies to all unwanted mutations appearing during breeding of conventional varieties or in plants obtained by NPBTs, including when the technique is used on a plant that has already been modified by an NPBT.

[21] See Section 7 of the opinion.

More generally, the Scientific Committee notes that breeders carry out a number of backcrosses when introducing a trait into a variety, and this should enable the risks associated with off-target modifications to be reduced significantly. If an unwanted effect associated with an off-target mutation is found, the Scientific Committee recommends backcrossing to remove it from the plant to be placed on the market.

For novel traits as defined in the opinion

An assessment should be carried out in the case of plants having a genuinely novel character. This assessment would depend on the trait introduced. Once the dossier had been considered, the assessment could be conducted, depending on the traits and the species, in a contained environment *(in vitro* or in a mesocosm to study the effects on ecological interactions and biodiversity[22] in particular) and/or in field trials.

For risks associated with faster breeding

Local management with, if necessary, gradual roll-out over time and space of plants with a *Novel Trait* should be suggested to control the pace of agro-ecosystem change that might result from use of these plants, especially with regard to dispersal in sexually compatible, related wild species.

For biomonitoring the Scientific Committee proposes the following management measures:

To limit any other risks not immediately identifiable, the Scientific Committee suggests introducing biomonitoring for *Novel Traits* (including those obtained by multiplex genome editing). This monitoring will make it possible to gain knowledge about the behaviour of these varieties when they are widely placed on the market at multi-year intervals and particularly about effects on biodiversity. The current biomonitoring system and networks for monitoring farming practice should be adapted to these requirements. After a period to be defined, and depending on the observations made, an assessment should be made as to whether the biomonitoring should be continued.

Lastly, the Scientific Committee stresses the need for long-term conservation and management of gene pools for each species that have not been modified by NPBTs.[23]

Proposals for intermediate options between the provisions of the EU catalogue and those of Directive 2001/18/EC

After outlining existing arrangements (Directive 2001/18/EC, requiring risk assessment for GMPs, and registration in the official catalogue, requiring assessment of varieties' value for cultivation, use and the environment), the Scientific Committee has considered possible intermediate options.

Subject to approval by public policymakers, the Scientific Committee suggests that breeders should provide the competent authorities with a dossier containing molecular and phenotypic data on each of their products.[24] Depending on the modification introduced and taking into consideration its history

[22] See Appendix VI.

[23] Biological resources centres will have to take account of development of varieties obtained by NPBTs.

[24] See molecular data required in Scientific Committee opinion.

of use,[25] the categorisation procedure might entail assessment for GMOs not exempt from assessment,[26] a new form of intermediate assessment, or exemption from specific assessment (as in the case of exempted GMOs and conventional breeding).[27] These options would make it possible to adapt the information requested and the assessments made to the technique used, the trait conferred on the plant, etc. Bodies such as the CTPS,[28] ANSES,[29] the InVS[30] and HCB could participate at national level in this categorisation procedure (which should be consistent with decisions taken at EU level).

Regardless of the current stage of development of NPBTs, the Scientific Committee believes that it is not just the techniques in themselves that should determine the assessment system, unless they result in GMOs within the meaning of Directive 2001/18/EC. For techniques producing plants and products that cannot be distinguished from others obtained by different methods, some of which may be unregulated, the Scientific Committee recommends assessment of these plants and products with reference to their traits, and particularly the novelty of these traits.

[25] If the phenotype exists and is used in agriculture.

[26] As provided for in Directive 2001/18/EC.

[27] The Scientific Committee notes, however, that assessment by the Technical Committee for Plant Breeding (CTPS) followed by registration in the French Official Catalogue are a prerequisite for placing the variety on the market.

[28] Technical Committee for Plant Breeding.

[29] French Agency for Food, Environmental and Occupational Health and Safety.

[30] French Health Monitoring Institute (now part of Public Health France).

1. Introduction

1.1. Background

On 22 February 2016 the High Council for Biotechnology was asked for an opinion by Ségolène Royal, Minister of the Environment, Energy and Marine Affairs, and Stéphane le Foll, Minister of Agriculture, the Food Processing Industry and Forestry, on the issue of new plant breeding techniques (NPBTs) (Appendix I). The HCB Board accordingly drafted a letter with guidelines for its two committees (Appendix II). An ad hoc working group (WG), whose composition was approved by the Board on proposals from the HCB President, the Scientific Committee and the Secretariat, was set up (WG composition in Appendix III). After meeting four times, the working group delivered a report intended to provide background for Scientific Committee discussions. The report was considered at the Scientific Committee meeting of 13 July 2016 and formed the basis of discussion at the Scientific Committee meetings on 13 July, 21 September, 27 October and 23 November 2016, which resulted in the drafting of this scientific opinion by the Scientific Committee.

Before we turn to the work process and the substance of the issues raised, the following points should be noted.

The HCB Scientific Committee's role is to clarify the scientific aspects of the issue referred for an opinion, together with regulatory and legislative options that are a matter of policy choice.

The Committee's scientific opinion is combined with the recommendation from the Economic, Ethical and Social Committee (EESC) to constitute the HCB opinion.

When reading the assessment below it must be borne in mind that the HCB Scientific Committee has worked on a referral concerned almost exclusively with identification of the risks associated with use of these new plant breeding techniques (NPBTs). For the purposes of an informed decision about cultivation of plants obtained by these techniques, this evaluation has also had to take into consideration scientific and agronomic advances throughout the value chain and the benefits that might emerge from the production of plants obtained by NPBTs.

It is important to note that this scientific opinion applies to cultivated varieties of plants of agricultural interest. **Consequently, NPBTs are here discussed with regard to breeding of cultivated varieties rather than modification of wild species**. This opinion concerns use of these techniques to breed plants of agricultural interest that will be grown beyond research laboratories. The HCB Scientific Committee thus notes that this opinion relates to plants that could be grown in the open field.[31] Intermediate plants not intended for registration in the French Official Catalogue of Plant Varieties and grown in the laboratory for research purposes or in containment greenhouses are not covered by this opinion. Modification of plants in the laboratory is subject to specific monitoring and reporting under the rules governing research and development work.

[31] For laboratory cultivation, French regulations (Decree No. 2011-1177 of 23 September 2011 on contained use of genetically modified organisms) require reporting of manipulations and appropriate containment of modified organisms.

The Scientific Committee notes that all agronomic practices, particularly those altering crop area or entailing use of natural or synthetic molecules, influence development of the biotopes populated by wild species.

1.2. Work process

This scientific opinion has been drafted to reflect discussion in the Scientific Committee's meetings.[32] The initial draft opinion was presented by the Secretariat on the basis of the working group's report. The discussion that took place in the meetings, including any points of contention, is reflected in the relevant sections. The Scientific Committee has also updated the fact sheets on all the techniques discussed that were appended to the Scientific Committee's interim report on NPBTs. This scientific opinion is structured according to the order of the questions sent to the Scientific Committee in the referral.

1.3. State of the art and questions concerning mutations and natural variation[33]

The rapid expansion in new plant technology, with design and use of new plant breeding techniques (NPBTs), raises a number of questions. The current NPBT debate in Europe hinges on the regulatory framework for products obtained by use of "new" genetic breeding methods, since there is doubt as to whether they come under the GMO directives.[34] It is important, even critical, to settle this question if these techniques are going to be adopted. It is also important for terms and descriptions to be clarified for all concerned, including supply chains and consumers.

It should be noted that, because of the subject's history,[35] the list of techniques discussed is heterogeneous[36] and the generic term "new plant breeding techniques (NPBTs)" may give rise to confusion. Thus:

[32] On 13 July, 21 September, 27 October and 23 November 2016.

[33] This section provides some basic information on molecular genetics. It should be borne in mind that, as in the rest of the opinion, the proposals it contains are restricted to crop plants.

[34] The EU directives on GMO use are (1) Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, and (2) Directive 2009/41/EC on the contained use of genetically modified micro-organisms.

[35] Initial report by COGEM in 2006, followed by establishment of a European Commission working group and publication in a journal with a high impact factor (*Nature Biotechnology*) of the findings of a parallel investigation by the Commission's Joint Research Centre (Lusser *et al*., 2012).

[36] List of NPBTs discussed by the European Commission working group: oligonucleotide-directed mutagenesis (ODM), zinc finger nuclease (ZFN) technology, cisgenesis (including intragenesis), grafting, agro-infiltration, RNA-dependent DNA methylation (RdDM), reverse breeding, and synthetic genomics. The reader is referred to the fact sheets describing these techniques.

While they all apply to plant breeding, the techniques considered are not necessarily specific to the plant kingdom: site-directed nucleases (SDNs) and genome editing, for example, are also frequently used for animals and in medicine;

These techniques are not necessarily new: grafting is an old technique, but the question arises of whether products obtained by grafting a non-genetically modified (non-GM) scion onto a genetically modified (GM) rootstock should be regarded as genetically modified;

- Some items on the list are not techniques as such but entail use of genetic engineering: innovative plant-breeding strategies (such as reverse breeding), for example. This then raises the question of their status under the regulations (the case of negative segregants);
- Further complexity comes from the fact that it is possible to have combinations of NPBTs (targeted cisgenesis with SDN-3 technology, for example).

Taking the NPBTs under discussion in the European Commission as its basis, this opinion concerns the following:[37]

i) Genome-targeting NPBTs

   (a) Site-directed nucleases (**SDNs**:[38] **ZFN**,[39] MN,[40] **TALEN**,[41] **CRISPR**[42]**/Cas9**)

   (b) **Oligonucleotide-directed mutagenesis** (ODM,[43] RTDS,[44] etc.)

ii) Epigenetic techniques

   (a) Gene expression control by RdDM[45]

iii) Factors related to use of genetic engineering techniques

   (a) Specific contexts in which genetic engineering techniques are used

      1. **Agro-infiltration**
      2. **Grafting** of a non-GM scion onto a GM rootstock or a GM scion onto a non-GM rootstock

   (b) New concepts associated with the nature of the modified sequence

[37] Techniques covered by individual fact sheets are shown in bold type. An additional fact sheet is devoted to 'conventional' transgenesis for comparison purposes.

[38] SDNs: Site-directed nucleases.

[39] ZFN: Zinc finger nuclease.

[40] Meganucleases (MNs) are not covered by a fact sheet in the appendix because the working group considered the technology to have been overtaken already by other targeting tools.

[41] TALEN: Transcription activator-like effector nuclease.

[42] CRISPR: Clustered regularly interspaced short palindromic repeats.

[43] ODM: Oligonucleotide-directed mutagenesis.

[44] RTDS: Rapid Trait Development System.

[45] RdDM: RNA-dependent DNA methylation.

Cisgenesis / Intragenesis

iv) Offspring of modified individuals in which genetic modification has been removed by segregation

(a) **Negative segregants**, produced through innovative breeding strategies (e.g. reverse breeding, various accelerated breeding methods, Seed Production Technology, etc.)

The question may arise as to the novelty of the traits conferred by these techniques. The Scientific Committee draws a distinction between two types of novelty:

- Introduction into a variety of a trait identified in another variety or another related or sexually compatible species: the idea is to enhance existing genetic diversity by introducing allelic states of interest. Consequently there is no addition[46] of genetic sequences or any modification of the function of the genes already present in the plant. **This will be called a "novel trait in the variety or species"**.
- Introduction of an entirely new trait into a variety and/or related species:[47] the novel trait stems from the fact that the gene has not been identified in the species in question or the modification of a gene already present introduces a new metabolic pathway or new function into the species. **This will be called a "*Novel Trait*" in this opinion.**

1.3.1. General characterisation of NPBTs

- Genome-targeting NPBTs

Molecular targeting of the genomic site for genetic modification is a key feature of a number of these new techniques (SDNs: ZFN, MN, TALEN and CRISPR/Cas9 (see the Scientific Committee interim report and the fact sheets on the HCB website).

Site-directed nucleases can be used to target selected DNA sequences for three purposes:

(1) Random mutation (insertion or deletion) of a single base pair or a small number of nucleotides (one to several dozen) **although targeted** at a specific site on the genome, **known as SDN-1**;

(2) **Allele conversion**, modifying part or all of a gene sequence, **known as SDN-2**;

(3) Targeted integration of a DNA sequence, **known as SDN-3**.

The diagram below shows how some of these techniques fit in with the existing landscape (Figure 1):

[46] It is important to clarify the concept of addition for cisgenesis and intragenesis. Genetic material may be introduced, but the genes inserted already exist in the species in a different allelic state or are present in certain varieties of the same species.

[47] This distinction is highlighted in Canada's GMO rules, which provide for assessment only in the case of varieties having a new trait not previously present in the variety or related species (http://www.hc-sc.gc.ca/fn-an/gmf-agm/index-eng.php).

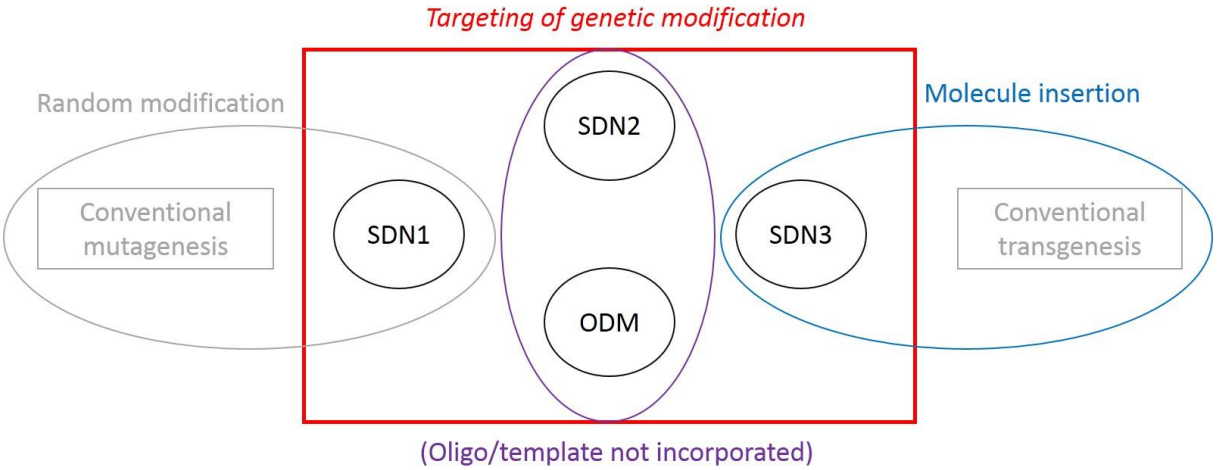

Figure 1. NPBTs: SDN-1 differs from conventional mutagenesis in that it targets a specific site on the genome, usually, but not automatically, leading to loss of function in the gene targeted (gene knockout). Nucleases are introduced into the cell to target a mutation site, but the nature of the mutation is not predefined. With SDN-2, a DNA template is introduced into the cell together with the site-directed nucleases, enabling the nature of the modification to be defined. The template itself is not incorporated into the genome. The same purpose can be achieved using oligonucleotide-directed mutagenesis (ODM, RTDS). SDN-3 allows targeted integration of a sequence. It is this targeting of the transgene insertion site that distinguishes the latter technique from conventional transgenesis.

- Epigenetic techniques

RNA-dependent DNA methylation (RdDM) uses epigenetic changes[48] to control (increase or reduce) expression of a given plant gene without altering its nucleotide sequence. This can cause variations in metabolic activity, for example. It is also possible to control gene expression in an organism interacting with the plant, enabling pathogens (for example) to be targeted. The Scientific Committee notes that new approaches are possible and that these epigenetic modifications can be achieved through expression or transient/stable transfer of a transgene or specific proteins (e.g. through agro-infiltration, CRISPR with a fusion protein having methyltransferase activity (Cas9-MT), or modification induced by a transient viral infection (VIGS)). With transient transfer,[49] the epigenetic alteration of gene expression control can be inherited over several generations.

- Other techniques

The other techniques are in fact methods: "**non-propagative**" agro-infiltration, grafting of genetically engineered plants, cisgenesis/intragenesis, and, for innovative selection strategies, removal of genetic material by segregation through conventional crossing (negative segregants), the status of which has to be clarified.

[48] Epigenetics describes the molecular mechanisms controlling expression of a genetically encoded trait. This opinion discusses modifications obtained by DNA methylation. Such DNA modifications are reversible, and, although they can be inherited between generations, whether they are retained will depend on the environment. Other modifications of DNA-associated proteins are possible.

[49] Longer-lasting expression is possible using transgenesis or SDN-3 targeted insertion for introduction into the genome. The non-permanent nature of the modification is again apparent if the transgene is removed by segregation.

1.3.2. Questions raised by NPBTs

In the following section, some experts wanted to contextualise the concepts of mutation and variation. One expert wanted the type of mutations observed to be addressed. Thus the discussion bore on whether or not mutations not found in nature could be obtained by using NPBTs. The question of the emergence and retention of these mutations was raised and gave rise to the paragraphs below (section 1.4).

With regard to the current EU regulatory framework on GMOs, questions about the status of the above NPBTs chiefly concern their similarity to techniques that come under the existing rules (mainly Directive 2001/18/EC). The European Commission is expected to rule on whether or not these techniques are considered to generate GMOs as defined by Directive 2001/18/EC and whether they should be regulated as such or be exempt from assessment.

At present a product has GMO status when obtained by techniques involving **insertion of new molecules of recombinant DNA.** The regulations cover **induced mutation**. Products of mutagenesis are exempt from assessment owing to a history of safe use.

The **targeting** capability of some NPBTS with regard to genome modifications could be put forward as an argument for easing the regulations concerning assessment requirements.

Multiplex genome editing capability, i.e. carrying out several genome modifications in a single stage principally in order to obtain a *Novel Trait* must also be taken into account.

The question of a modification's **transmission** and **heritability** should also be considered:

(1) Transient presence (of short duration, from a few hours to a few days, in a cell and not inherited) as opposed to heritability of the genetic modification. This also covers modification of somatic cells as opposed to germ cells (modification of somatic cells can also be inherited by vegetative propagation);

(2) Transient presence as opposed to heritability of the epigenetic consequences of a genetic modification (epigenetic changes can be induced without stable insertion of genetic material in the genome; with stable insertion, however, they can be inherited after elimination of the inducing gene by segregation);

(3) Transient presence as opposed to heritability of the recombinant DNA needed to obtain the mutation.

Another set of questions concerns **detection** of products obtained by a particular technique. If molecular detection technology is unable to tell the difference between the techniques used to obtain products, non-DNA **traceability** methods (technical or administrative traceability) could provide some information.

There is also the question of **management methods** for potential risks. Where they exist, risks specific to NPBTs will have to be identified and appropriate and proportionate management methods suggested.

As noted above, EU legislation is being debated against a background of continuously evolving genetic engineering techniques. The question of the need for assessment arises, particularly if one organism cannot be distinguished from another obtained by practices not subject to assessment.

1.4. Considerations regarding natural genetic variation in a plant species

For products obtained by NPBTs there is the question of differences between natural variability and variability obtained by these techniques. It is imperative to define the concept of variability in order to understand how to assess the risks and benefits associated with both conventional and new techniques.

Variability is relevant to addressing the issues of assessment:

- In terms of risk, are molecular changes induced by NPBTs qualitatively and/or quantitatively different from those resulting from natural variation or conventional plant breeding techniques?
- In terms of identification/detection, how can events associated with use of NPBTs be distinguished from natural variation or conventional breeding?
- Both these questions also arise for presence of additional (off-target) mutations.

1.4.1. Origin of genetic variations

In biology, genetic transmission of traits between generations is based on transmission of the genome (nuclear and cytoplasmic). The genome's primary structure, i.e. the sequence of nucleotides constituting the DNA molecule, is the carrier. Epigenetic changes, which modify the chemical structure of the nucleotides (or chromatin) but not their sequence, influence genome expression.

Genomes are susceptible to modification, ranging from change in a base pair up to major modifications such as deletion, duplication, insertion of large DNA fragments or chromosomal rearrangements, which affect the genome's primary structure. These modifications can result from DNA replication/repair errors, action of transposable elements, or chromosomal recombination events related to sexual reproduction.

This phenomenon of natural modification of the genome is universal and to be found in all living organisms (plants, animals and microorganisms) at a rate that depends on the organism (e.g. one mutation for approximately 100 million base pairs per generation in Thale cress, *Arabidopsis thaliana* (Lynch, 2010)). Consequently, for a plant with a small genome such as *Arabidopsis thaliana*, the genome of each new seed contains one mutation on average compared with the genome of the seed that generated it (Ossowski et al., 2010). Applying linear extrapolation to a genome 120 times larger, such as that of wheat, the figure would be roughly 120 mutations per seed. In a hectare of wheat containing around one million plants, study of the genomes of all the seeds harvested would therefore show at least 120 million mutations compared with the genomes of the seeds sown originally. The upshot of this theoretical calculation is that, statistically speaking, in all the genomes of the grain population harvested in a hectare of wheat, every gene would contain one mutation in its DNA sequence by comparison with the grain sown.

In other words, by growing a hectare of wheat, a farmer could, statistically speaking, obtain one mutation in each wheat gene (in reality the distribution of these mutations is uneven (see Section 1.4.3)).

A large proportion of these changes cannot be linked to or correlated with phenotypic variations with a measurable impact on the organism or the ecology of the population to which the organism belongs. At present most of these mutations are thus described as “silent”, either because they actually are, or because our current methods of observation do not show their effects.

For wheat, and all crop varieties in general, if the harvest is consumed in its entirety these mutations cannot be expressed in offspring. If part of the harvest is resown or used for breeding purposes, some of the mutations will therefore be present in the progeny. Mutations affecting the phenotype (“non-silent” mutations), if not lethal, will usually not persist in the progeny unless they are selected and offer a trait sought by the breeder. “Silent” mutations (i.e. mutations not affecting the phenotype) will evolve in the same way as mutations in non-crop plants, which are described in the following section.

This argument concerning occurrence of natural mutations in wheat applies to all living organisms. The DNA base sequence is susceptible to mutation, and the gene pool of every species[50] contains a large number of variations, some of which are silent whilst others are not. These mutations are either present in the gametes and therefore transmitted to offspring or else present in the genome of somatic cells and thus not transmitted.

Similarly, work on epigenetic modifications (DNA methylation, modification of associated histones) has shown that transmissible epigenetic changes can exist that are subject to variation within populations.

1.4.2. Fate of genetic variations: natural evolution (no human intervention)

The only potential limit to the number of genetic variations is the rate of natural mutation. But in reality, in a given environment, variation in natural populations is the result of a balance between (1) processes that tend to increase variation (mutation and, similarly, migration between different populations), and (2) processes that tend to reduce variation: genetic drift (accumulation of variations being limited by finite population size) and natural selection.

The silent mutations appearing in non-coding, non-regulatory regions, and those appearing in coding regions (which do not induce amino acid substitutions and therefore changes in the protein sequence encoded by these genes), are considered to be “neutral”: they do not alter the phenotype and are not affected by natural selection. According to the neutral theory (Kimura, 1984), genetic diversity, defined as the proportion of variable sites between two randomly selected sequences in a population, is explained by mutation rate and population size. It is generally between 0.01% and 5% (Gouesnard et al., 2005).

1.4.3. Characteristics of variation within genomes

Coding sequences and regulatory non-coding sequences have a rate of change that is on average slower than that of non-coding sequences (since negative selection predominates), and their

[50] Two individual human beings are set apart from each other by some three million genetic variations, and up to 1500 somatic mutations per cell are found in a single individual (*Science*, 25 September 2015, Vol. 349, Issue 6255).

arrangement in the genome is stable within a species. Conversely, non-coding sequences, which do not control gene expression and are in the majority (up to 98% of the genome in maize, for example), change at rates that are much higher on average, and they often contain transposable elements.

As for coding sequences, the degree of variation changes from one gene to the next and is influenced by gene function and natural selection. The latter depends on variation in the overall environment and/or the gene environment in the genome. Recombination constantly produces new gene combinations and therefore new phenotypic variations (most traits are multigenic: their value is determined by combination of a number of genes).

1.4.4. Intra- and interspecies variation

Genetic variation among individuals and populations of the same species is usually less than genetic differences between species. Nevertheless, species are not independent, or even necessarily separate entities. Closely related species (having diverged recently on the evolutionary time scale) share a proportion of the variations inherited from their ancestral species. Moreover, hybridisation between different plant species is possible, with new variations thus being exchanged between species. However, this is still uncommon between crops and wild species (Andersson and de Vicente, 2010; Dempewolf et al., 2012).[51]

1.4.5. Human selection and crop evolution

Genetic variation through continually occurring natural change in the genome is the basis for evolution of organisms. It is also on this genetic variation within and between species that plant breeders rely to improve their plants.

Finding the best combination of traits for a particular species and given environment has been the main concern of plant and animal domestication and breeding for over 10,000 years. The huge number of combinations producing genetic variation through natural mutagenesis offers many breeding options, but this is also a problem, since it may be necessary to eliminate variations that counteract the effects of selection. Advances in plant genomics (Kole et al., 2015) might perhaps, in years to come, constitute an alternative, a complement to NPBTs or a factor in combination.

One member of the Scientific Committee wished to add that the distribution of targeted mutations differs from that of natural mutations on at least three counts.

- *Location on the genome,*
- *Type of mutation depending on SDN technique (proportion of insertions, deletions and substitutions, and their outcomes in terms of silent or non-silent mutations);*
- *Their adaptive value for cultivation escapes, or related species, sexually compatible with the cultivated variety obtained by an NPBT.*

[51] Although it is less unusual between wild plants (Whitney et al., 2010)

The distribution of mutations obtained by conventional mutagenesis is itself different from that of targeted mutations and natural mutations.

1.4.6. Targeted mutations, off-target mutations and natural variation

The changes induced by SDN techniques are derived from a double-stranded break in DNA.

- In the case of SDN-1, the plants selected show a targeted mutation, mainly in the form of micro-deletions or micro-insertions, and these changes are usually associated with gene silencing. Natural variants of the silenced gene have greater molecular diversity. These mutations include not only micro-deletions and micro-insertions but also substitutions, translocations and other rearrangements.

- In the case of SDN-2 and ODM, the mutations introduced reproduce variations observed as part of genetic diversity, which have been selected to be associated with a phenotype of agricultural interest. In most cases, the same phenotype can be obtained from different mutations.

Thus, for a given gene, the mutations from SDN-1, SDN-2 or ODM may or may not be identical to those found in cultivated varieties.

A member of the Scientific Committee raised the issue of the characteristics of off-target mutations for SDNs. The issue related to the fact that they could differ from mutations found in the absence of manipulation and could affect regions of the genome with little natural variation, particularly uncharacterised regions. The Scientific Committee notes that, as explained elsewhere in the opinion, off-target mutations have the following characteristics:

- They are found in sequences similar to the target sequences (1 to 5 nucleotide differences depending on the technique). They are therefore computationally predictable in known genomes, or can be identified by sequencing modified cells or using dedicated techniques (involving some degree of complexity).
- The biochemistry of off-target mutations is the same as that of natural variations. Since SDNs produce double-stranded breaks in DNA, physiological repair systems are called into play.
    - The fact that there is little variation in some regions of the genome is accounted for by the functional importance of these regions (negative selection pressure). Effects due to off-target mutations in these regions would therefore in all likelihood be associated with a phenotype that the breeder could choose whether or not to keep.
    - Some types of mutation at given sites (for example, variations in the number of repetitions in repeated regions) are influenced by the mechanisms involved in producing these mutations but are not indicative of any particular risks. An SDN-induced break in these regions would therefore have the same consequences as a natural break followed by physiological repair.
- For SDN-2 and SDN-3 the consequence of an off-target mutation will very probably be the same as for SDN-1, since the probability for the template DNA to recombine in the area of the

DNA break is very small. This also applies to off-target mutations found in ODM, where sequence homology is necessary for oligonucleotides to work.

2. Definition of terms and techniques

To comprehend the issues discussed in this opinion, it is essential to understand the techniques. The Scientific Committee has therefore produced a set of fact sheets.[52]

These fact sheets have been updated with additional information from the working group and from the Scientific Committee's meetings. For each technique they give a cellular and molecular description, practical details, possible uses, pros and cons in relation to existing techniques, detection and traceability methods, developmental stages and additional background information. They can be found in Appendix VII of this document.

For reasons of consistency the HCB Scientific Committee has chosen to give certain terms that might be used in other contexts a specific meaning that is explained in the glossary in Appendix VI. To avoid any confusion, the Scientific Committee would like to clarify that this glossary applies only to this opinion.

2.1. Multiplex genome editing and simultaneous production of multiple site-specific modifications

SDN techniques can be used to make more than one modification to the genome at the same time.

At one locus

With SDN-2, use of a "repair template" containing a combination of several mutations/insertions/deletions at the same locus can produce a plant that it would be very difficult or even statistically impossible to obtain by random mutagenesis or selection of natural mutations. It should be noted that several mutations can occur naturally at a given locus. Multiplex genome editing also enables modifications to be traced.[53] Apart from this, such editing could have the benefit of modifying the function of a gene: this would then be a *Novel Trait*. Similarly, the SDN-3 technique allows insertion of more than one transgene at just one locus. All these transgenes will be transmitted to offspring as a single locus.

At multiple loci

SDN techniques can be applied simultaneously to multiple regions of the genome by introducing multiple nucleases and/or guide RNAs and multiple repair templates (Raitskin and Patron, 2016). It is thus possible to obtain controlled genetic modifications of multiple genes or sequences. For example,

[52] These fact sheets have been updated with additional information from the working group and from the Scientific Committee's meetings.

[53] Traceability through combination of a specific DNA sequence without changing the encoded protein sequence.

this would allow simultaneous silencing of a gene's different alleles in polyploid plants (such as hexaploid wheat) (Wang et al., 2014), targeting of gene families, and modification of genes involved in the same metabolic pathway.

It would also be possible in theory to obtain novel products[54] by modifying a series of genes or through mutagenesis of a gene to change its function.

It is important to note at the moment and in the near future that 1) the ability to deliver SDN effectors easily (whether for SDN-1, -2 or -3) is a significant limitation on multiplex genome editing capability, and 2) creation of new functions falls mainly within the sphere of research.

2.2. Boundaries between SDN-1, -2 and -3

Classification of the various genome modification strategies into SDN-1, SDN-2 or SDN-3 facilitates description of the characteristics associated with each technique. These techniques have been a subject of debate, with the Scientific Committee in agreement that:

- SDN-2 differs from SDN-1 through use of a template allowing modification, by recombination, of the gene targeted by the nuclease-induced break.
- SDN-2 differs from SDN-3 because in the case of SDN-2 the modified gene is present in the plant, remains in its location in the genome and keeps its copy number.
- The boundary between SDN-2 and SDN-3 is not necessarily clear. With SDN-2, the new DNA sequences obtained could lead, for example, to formation of new RNA (even short, such as small RNA) or new forms of RNA expression control, as could SDN-3. The level of similarity used in order to distinguish SDN-2 from SDN3 will have to be determined on case by case if this is required for regulatory purposes (see Section 7).

2.3. Considerations concerning delivery and effector insertion in the target cell

The question of vectors and effector insertion/persistence in the cell is fundamental to any discussion of risks and traceability for varieties obtained by NPBTs. Vector/effector persistence can affect risk (see Section 5.2.1) but can also, in some cases, be used to identify a product obtained by these techniques (see Section 3.3).

At the moment there are a number of delivery methods for introducing effectors.

[54] See paragraph on novelty in the introduction (Section 1.3).

2.3.1. Transformation using Agrobacterium tumefaciens *bacteria*

Agrobacterium tumefaciens bacteria cause crown gall disease in some plants. When these bacteria are used for plant transformation, the Ti-plasmid[55] T-DNA genes are replaced and the strain is unable to cause the disease (Simpson et al., 1986).

Agrobacterium carrying the construct to be transferred is placed in contact with the plant cells and transfers recombinant T-DNA into their nuclei. Various techniques (see Appendix VII) are used to select the transformed cells and then regenerate them into whole plants. For some species it is possible to inoculate the floral organs with *Agrobacterium*, allow the fruit to grow and then select the transformed seeds (obtaining up to several per cent of transformed seed out of all the seed produced (Bechtold et al., 1993)). For NPBTs, *Agrobacterium* is placed in contact with tissue that will not be used for reproduction but be ground to extract a metabolite of interest (vaccine, etc.).

2.3.2. Direct transformation

Direct transformation techniques cover all the methods employed to introduce macromolecules into plant cells by chemical or physical means without using *Agrobacterium*.

- Protoplast transformation

Plant cells have their pecto-cellulosic walls removed by enzyme digestion (thus becoming protoplasts), and DNA, RNA or proteins are introduced through the cell membrane using molecules (such as polyethylene glycol) or electric shocks that transiently weaken this membrane, making it permeable. In the case of CRISPR/Cas9, the Cas9 protein and guide RNA can be introduced directly (Woo et al., 2015), and therefore no DNA is inserted.

- Biolistics

Microscopic gold or tungsten particles coated with DNA or RNA are shot into plant cells *in vitro* (bombardment). The molecules on these particles are projected into the cell nucleus, upon which they act. Trials are under way with bombardment using pre-assembled nucleoprotein complexes.

It is possible, after selection, to regenerate whole plants from the transformed cells. If a flower bud is bombarded rather than individual cells, it is possible to grow the plant until seed is produced and then to select the transformed seed.

- Whiskers

Needle-like metal fibres (whiskers) coated with DNA are mixed with plant cells at high speed, impairing the cell membrane and allowing the DNA to penetrate the cytoplasm and then the nucleus. This technique has been used for maize (Kaeppler et al., 1990; Petolino and Arnold, 2009), rice (Terakawa et al., 2005), and some forage plants and turf plants such as bent (Asano et al., 1991), but it is not widespread.

[55] The endogenous bacterial sequence containing the genes responsible for crown gall disease.

2.4. Methods for selecting modified cells and plants and deleting marker transgenes

Genetic engineering methods are often inefficient, and most of the cells obtained are not modified. It is therefore necessary to use **methods for selecting modified cells**. This can be done either by using a chemical selection agent (to eliminate untransformed cells) or by molecular screening of cells, tissue or plants (with PCR, for example). Selectable marker genes may be genes conferring resistance to a selective agent (herbicide, antibiotic), metabolic markers (use of mannose as a sugar, for example: PMI[56]) or colour markers (GUS[57] or GFP[58]) (Breyer et al., 2014).

To avoid the selectable marker remaining in the end product, it can subsequently be removed (Gleave et al., 1999; Yau and Stewart, 2013) using various strategies such as negative marker selection by crossing or marker excision using the Cre-*lox* system or the R/Rs system from *Zygosaccharomyces rouxii* (rare instances of excised DNA being maintained as episomes in the next generation have been reported (Srivastava and Ow, 2003)). These strategies thus often require transgenesis ("negative" segregation monitoring to eliminate the transgene).

2.5. Whole-plant regeneration[59]

Although transformation is carried out on culture cells, a whole plant has to be regenerated. However, for the time being, transition from cell culture to whole-plant regeneration has been perfected for only a small number of cultivated varieties. This transition can lead to genetic, epigenetic or phenotypic variations (the latter resulting from genetic and epigenetic changes) known as somaclonal variation (Anderson et al., 2016; Jiang et al., 2011; Kaeppler et al., 2000; Wei et al., 2016) unrelated to the desired modification.

Although induced genotypic variations are undesirable because associated with an unwanted phenotype, it is possible to eliminate them from the selected plant by backcrossing with the original variety or to select plants not showing such variations after regeneration.

[56] PMI: Phosphomannose isomerase.

[57] GUS: Beta-glucuronidase colours appropriate substrates.

[58] GFP: Green fluorescent protein, which fluoresces when exposed to a given wavelength.

[59] This stage is not subject to regulatory assessment if used for clonal propagation. Somaclonal variation can also be used to produce new varieties. The few commercial varieties obtained by somaclonal variation (Chawla, 2009) are not considered to result from induced mutagenesis for the purposes of Directive 2001/18/EC.

2.6. Transient or stable plant transformation

The different delivery methods for the components required by SDN technology (effectors), together with their chemical characteristics (DNA, RNA or protein), determine whether they will be stable or transient. If one or more of the components needed for SDN techniques is present in a stable, heritable form, the plant is transgenic. To prevent the release of a GMO, the absence of components or component parts in the plant obtained must therefore be proved and documented. Intermediate products/plants generated while using the technique should be distinguished from the finished products/plants obtained at the end of the process.

2.6.1. SDNs

When using SDN-1, SDN-2 and SDN-3 techniques, it is important to distinguish the desired DNA modification itself, which is stable in the genome over generations, from the input of the components needed to bring about the modification; the latter are introduced transiently into the plant cell in the form of DNA, RNA or proteins (as guide RNA in CRISPR/Cas9, for example).

The components can be introduced through:

- Transient delivery of a protein: Introducing effectors in the form of proteins leads to a brief presence of proteins in the cell and is not part of nucleic acid transfer.

- **Transient transgenesis**: Introduction of a DNA or RNA fragment that will not be integrated in the genome and will not be transmitted. This strategy results in transient presence in an intermediate organism of genes encoding effectors. As in the case of effector delivery in protein form, this transient transgenesis results in a brief presence of effector proteins and RNA in the cell (for a few hours) and is not part of nucleic acid transfer (Liang et al., 2015).

- **Transgenesis using autonomous replicons**: Introduction of a DNA or RNA fragment that replicates autonomously. This is the case, for example, for (RNA or DNA) virus sequences able to replicate that can be used to amply and express CRISPR guide RNA (VIGE: virus-induced genome editing) (Ali et al., 2015; Baltes et al., 2014; Čermák et al., 2015; Yin et al., 2015). While some viruses that could be used to introduce effectors are seed-transmissible (Kil et al., 2016), most are not, and it is easy to ensure that the offspring do not carry the virus sequence.

- **Transgenesis with integration then eventual removal:** Integration of a DNA fragment in the genome through stable transgenesis. The transgene can then be eliminated by two techniques: crossing (negative segregation) or excision (see Section 2.4). If CRISPR is used there is the possibility of self-excision, consisting in a construct that includes additional guide RNA resulting in transgene removal (Schaeffer and Nakata, 2015).

2.6.2. RdDM

There are a number of techniques for obtaining double-stranded regulatory RNA:

- **Transient transgenesis:** Introduction of a fragment that will not be integrated in the genome and not replicate independently. This leads to transient presence of the transgene in an intermediate organism. Induced methylation can be stable over several generations.

- **Transgenesis using autonomous replicons:** Delivery of a DNA or RNA fragment that replicates autonomously. This is the case, for example, for (DNA or RNA) virus sequences that are able to replicate, known as VIGS (virus-induced gene silencing) (Martín-Hernández and Baulcombe, 2008; Peele et al., 2001).
- **Integrated transgenesis and negative segregation:** Integration of a DNA fragment in the genome (transgenesis). The transgene can be eliminated by crossing or excision.

2.7. Relationship between DNA sequence modifications and phenotype

The question of the consequences of genetic modifications for phenotypes is an important one. It is not desirable for a sequence modification to bring about unwanted alterations in the plant that might then entail risks if the plant is grown or eaten. Significantly, the Scientific Committee nonetheless notes in this respect that:

- Genetic modifications introduced into crop plants will mostly be modifications of sequences that are either known or which exist but have not been molecularly characterised. These modifications are intended to reproduce a variation associated with a phenotype of agricultural interest. The Scientific Committee recommends that modifications whose effects are novel[60] (because of a new gene function, for example) should be carried out in the laboratory prior to any cultivation. Agricultural use of a particular variation will have to be studied on a case-by-case basis.
- It is possible to find phenotype modifications caused by mutations in functional non-coding sequences. The sequences that will be used must therefore be identified and characterised. This is a prerequisite if they are to be modified for the purposes of plant breeding.

[60] See definition of novelty in Section 1.3.

3. Assessment and traceability methods for plants and products obtained by NPBTs (Point 1 of the referral)

In this section the Scientific Committee discusses the presence or absence of effectors in SDN technology and the difference between the effector-free end product and the intermediate product transiently expressing effectors.

3.1. Background

3.1.1. Regulatory environment

GMOs for contained use

As long as they are not intended to be released and placed on the market, genetically modified plants are not traced under Directive 2001/18/EC. However, in France they are covered by reporting for contained use of GMOs,[61] which provides for laboratory traceability through a register of GMOs. Thus if the plants grown in a contained environment are to be used to breed plants placed on the market, a complete "genealogy" of the variety can be provided.

Released GMOs

At present, traceability of GMOs and derived products from them is covered by Regulation (EC) No 1830/2003. This requires tracking of genetically modified seed yielding products intended for industrial or food processing for food or feed consisting of, containing or produced from GMOs, including additives and flavourings. For all these products, throughout their supply chains, operators must inform their customers in writing of the presence of GMOs, thus allowing them to use appropriate labelling.

In France, checks are carried out by the DGCCRF[62] on the basis of annual sampling plans for assays to detect, identify and quantify any GMOs and thus check labelling compliance. Similarly, the DGAl[63] has an annual seed monitoring plan.

To carry out these checks the competent authorities use methods and molecular tools that the Scientific Committee wishes to clarify.

3.1.2. Definitions

The Scientific Committee would like to define what it understands by detection and traceability of plants and products derived from plants obtained by new plant breeding techniques (NPBTs).

[61] Reporting regulated within the scope of Directive 2009/41/EC.

[62] Directorate General for Competition Policy, Consumer Affairs and Fraud Control (Ministry for the Economy).

[63] General Directorate of Food (Ministry of Agriculture).

- **Detection:** Ability to confirm the presence of an entity obtained by NPBTs in a given sample. The accuracy of the information on the nature and quantity of the entity present will depend on the approach used, the detection methodology and the extent of available knowledge about the entity. For each technique, there is a cut-off value below which detection becomes impossible. This is not a fixed value, and it can be brought down with individual improvements to each detection technique.
- The **limits of detection** of a known modification will depend on the NPBT and the technical limits of the methods used for detection. Detection is made even harder by dilution in the case of mixtures.
- **Detection by screening:** Ability to recognise the presence of genetic elements revealing the existence in the sample of an entity obtained by NPBTs, without necessarily allowing exact identification.

  The genetic elements investigated may reveal part of a modification sequence or the technique employed to generate it.

  In the case of GMOs, they are mainly the genetic elements constituting the transformation cassettes. In some cases the elements detected may suggest the presence of a known GMO whose identity must be verified by a specific identification test. Detection by screening can reveal the presence of partially or wholly unnotified transformation events.[64]
- **Identification:** Ability to be certain that an entity obtained by a given NPBT is present in the sample tested.

  For GMOs, this is based on detection of junction fragments between the genetic elements making up the transformation cassettes (construct-specific detection tests) or between the insert and the surrounding plant genome (event-specific detection tests).
- The **limits of identification** for an unknown technique or modification will depend on the NPBT and in particular the possibilities of natural genome variation (see Section 1.4).

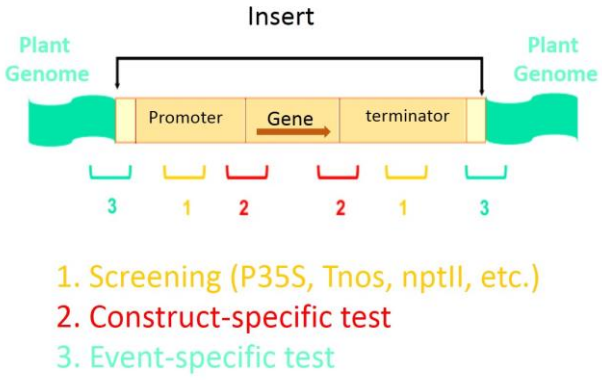

Figure 2: Potential targets for GMO detection/identification by PCR amplification. The oligonucleotides used for PCR are shown below. In the case of NPBTs this detection strategy can be used for SDN-3, intragenesis and cisgenesis (Type 1 and Type 3 oligonucleotides for the latter).

[64] Because the number of transgene sequences is "relatively" limited at present.

- **Quantification**: Ability to measure the quantity in the sample of an entity obtained by a given NPBT by comparison with the total genomic quantity of the species to which it belongs. For GMOs this quantification is necessary in order to be able to apply the labelling rules (mandatory if the threshold for authorised unintended presence is exceeded (Regulation (EC) No 1829/2003)).

- **Traceability:** Ability to trace the history, location and uses of an entity obtained by NPBTs using registered identification documents. Traceability systems must comply with national and international standards (ISO 9000-2005, ISO 9001-2008, ISO 22000, NF ISO 22004, ISO 22005-2007) and are designed to be as uniform as possible within supply chains. They are based on standardised records, which have to be kept for a given length of time (usually at least 5 years). In the EU, GMO traceability is based on assignment of a unique identifier for each authorised event, enabling all its uses to be tracked ("from farm to fork") when it is being placed on the market (Regulation (EC) No 1830/2003 and Regulation (EC) No 65/2004).

These concepts are addressed for GMOs in the HCB opinion of 17 January 2012 on coexistence.[65]

3.1.3. Scientific Committee approach

First of all, the Scientific Committee would like briefly to recall the genetic and phenotypic methods available for detecting GMOs. These methods will form the basis for the subsequent discussion of detection of plants and products obtained by NPBTs, but new approaches could be developed.

The Scientific Committee has considered methods of detecting and identifying unintended modifications induced by some NPBTs at the **delivery and somaclonal variation** stages. Delivery is used for conventional transgenesis and some NPBTs (see Section 2.3) but not in conventional breeding methods involving natural variation or plants obtained by mutagenesis.

The Scientific Committee has discussed **detection of effectors** used for some NPBTs, whilst noting that these effectors have to be removed from the plants before the latter are placed on the market; if this is not done, the plant could be considered to be transgenic because of the integration of exogenous sequences.

Each type of NPBT has been studied in individual fact sheets (Appendix VII). The summary tables at the end of this section are preceded by a list of biological information that the Scientific Committee would deem necessary to facilitate traceability of a plant/product obtained by NPBTs.

Plant/product detection, identification and traceability are there addressed in terms of the following three questions:

- Is the modification detectable in plants and their products if it is documented?

[65] http://www.hautconseildesbiotechnologies.fr/fr/avis/avis-sur-coexistence-definition-conditions-techniques-relatives-a-mise-culture-recolte-stockage

- Can the technique used to obtain this modification in the plant and its products be identified without documentation?
- If detection is possible, can it be used to find adventitious presence in mixtures of varying complexity during the production, processing and distribution process?

3.2. Detection of plant and products derived from GMOs

On the market, it must be possible to detect and identify GMOs and derived products in order to ensure that they are traceable throughout the food chain. To this end, an identification method that has been developed by the notifier and subsequently validated is required whenever an application is submitted for authorisation of any new genetic transformation event in Europe.

GMO detection targets can be transgenic DNA sequences inserted in the genome, the RNA transcribed from these sequences, proteins obtained by RNA translation or metabolites obtained by metabolism. Metabolites can be detected by specific biochemical assays (e.g. lauric acid for oilseed rape, or beta-carotene for rice). Recombinant proteins can be detected by enzyme assays (herbicide degradation, for example) and immunoassays (ELISA and strip tests). Transgenic nucleic acid sequences can be detected by qualitative or quantitative real-time PCR, Southern or Northern blotting, DNA-chip hybridisation and first-, second- or third-generation sequencing (next-generation sequencing, NGS). Multiplex methods are possible for simultaneous detection of multiple targets with different PCR and sequencing techniques (e.g. ligation-mediated PCR (Holck et al., 2009)).

To optimise the screening stages, PCR detection systems using pre-spotted plates (PSP) combined with decision-support systems have also been developed, such as that used by the Joint Research Centre (JRC) to detect all GMOs authorised in the EU in a single PCR experiment (Rosa et al., 2016) or a decision-support system combining traceability and analytical data (Bohanec et al., 2017).

Unlike other methods, detection of transgenic DNA sequences is not affected by the level of transgene expression, which can vary according to the tissues or organs tested, the plant's developmental stage, the genetic background or the environment. On the other hand, it may be affected by the proportion of the genetic component of the DNA carrying the transgene in the organ of the plant tested (and by the transgene copy number), for example in the seed of a non-transgenic plant fertilised by a transgenic plant (see coexistence opinion[66]).

Detection of unauthorised GMOs is based on screening and has to prove that the genetic elements detected do not come from authorised GMOs or non-GM sources (viruses and soil microorganisms, etc.), and this can sometimes be quite complicated. Ease of identification depends on the extent of available knowledge about the transgene sequences being investigated. Identification is easier if information is available (for example, about an event that has been assessed but is not authorised in the EU, or an event not authorised in the EU that contains a genetic construct similar to an authorised event obtained by the same breeder). In the case of whole plants, the reasons for which a sample is suspected of containing a GMO (phenotype, resistance involving certain types of gene) also provide the information to guide identification. Conversely, if little information is available, identification may

[66] http://www.hautconseildesbiotechnologies.fr/fr/avis/avis-sur-coexistence-definition-conditions-techniques-relatives-a-mise-culture-recolte-stockage

entail laborious cloning and sequencing stages, which is not routinely possible at present but can only be done in exceptional circumstances, as in the case of litigation.

Detection of unknown GMOs for which no information is available, whether for the sequence or the recombinant protein obtained, is also based on screening, but this cannot detect GMOs containing only genetic elements not yet described in testing laboratories' databases. As regards sensitivity, the quantity of genetic material present in the sample tested may affect detection capability.

Approaches based on blind comparison of omics data (whole-gene sequencing, proteomic and transcriptomic data) for the sample tested and its unmodified equivalent are being studied (Holst-Jensen et al., 2012) but still seem hard to envisage as a matter of routine. Developments in technology have indeed permitted a gradual reduction in the production time and costs for this type of data but have not removed the problems relating to the time and skills required for the subsequent bioinformatics analysis or the problems relating to existence of reference genomes, environmentally induced plant variation or the effect of physiology on levels of gene expression.

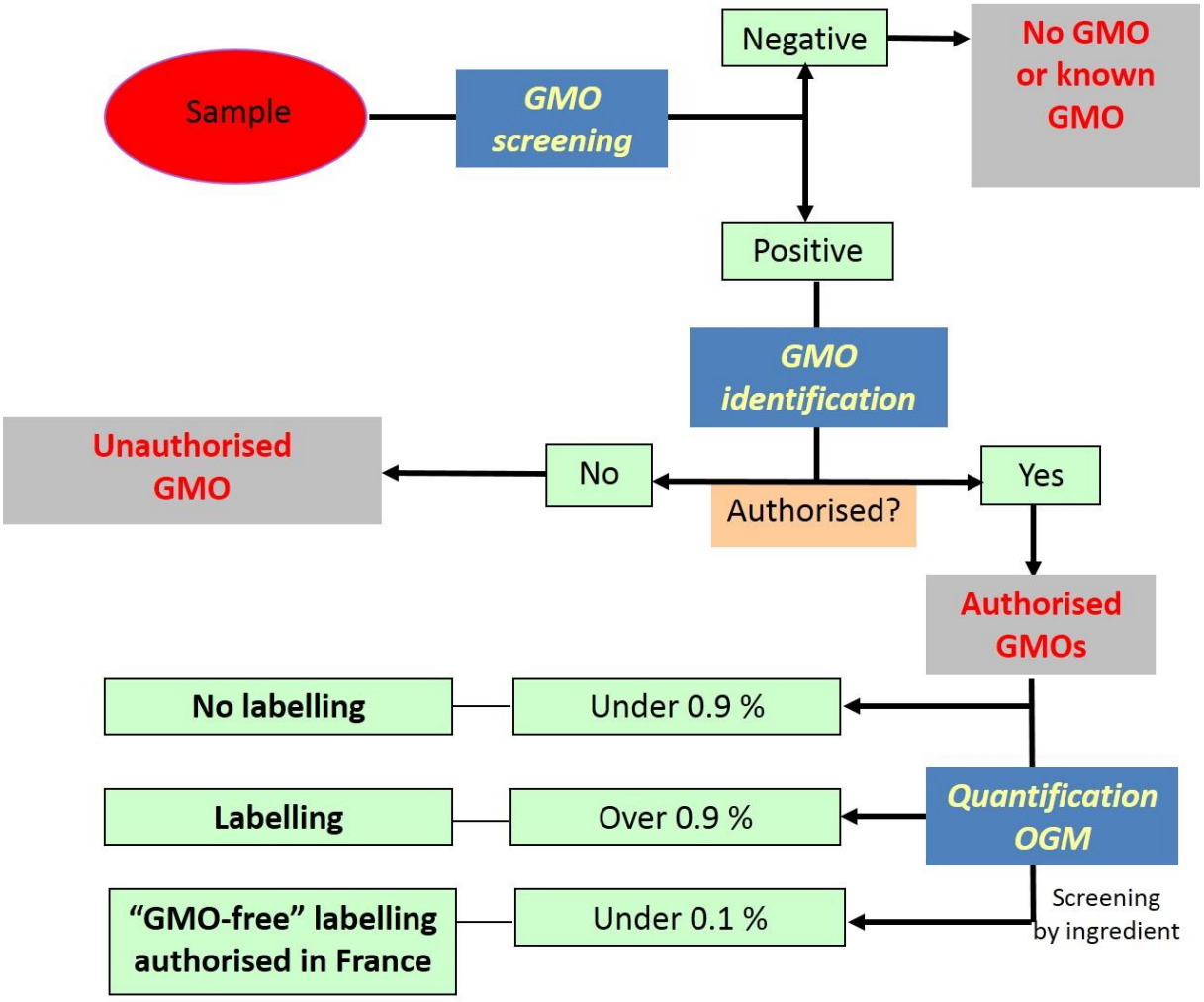

Figure 3: Sample testing procedure to find GMOs and consequences for labelling. A sample is considered to test negative for GMO presence below the limit of detection for the detection technique used and positive if this limit is exceeded. The 0.9% and 0.1% thresholds shown above reflect French GMO regulations.

3.3. Detection of delivery technique

For a notified genetic modification, detection can focus on the modification itself. However, in addition to the target modification, methods employed to obtain the genetic transformation (delivery, transformation, selection) may sometimes leave traces that can be used for detection.

3.3.1. Agrobacterium

Agrobacterium is often used to introduce the components for SDNs, cisgenesis, intragenesis, negative segregants and RdDM. The Scientific Committee has discussed possible persistence of small DNA fragments from *Agrobacterium* in the target zone (Brunaud et al., 2002) and elsewhere in the genome (Schouten and Jacobsen, 2007).

Depending on the size of the fragments, they could be detected by Southern blotting (>50 or 100 bp, depending on the size of the genome, etc.) or PCR (>20 bp). In the case of small fragments (<20 bp) next-generation sequencing should enable them to be identified, within certain limits.

The number of these fragments can be drastically reduced by crossing with a non-transgenic plant if these fragments are not genetically linked to the genes involved in the traits selected. While the Scientific Committee therefore agrees that these fragments may exist, it nevertheless finds that using them for plant identification or traceability would be difficult and unreliable. On the other hand, if insertion of these fragments were documented, they would be easily detectable. Once identified in a variety, they could be traced.

In the same vein the Scientific Committee has discussed possible persistence of *Agrobacterium* in transformed plants over one or two generations. *Agrobacterium* associates naturally with plants. The Scientific Committee agreed that the presence of *Agrobacterium* with a modified Ti plasmid could not always be used to detect genetically modified plants, since most of the bacteria were usually eliminated by antibiotic treatment in the laboratory after the transformation stage and their presence would be minimal or non-existent after one or two generations of plants. They would definitely be absent from the seed that was marketed. Although natural unmodified *Agrobacterium* might be detected, this would not be proof that the plant was transgenic, since these bacteria are naturally present in soil. The Scientific Committee concluded that presence of *Agrobacterium* with a modified Ti plasmid could not be used to classify plants as genetically modified.

3.3.2. Direct transformation (protoplast, biolistic, etc.)

Direct transformation techniques are not detectable in the plant thus obtained. However, fragments of the vectors inserted can be detected in the same way as for *Agrobacterium*.

A report has shown that in plant protoplasts transfected with a plasmid encoding the nuclease and guide RNA, in 0.06% to 0.14% of cases small insertions of plasmid DNA were observed at the cleavage site (Kim and Kim, 2016). However, given the randomness of the insertion and the rareness of these events, and in view of the fact that these plants would certainly not be selected by breeders, these events cannot be used for detection.

3.3.3. Viruses

If a viral replicon is used to express the components of modifications (for example, an RNA or DNA virus to express the guide sequence in the CRISPR SDN technique (Yin et al., 2015)), its detection, if it persists, could provide evidence of the technique used.

3.4. Effector detection[67]

If the effectors for SDN and RdDM techniques are not removed, the end plant will inevitably be covered by GMO regulations. Effector detection is thus a crucial step (see Section 7). Effector detection methods can also be used for monitoring programmes, particularly regarding SDN-1, SDN-2 and SDN-3 techniques.

Effectors can be detected by PCR and RT-PCR[68] if the effector sequences are known (notification or database listing all guide RNA and nuclease sequences used). Frequent use is made of some effectors, such as Cas9 and its tracrRNA, various meganucleases, and nucleases such as TALENs and ZFNs. It should be possible to use parts of these sequences that have been kept, taking account of codon modifications according to the species. Use of primer mixtures can help to identify even partially modified effectors (truncated or silenced). This question of effector detection must be reviewed in the light of technological developments. It is essential to be able to detect any effectors persisting, as in the case of non-transient DNA delivery, for example.

3.5. Overview of detection of plants and products obtained by NPBTs

3.5.1. A supporting document for NPBT detection, identification and traceability

The Scientific Committee has agreed on a list of biological information needed to facilitate traceability of a plant/product obtained by NPBTs. In this opinion, the term **documentary traceability** refers to this list (it would not be mandatory to use this list in full on labelling):

Supporting document for a product obtained by NPBTs:

Mandatory biological information for traceability:

- Species and variety;
- Breeding method;
- Delivery method (if applicable);
- Tissues targeted by modification;
- Trait(s) modified or introduced;
- Phenotyping method;
- Target region sequences (before and after modification) and chromosome location;

[67] See definition of effector in the introduction.

[68] RT-PCR: Reverse transcription of RNA, followed by PCR.

- Presence or absence of effectors needed for SDNs (if applicable);
- Unique identifier, as per the standard, if possible.

3.5.2. Summary tables

See next pages.

Table 1: Summary of options, by technique, for detection and identification of breeding method

Techniques / ***Issues***	SDN-1 end product (i.e. effector-free) (Site-specific mutation)	SDN-2 end product (i.e. effector-free) (Allele conversion)	SDN-3 (Sequence insertion)	ODM (Oligonucleotide-directed mutagenesis)
Detection of DNA modification[69]	Yes	Yes	Yes	Yes
Stable insertion of foreign DNA	No	No	Yes	No
Identification of breeding method	No	No because comparable to a natural variant. Possible if modification is combined with a molecular signature.[70]	Yes unless there is a natural equivalent. Sometimes possible if combined with a molecular signature.[70]	Generally no. Possible if modification is combined with a molecular signature.[70]
Breeding by a technique listed in Annex 1 B of Directive 2001/18/EC	Yes	Yes	No unless the modification could occur naturally.	Yes
Field coexistence: detection	Yes[71] and if phenotype and trait are not present in growing area.	Yes[71] and if molecular signature, or if phenotype and trait are not present in growing area.	Yes:[71] GMOs.	Yes[71] and if phenotype and trait are not present in growing area.

[69] Identification of a genetic modification in an organism's genome does not indicate how it was obtained.

[70] A molecular signature would consist in inserting a predefined pattern of nucleotides unlikely to be found in nature. It would thus be possible to ascribe a mutation to a technique rather than just selection of a natural variant.

[71] Yes if the modification is transferred, which will depend on the plant's method of pollination.

Techniques / ***Issues***	***Non-GM scion on SDN-1, SDN-2 or ODM rootstock***	***Non-GM scion on SDN-3 or transgenic rootstock***	***GM scion (SDN-1, SDN-2, SDN-3, ODM, cis- & intragenesis) on non-GM rootstock***
Detection of DNA modification	Yes for rootstock. No for scion and fruit.	Yes for rootstock. No for scion and fruit.	Possible for scion. Refer to modification techniques (see table on previous page).
Stable insertion of recombinant DNA	No	Yes for rootstock.	Possible: SDN-3, cisgenesis and intragenesis.
Identification of breeding method	No for whole plant, except for rootstock with molecular signature.	Possible for rootstock.[72] No for scion and fruit.	See table on previous page.
Breeding by a technique listed in Annex 1 B of Directive 2001/18/EC	Yes	No, apart from some types of cisgenesis.	See table on previous page for each technique.
Field coexistence: detection	Yes for the transgenic part requiring authorisation.	Yes for the transgenic part requiring authorisation.	See table on previous page for each technique.

[72] See SDN-3 column on previous page for specific detection problems.

Techniques / ***Issues***	***RdDM*** (RNA-directed DNA methylation)	***Agro-infiltration***	***Cisgenesis*** (introduction of DNA from a sexually compatible species)	***Intragenesis*** (introduction of recombinant DNA from a sexually compatible species)
Detection of DNA modification	Yes and **technically complex.**	Yes	Yes	Yes
Stable insertion of recombinant DNA	Depending on technique	No	Yes	Yes
Identification of breeding method	Yes if transgenesis. No if RNA, CRISPR.	Yes, transiently.	Yes if transgenesis by *Agrobacterium*. No if the genetic modifications could occur naturally	Yes
Breeding by a technique listed in Annex 1 B of Directive 2001/18/EC	Yes	No, but not relevant: mostly transient expression.	Possible in some cases.	No
Field coexistence: detection	Unstable heritability and varying contribution to gamete profile depending on plant.	Not relevant owing to contained use.	Transfer depending on plant's pollination method.	Transfer depending on plant's pollination method.

3.5.3. Conclusions

In most cases if information about the modification is available, plants and products from plants obtained by NPBTs can be identified. However, it will not always be possible, especially with no context, to be certain that the modification found actually results from use of an NPBT rather than being a natural modification, or even obtained by another technique: carrying out checks without traceability data is likely to produce a large number of false positives. A sample is considered to test negative for presence of a plant obtained by an NPBT if it is below the limit of detection for the detection technique used and positive if this limit is exceeded.

Lastly, in some cases (negative segregants or some types of grafting in particular), even with the help of molecular traceability data, it will not be possible to identify products of plants obtained by NPBTs.

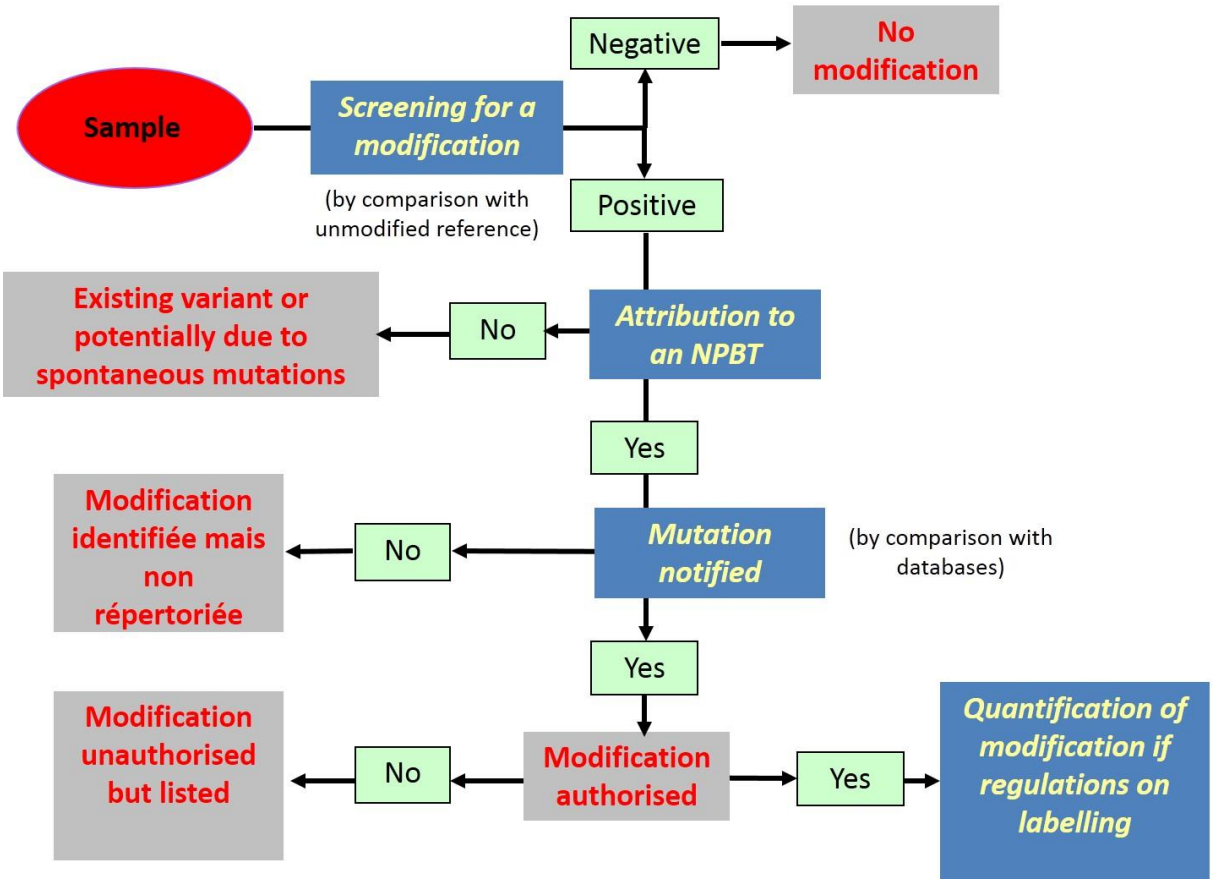

Figure 4: Potential sample testing procedure to find a modification induced by an NPBT.

4. Implications for supply chain coexistence (Point 2 of the referral in connection with the previous point)

For this section the following points were discussed in the Scientific Committee's working sessions: the commercial and non-environmental aspects of individual supply chains and their coexistence (as opposed to coexistence of GMOs and the environment).

Coexistence seeks to allow each of the parties involved in the different supply chains (agricultural, seed, food-processing) to ensure compliance with their own rules. At present, coexistence in France concerns three types of products:[73]

- Containing GMOs (over 0.9%),
- Not containing GMOs (under 0.9%),
- GMO-free (under 0.1% or under 0.9% in France), including the organic supply chain.

The object is not to protect health or the environment, since authorised plants have already been assessed, but to organise production in such a way that supply chains that do not wish to use NPBTs or their products are able to avoid doing so.

4.1. End product versus plant breeding method

For supply chains interested only in the end product: i) If varieties are obtained by NPBTs[74] whose products result from the genetic diversity of the species itself or closely related species,[75] and ii) inasmuch as the properties of these plants are indistinguishable from those that could be obtained by crossing or other exempted techniques, the question of coexistence does not arise, since there can be no distinction.

For NPBT-derived varieties that could not have been selected from the genetic diversity of the species or closely related species,[76] the question of coexistence does arise.

It should be noted that some supply chains interested only in the end product, whilst prohibiting certain traits such as herbicide tolerance or *Novel Traits*, could lay down their own specifications. If these supply chains are officially recognised, specific measures could be proposed.

For supply chains interested in the plant breeding method: While it is not always possible to detect and identify a modification, it is nevertheless possible to trace it with a documentary record. Here again, these supply chains will have to provide specifications. As for the risk of adventitious presence of the modification, a best-endeavours obligation could be considered (volunteer monitoring, separation distances, buffer zones, border removal, segregated supply chains for seed production,

[73] Regulation (EC) No 1830/2003 (EC, 2003) and Decree No. 2012-128 of 30 January 2012 on "GMO-free" labelling of foodstuffs (see Figure 3).

[74] SDN-1, SDN-2, RdDM and in some cases intragenesis and cisgenesis.

[75] Closely related sexually compatible species with which hybridisation is possible.

[76] See previous footnote.

etc.), whereas an absolute obligation could be hard to enforce owing to lack of routine detection methods.

4.2. If Directive 2001/18/EC were to be interpreted as excluding plants obtained by some NPBTs

If Directive 2001/18/EC were to be interpreted as excluding plants obtained by some NPBTs, there would not at present be a specific supply chain for these plants. However, each supply chain could lay down its own specifications within the limits of traceability techniques (including documentary traceability) without affecting the rules.

4.3. If Directive 2001/18/EC were to be interpreted as including plants obtained by some NPBTs

If the European Commission were to include plants obtained by some NPBTs in Directive 2001/18/EC or were to introduce another specific interim regulation, the question of detection would become critical. It would then be necessary to distinguish between modifications that had been notified and those that had not. The situation would also vary according to whether genetic modifications were to be detected in a crop in France or in an imported batch. Last but not least, the situation would depend on the type of NPBT used.

If a modification is identified (by its molecular description, for example), detection is possible – although without systematic attribution to a technique – for some NPBTs such as SDNs, ODM, intragenesis and cisgenesis, and it will be very difficult for RdDM and grafts on GMOs (in most cases) and virtually impossible for negative segregants.

If a modification is registered in data banks, detection is possible for genome editing techniques (mutation, “methylation”) as well as negative segregants and grafts if a screening method has been developed, particularly with high-throughput sequencing. But it will sometimes be impossible to tell whether the modification observed has actually been obtained by using an NPBT.

If genetic and phenotypic modifications are not registered in data banks, the technical limitations outlined above would make it harder, and even for some NPBTs virtually impossible, to detect presence, whether adventitious or not, of products obtained by NPBTs in an “NBPT-free” supply chain. As has already been done for existing GMOs (Holst-Jensen et al., 2012; Petrillo et al., 2015), developing appropriate detection methods and data banks therefore seems a prerequisite for separate supply chains with labelling to match.

The Scientific Committee notes, however, that the type of modified products and the regulatory thresholds for their presence will affect detection capabilities, with detection becoming impossible in many cases.

5. Direct risks to health and the environment associated with novel characteristics of products obtained (Point 3 of the referral)

The Scientific Committee discussed the mainstreaming of GMO cultivation and consumption over the past 20 years, together with the impact on human, animal and environmental microbiota. For each of the risks identified it further examined whether it was a direct risk and associated with the products' novel characteristics. The definition of off-target modifications was clarified. The possibility and potential consequences of undetectable persistence of active effectors was discussed.

The HCB Scientific Committee will here address foreseeable risks, since it is impossible to discuss development risks[77] at this point as they are unforeseeable by definition. We may assume that future developments in scientific and technological knowledge will alter our understanding of the risks and bring about a change in the management measures needed as a result.

Risk is the consequence of an identified danger (or source of damage) and exposure to this danger (likelihood of damage occurring).

Under Directive 2001/18/EC (Annex II) and Regulation (EC) No 1829/2003, any potentially harmful characteristics associated with a genetically modified (GM) plant are assessed by comparing them with the characteristics of an equivalent non-GM plant, used as a comparator. The biological relevance of statistically significant differences is used to formulate risk hypotheses which are subsequently tested.

- For assessment of health risks associated with a genetically modified plant for food use, the biological relevance of a difference between the GM plant and its non-GM equivalent is determined by comparison with a batch of non-GM reference varieties with a history of safe use.
- For environmental risk assessment, the comparative assessment procedure is recommended, but because the concept of safe use does not apply to the effects of agriculture on the environment, equivalence limits are determined not by limits of variation but by limits of concern.[78] In other words, any characteristics of the GM plant not measured in connection with environmental protection goals will be subject to an in-depth risk assessment. One recurrent problem is how to define these goals.

Exposure assessment is based on a pre-estimate of the crop area and consumption of products obtained from it. This parameter hinges on farmers' adoption of the modified variety/varieties.[79] This adoption, which is often hard to predict, varies according to sundry social and economic parameters, the impact of climate and also the strategies of the various parties involved in the supply chains (Bonny, 2008). This is true for all farming, whether or not it entails use of genetically modified varieties.

According to Directive 2001/18/EC (Annex II), **direct risks** refer to primary effects on human health or the environment which are a result of growing the GMO itself and which do not occur through a causal

[77] A development risk is defined as a risk that is unknown when a product is launched and which becomes apparent through a subsequent growth in scientific and technological knowledge over time.

[78] This term denotes the limits beyond which a given characteristic will be of sufficient magnitude to cause environmental harm (according to EFSA, 2010). These limits are defined in relation to environmental protection goals.

[79] The Scientific Committee notes that exposure relates to the trait rather than the variety; thus if the same trait is present in a number of different varieties, adoption of all these varieties must be taken into account.

chain of events. **Indirect risks** refer to effects on human health or the environment occurring through a causal chain of events, through mechanisms such as interactions with other organisms, transfer of genetic material, or changes in use or management.[80]

The "novel characteristics of products obtained" are twofold: those resulting directly from the desired traits and those resulting from unintended effects of the techniques used to obtain these traits.

The points to consider are:

- Delivery method (when used);
- The fact that modifications are directed (genetic directionality);
- The option of making several modifications at the same time (multiplex genome editing).

In addition to the product obtained, the genetic engineering process must thus be taken into account in risk assessment, for two reasons:

- There may be risks associated with the modification technique, for example the novelty constituted by delivering proteins or nucleic acids to plant cells by comparison with traditional selection;
- The much greater effectiveness of these techniques in generating varieties with one or more traits with potential monetary value for agriculture, combined with the faster pace of innovation that farming will be facing (since adoption of innovation is not directly connected with use of NPBTs but depends on a number of social and economic factors), could lead to a wider choice or supply of varieties[81] and potentially to wide adoption by farmers and therefore substantial exposure.

The HCB Scientific Committee notes that these risks are not mutually exclusive.

The HCB Scientific Committee wishes to point out that although the referral relates to direct risks associated with novel characteristics of the products obtained, it has identified a number of indirect risks and risks not associated with these novel characteristics. As with all human activities, farming and placing varieties on the market has risks. Such risks apply as much to NPBTs as to varieties obtained by traditional methods (crossing), regulated methods (transgenesis) or unregulated methods (induced mutagenesis). These indirect and/or non-specific risks are listed in Appendix V. The Scientific Committee also notes that traits would be introduced for their agricultural value, which it is therefore important to consider in assessment.

5.1. Risks arising from desired traits

The Scientific Committee would like to stress the following three points:

[80] For example, toxicity and the plant's effect on non-target organisms or soil microorganisms would be direct risks. Examples of indirect risks would be onset of tolerance to a toxin or emergence of herbicide-resistant plants through selection or gene transfer.

[81] Introduction of different rates of early maturing in more varieties and/or introduction of the same trait into several species used in crop rotation.

- Use of NPBTs does not automatically mean that novel traits will be obtained (not restricted to novelty).[82] The particular merit of SDN-1, SDN-2 and ODM techniques lies in the ability to introduce the characterised allele of one variety into a different variety in just a few stages.
- Some traits obtained by these techniques could be obtained by other techniques not covered by GMO regulations (crossing of natural variants or random mutagenesis) or by regulated techniques (transgenesis): disease-resistant or herbicide-tolerant plants, for example (duplication of techniques).
- Some techniques require introduction of foreign genetic material transiently. Persistence of the (RNA, DNA or protein) effectors used for the modifications, and of the vector (*Agrobacterium*, virus, etc.), is a risk specific to these techniques (see Section 5.2). The absence of these molecules and vectors must therefore be verified. If the such molecules persist, which the Scientific committee considers undesirable since technically avoidable, an assessment of the risks associated with this persistence should be carried out.

Once the absence of vectors and effectors has been verified, the direct risks are common to all genome modification techniques and to crossbreeding (see Appendix V). Uncertainty concerning these risks will logically increase as the gap with the species' intrinsic genetic variability grows and the trait's novelty becomes established.

On the other hand, the Scientific Committee notes that, although this is not included in the terms of reference (direct risks), changes in farming practices and food-processing procedures connected with the new traits (indirect risks) should be assessed on a case-by-case basis, since they can themselves, like all human activities, have consequences for the environment and human health. Similarly, although outside the scope of the referral, the question of the expected benefits of use of NPBTs should also be taken into consideration by policymakers.

5.1.1. Risks associated with modification of crop plants not previously genetically modified

These are risks generally associated with plant breeding but which might become more frequent in connection with NPBTs, owing to the latter's ease of use. NPBT development, combined with diminishing costs (this again being contingent on the associated regulatory costs), could lead to modification of species not previously modified by transgenesis.

The (state of the art) assessment context for associated risks would then relate to the species rather than just the traits modified.

By way of example, mention may here be made of the risks associated with dispersal if crop plants have wild breeding partners. The issues relating to dispersal risks and hybridisation with species locally present in the wild have not necessarily been taken into account for species for which there are no transgenic varieties. Although an assessment method has already been established for other species, this must be adjusted case by case for each new species for which NPBTs might thus be used.

[82] See Section 1.3 above.

The Scientific Committee notes that the technical capacity to regenerate plants after transformation severely restricts modification of new species, since regeneration methods have been developed for only a small number of crop species.

5.1.2. Risks associated with potential novel traits

The Scientific Committee distinguishes novel traits (see Section 1.3) in a variety or species[83] from traits that are not present in nature and which could be obtained using synthetic biology, for example.

5.1.2.1. Novel traits in a species[84]

A novel trait in a species,[85] for the purposes of this discussion, is a trait not present in varieties of the same species currently authorised for sale (and therefore registered in the EU catalogue), although the trait may be present within the species' genetic diversity. Since a novel trait can be selected using conventional breeding methods, induced mutagenesis or transgenesis, the HCB Scientific Committee believes that genome-editing and epigenetic modification techniques in particular allow faster emergence of novel traits in varieties, although this risk is not specific to NPBTs.

Among new traits currently being developed, we may mention the following (Ricroch and Hénard-Damave, 2015):

- Biotic stress resistance: new types of disease- and pest-resistance, new types of herbicide-tolerance;
- Abiotic stress tolerance: drought, salinity, water submergence;
- Efficient use of soil nutrients;
- Nutritional quality: lower antinutritional-compound content, higher nutrient content (e.g. vitamins, minerals and fatty acids);
- Therapeutic molecule production.

As well as:

- Petrochemical substitutes;
- Phytoremediation.

[83] For these novel traits, Canadian Directive 94-08 draws a distinction between qualitative novelty (the new trait is not present in stable, cultivated populations of the plant species) and quantitative novelty (the trait in the plant species is present at a level significantly outside the range of that trait in stable, cultivated populations of that plant species).

[84] See Section 1.3 above for definitions of novelty.

[85] Which must be differentiated from a novel trait in general (see Section 1.3).

In the case of cisgenesis and intragenesis, the fact that the transgene comes from a sexually compatible species or variety does not mean that plant quality and toxicity (for Solanaceae: tomatoes and potatoes, for example) can be ignored. However, these risks are similar in nature to those resulting from introgression of the desired gene from the wild plant into a crop plant by hybridisation, as currently happens with conventional breeding.

Mention may also be made of the potential risks of acclimatising species to environments where they cannot grow naturally (because of salinity, drought or contaminated soil, for example), which could lead to their adoption in new areas. As with any new farming practice, these impacts would then have to be assessed and/or monitored by the public authorities on a case-by-case basis.

New traits for crop varieties could also emerge as a consequence of more knowledge about functional genomics. In both basic and applied research, genome editing technology should itself speed up knowledge acquisition in functional genomics in the laboratory, particularly for multigenic traits (Liu et al., 2016).

5.1.2.2. Novel traits (synthetic biology)[86]

A function not performed by the gene either in the variety or in any other related species obtained by molecular evolution must undergo specific assessment (see Section 7).

5.2. Risks due to unintended effects inherent in NPBTs

5.2.1. Unintended effects associated with effector persistence

A number of the techniques mentioned require breeding of intermediate plants into which effectors from other species have been introduced. Techniques generating SDN-1, SDN-2 and SDN-3 modifications use nucleases and possibly guide RNA (as in CRISPR/Cas9 systems). It should be noted that:

- Persistence of nuclease expression may result in a larger number of off-target modifications (Yee, 2016);
- Persistence of guide RNA alone does not seem to be associated with any specific risks;
- Persistence of a nuclease (such as Cas9) and guide RNA together may result in a larger number of off-target modifications;
- Moreover, crossing of plants containing these effectors (for example, a plant containing Cas9 with a plant containing guide RNA) may result in genetic modifications in offspring;
- Lastly, in the particular case of a sequence recognised by guide RNA being homologous to a zone in which a transgene encoding a nuclease and guide RNA is

[86] See Section 1.3 for definition.

stably inserted, such situation could lead to gene drive. Thus, gene drive is highly unlikely if not specifically intended. It is therefore a special type of transgenesis[87] and must be assessed as such. Gene drive is not considered here.

Release into the environment of "intermediate" plants expressing a nuclease transgene is neither necessary nor desirable in the case of a technique that can be used in a contained environment.[88] If an effector persists in DNA form, the plant obtained will meet the definition of a GMO and must be authorised under Directive 2001/18/EC. The Scientific Committee considers that effector persistence is undesirable and that applicants must ensure that commercial strains are effector-free.

Consequently, the Scientific Committee insists that absence of effectors in DNA, RNA or protein form be proven prior to authorisation of placing on the market.

Similarly, in the case of modification using **RdDM** (RNA-dependent DNA methylation) there would be the risk that the transgenic parent's transgene (whether stable or not) might be inherited.

In the case of **agro-infiltration** the risk would be that the transformation could be stably inherited by the plant's offspring in the environment. These would then be GMOs for the purposes of Directive 2001/18/EC.

In the case of **non-GM scions on GM rootstocks** it must be verified that no transgenes or unwanted products have transferred to the non-GM part and its offspring. The rootstock must be assessed in its own right.

5.2.2. Risks associated with off-target modifications and unintended genome modifications

The risk associated with unintended genome modifications is not specific to NPBTs. It can also arise from introgression of a trait through conventional breeding or use of induced mutagenesis, producing modifications that are not wanted by breeders, or even known to them. However, the HCB Scientific Committee has nevertheless wished to explore this risk in this section.

Techniques using targeted nucleases (SDNs) and transgenes (negative segregants, negative marker selection, cisgenesis, intragenesis), ODM, RdDM and agro-infiltration carry the potential risk of genome modification other than the modification originally desired. The same risk exists with transgenic plants and other unregulated plant breeding methods. The Scientific Committee will discuss unwanted modifications specific to certain NPBTs irrespective of the related technique (delivery, protoplasts or regeneration) in this section, and, in the following, unwanted modifications that may also be found in transgenic GMOs or exempted techniques.

[87] Gene drive inheritance is unusual because the transgene is inherited by a population much faster than in the case of Mendelian inheritance, although the gene drive regions are subject to natural mutation that may stop this gene drive.

[88] Despite the absence of data proving an effect, the HCB Scientific Committee cannot rule out the possibility that such plants may pose a risk to health and the environment.

In the case of **site-directed genome editing** techniques **(SDNs, ODM, RdDM)**, **off-target** modifications may occur. Other genome engineering techniques are not targeted,[89] and although they induce a number of unwanted random modifications, these cannot be called "off-target". In the case of SDNs, the nucleases may act on other sites of the genome, particularly those with sequences similar to the target sequence. For CRISPR, off-target mutations have been found on sites differing by up to five base pairs from the guide RNA (Fu et al., 2013; Jinek et al., 2012; Tsai et al., 2015) as have genome rearrangements in the case of cleavage at two separate sites (Pacher et al., 2007). These specificity problems also exist with other nucleases (Hendel et al., 2015; Lin et al., 2014).

The types of off-target modification with SDNs, ODM and RdDM depend on the technique used and the mode of delivery. They are generally much fewer in number than the mutations obtained by chemical mutagens and radiation (unregulated techniques) and of the same order of magnitude as mutations occurring naturally in plant germ cells.

These off-target modifications are found mainly in the case of persistent expression of nucleases (Yee, 2016) and demethylases. The Scientific Committee notes that techniques are moving towards short-term modification-enzyme expression systems with nuclease combinations having improved specificity. The risk of off-target mutation is thus steadily decreasing. In the particular case of CRISPR/Cas9, the rate of off-target modifications depends on Cas9 and guide RNA dosage and an optimal choice of guide RNA (Hsu et al., 2013; Pattanayak et al., 2013). Optimisation of these parameters thus reduces the risk (Peterson et al., 2016).

It is possible to limit off-target mutations through choice of guide RNA, but to do this the plant's genome sequence must be known. Given the range of natural genetic variability, the reference sequences will not necessarily match the sequence of the variety under consideration. Non-coding sequences are not always known precisely, especially if they are not functional. It is therefore hard to identify possible off-target mutations in non-functional sequences, which do not have foreseeable consequences. Verification by whole genome sequencing is difficult in crop plants because of the size and diversity of sequence repeats in these genomes.

The off-target mutations induced are therefore hard to routinely quantify precisely for crop plants' large genomes. Backcrossing during the selection process can eliminate most off-target mutations (apart from those that may be genetically linked to the desired trait, but these can be found by sequencing). Backcrossing is nevertheless technically difficult to use for perennials such as fruit trees or for plants that reproduce mainly through vegetative propagation. Moreover, off-target mutations are not easily distinguishable from the natural mutations found in all living organisms, including plants.

Reducing off-target mutations is an active field of research. The frequency of off-target mutations can be reduced by using new CRISPR/Cas9 systems, to such an extent that it has recently been reported that it is impossible to distinguish these mutations from natural variation in cultured human cells (Kleinstiver et al., 2016). These nuclease variants retain on-target activity comparable to the original

[89] DNA insertion techniques using *Agrobacterium* cannot target an insertion region in the genome, while techniques using chemical or physical mutagens induce random localised mutations in the genome.

nuclease (70% to 140% according to the paper) with no detectable off-target activity. Other assays for the model plant *Arabidopsis thaliana* have shown similar results (Peterson et al., 2016).[90]

Although it is often possible to remove off-target mutations by backcrossing with the genetic background of varieties that are going to be marketed, if the techniques cost very little and for perennials such as fruit trees, where backcrossing is harder to use, a breeder might find it worth transforming elite varieties directly without resorting to a donor parent (thus saving considerable time). In this case, possible specific off-target mutations of the commercial genetic background would also be removed by backcrossing (except perhaps for some perennials with too long a generation time).

It is theoretically possible to assess and forecast off-target mutations through bioinformatics analysis of sequences showing some homology to the target sequence. However, this can only be done with an exhaustive knowledge of the genome of the plant to be modified.

Off-target mutations with a phenotypic effect will be detected when the plant obtained is cultivated.[91]

The Scientific Committee has discussed the possibility of suggesting to breeders that identified regions of the genome should be sequenced in the laboratory for a few years in order to measure the frequency of off-target mutations when using SDNs and ODM. This would make it possible to gather more information about these off-target effects and restrict uncontrolled use of these techniques. However, it does not always seem possible at present to make such a record, since it would be necessary to know the full sequence of the variety grown rather than just of the model variety for the species.

5.2.3. Risks associated with combining targeted modifications

Some NPBTs open up the possibility of modifying several genes at the same time (Qi et al., 2016)). This multiplex gene editing represents a special use of such techniques and raises specific questions about risk assessment. Thus, mutation of all the genes in a gene family or metabolic pathway (metabolic engineering) may allow emergence of *Novel Traits*[92] and expression of pleiotropic effects and therefore raise questions about the impacts and risks associated with these effects.

Mutation of a set of genes could lead to changes in expression of other genes or metabolic pathways through regulatory pathways (modification of control loops, “compensatory” effects). These changes

[90] Two translocation events were found in this paper, without its being possible to link this directly with use of the technique.

[91] The Scientific Committee wishes to point out that the risks associated with the effects of off-target modifications in crop plants are not therefore of the same nature as those that may appear when these techniques are used in human therapy. Off-target mutations in patients can have a serious impact on the functioning of their bodies and thus on their health; in this case it is thus essential to use only techniques with off-target effects approaching zero. The fact that human genome sequencing is possible, fast, and easy to analyse permits this type of validation. For plant breeding, if an unwanted mutation event were to compromise a plant’s viability, the plant would simply not be selected by the breeder for the breeding programme.

[92] See Section 1.3.

might not be detected in the phenotypic assessment of a variety if, for example, they modify some compound level or sensitivity to particular non-target pathogens, since phenotyping is not exhaustive.

A number of traits each controlled by genetic or epigenetic modification of a locus or by a transgene (cisgene or transgene) can be combined in a single variety. Combination of a number of genetic and epigenetic modifications does not necessarily produce the sum of their individual phenotypic modifications (phenomenon of epistasis) (Phillips, 2008).

5.3. Risks associated with potential acceleration of breeding owing to efficiency and technical ease of use of NPBTs

The Scientific Committee notes that the pace and efficiency of some new plant breeding techniques represents a new factor. However, it wishes to point out that any potential acceleration for crop plants will depend on the ability to regenerate a whole plant from a modified cell (see Appendix V) (Germana and Lambardi, 2016). For crop species, these targeted methods break with the process of biological development based on random production of variants and subsequent selection (selective breeding of domesticated species). NPBTs guide the breeding of traits in crop species, which is thus much less restricted by the low probability of recombinations and random mutations for the purposes of the intended trait modifications.

NPBTs make it possible to introduce targeted improvements into a given variety and can accelerate the effect of breeding in crop species by targeting modifications of these plants at a number of sites within a gene or in multiple genes simultaneously (Cambray et al., 2011; Esvelt and Wang, 2013; Woodruff and Gill, 2011) (see multiplex genome editing). These targeted, rather than random, modifications constitute a significant change in the breeding process. With natural genetic variation in a species, a number of mutations (polymorphisms) causing amino acid substitutions can often be found in a given protein (see Section 2.1). On the other hand, the probability of finding a given mutation when sampling natural populations is very low (between 0.01 and 0.0001 (Wakeley, 2009; Watterson, 1975)). If we wait for a mutation to appear at random (that could subsequently be selected for breeding) and if we are able to screen an extensive population of a billion individuals, it is probable that a predefined new mutation will be found in a given generation. In such a population, the probability of combination of two specific separate mutations would then be virtually zero (squared, the probability for a billion individuals is in the region of 10^{-9}). New site-directed mutagenesis techniques are changing the pace and possibilities of plant breeding, since they make it possible simultaneously to combine a number of mutations defined by earlier experiments.

What NPBTs therefore have in common is that they are able to speed up the breeding of crop plants. The resulting impact cannot be assessed purely on a case-by-case basis but must also be assessed as a whole.

This acceleration of the breeding process for new varieties may be a factor in agronomic improvement but can also hold risks. While NPBTs would speed up adoption of new varieties obtained by these techniques, having an impact on agricultural ecosystems, this could lead to additional adjustment problems for biodiversity and associated ecosystem services. Similarly, cultivation of new varieties or species in currently unfarmed areas could alter the ecological characteristics of these environments. Yet biodiversity in both farmed and unfarmed areas supports ecological functions that are essential for human communities, in particular regulating and supporting ecosystemic services (Millennium

Ecosystem Assessment, 2005[93]). Various economic, sociological and ecological effects on farm production and food-processing systems may be expected, depending on the nature of the traits modified and/or the environments where the new varieties are introduced. Given the range of situations that might occur, it would be useful, if new varieties contain *Novel Traits*, for these innovations to be monitored with regard to ecological, agro-ecological, economic and societal impact.

6. Management measures required to prevent and limit risks to health and the environment associated with use of products obtained by NPBTs if such risks are identified (Point 4 of the referral, in connection with Point 3)

Assessment

Management measures must be introduced in the light of the results of an assessment conducted on the basis of the proposals set out in Section 7 once the public authorities have taken appropriate action.

Biomonitoring

Management measures could be proposed to supplement any case-specific monitoring, crop containment or operator protection measures that might prove necessary to prevent or mitigate known or strongly suspected risks associated with unintended effects expressed in some of these new crop varieties. In some cases biomonitoring might be necessary. This would assume that the crops are recorded in a parcel register (the security of these parcels and of farmers being guaranteed by the public authorities) of the same kind as that introduced for the first Bt maize crops by the 1999 Outline Farming Act.[94] This network could be extended to monitoring of unintended effects of cultivation of some new varieties.

Local management of these new plants, with gradual roll-out over time and space, might be suggested to control the pace of agro-ecosystem change that might result from NPBTs.

Screening of plants expressing effectors could be done as part of screening of authorised and unauthorised GMOs.

Maintaining a modification-free gene pool

The Scientific Committee notes that it is important to conserve pools of genetic resources unmodified by NPBTs.

Lastly, the Scientific Committee points out that, owing to the complexity of the issues involved, advances in knowledge will make it possible to take better account of risks and their consequences.

[93] http://www.millenniumassessment.org/en/

[94] See glossary for further information.

7. Proposal of intermediate measures between the provisions of the EU catalogue and those of Directive 2001/18/EC that seem advisable for regulating use of NPBTs on EU territory, incorporating assessment of the socio-economic implications (in connection with Point 7).

In this section, procedures and the need for mesocosms were discussed during the Scientific Committee's working sessions.

7.1. Outline of the two systems in question

A brief outline is provided below, for the purposes of comparison, of the registration procedures for a catalogue (Section 7.1.1, taking the Official French catalogue as an example) and the procedures arising out of Directive 2001/18/EC on GMOs (Section 7.1.2).

7.1.1. Registration in the Official French Catalogue

There are two official catalogues at the national level in France: the Official French Catalogue of Plant Varieties, managed by the Technical Committee for Plant Breeding (CTPS), and the EU catalogue, which is the sum of national catalogues. Since the requirements for registration in a national catalogue may vary from one country to another, reference is here made to the Official French Catalogue.

In France, marketing of varietal seed requires authorisation. This is provided through registration with the Official French Catalogue, the purpose of which is to guarantee users seed that is of sound and fair merchantable quality. Once a new variety has been produced, it must undergo a series of tests to check that it meets the three requirements of distinctness, uniformity and stability (DUS), as well as the requirements of value for cultivation, use and the environment (VCUE). Thus for some species, assessment of cultivation covers yield and growth characteristics, while assessment of use may cover protein and antinutrient content, and environmental assessment may cover resistance to certain pests to reduce pesticide use and resistance to abiotic stresses to reduce use of resources (water, nitrogen, phosphorus, etc.). The VCUE tests are specific to each species.

By way of example, peas will be assessed for seed yield, protein content, antitrypsin factors, 1000 kernel weight, cold resistance (winter peas only) and lodging resistance, while wheat will be assessed for habit of growth, cold resistance, lodging and outgrowth resistance, suitability for early sowing, resistance to certain diseases (brown foot rot, rust (brown and yellow), glume blotch, eyespot), yield, end-use quality, processing characteristics, etc.

7.1.2. EU system specific to GM plants

Directive 2001/18/EC regulates cultivation and placing on the market of genetically modified plants (if not excluded under Annex I B). An assessment of risks to health (allergenicity, toxicity and nutrient

composition) and to the environment (direct and indirect risks) must be carried out.[95] It covers the transgene insertion event and allows authorisation of import or cultivation of plants containing such events whatever their genetic background (see glossary, Appendix VI).

Thus a variety obtained by a conventional breeding technique will be assessed by the Technical Committee for Plant Breeding (CTPS) for registration in the Official French Catalogue, while a variety obtained by genetic engineering will be assessed first according to the criteria laid down by Directive 2001/18/EC and then by the CTPS.

7.2. Discussion of intermediate arrangements

7.2.1. Concept of difference/equivalence leaving aside the trait introduced

Whether for DUS/VCUE assessment by the CTPS or assessment under Directive 2001/18/EC, the bulk of assessment consists in comparison with similar unmodified varieties. However, the concepts of difference and equivalence, leaving aside the new trait itself, are still complex. Which reference plants are the most legitimate? Which exact measurement methods should be used to decide between difference and equivalence: phenotypic tests (including omics tests[96])? What range of genetic, phenotypic and environmental variation should be included in these assessments?

The phenotypic testing carried out by the CTPS, although it cannot be exhaustive, has so far shown itself to be effective in terms of environmental and food safety.

In future, multi-omics data analysis could be another means of deciding on difference or equivalence between two varieties. It might provide a better understanding of unforeseen direct and indirect risks. However, this field is not yet sufficiently developed in terms of standardisation of data, databases and designations to be used as a method of measuring at present. It might eventually supplement current phenotypic testing.

7.2.2. Procedures: A system based on case-by-case appraisal of the need for specific assessment

The Scientific Committee suggests case-by-case appraisal according to the product obtained and the techniques used to obtain it, based on a declaration by the breeder.

Thus the Scientific Committee suggests that the breeder should submit a descriptive document (a brief guide) to the assessment body.

[95] In France the health assessment is carried out by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) and HCB and the environmental assessment by HCB.

[96] Omics tests include tests that can be considered phenotypic: proteomic, metabolomic, high-throughput imaging, etc.

This body will then assess the information enabling plants obtained by NPBTs to be identified and assessed. The vade mecum should be included in the assessment dossier and would initially help to orient assessment of the genetic modification (Figure 5).

This brief guide should provide the following information where applicable:

- Species,
- Identification of event and plant material prior to modification by an NPBT,
- Breeding method,
- Delivery method,
- Tissues targeted by modification,
- Trait(s) modified or introduced,
- Molecular characterisation of event,
- Target region sequences (before and after modification) and chromosome location,
- Presence or absence of components needed to use the technique,
- Phenotypic tests (phenotyping methods for trait modified or introduced),
- Impact on health and the environment,
- Any information that the breeder considers relevant to add.

As a first step, this descriptive document would enable the genetic modification to be classified according to the framework set out below. It is at this stage that it must be determined whether a plant/product has been obtained by SDN-2 or SDN-3, for example. If effectors (components needed to carry out the technique) are present in the plant/product, it would de facto be considered a GMO.

This assessment would also be based on a review of state-of-the-art knowledge about the product, an analysis of its impact in terms of the list of identified risks (e.g. risks/benefits of a modification of the biochemical composition of the plant or harvested plant parts) and study of the metabolic pathways affected by the genetic modification, which would provide a better understanding of the new trait's expected and unforeseen effects. Consequently, guidelines for the applicant should be available, specifying the information required for the final dossier in each case.

The body that will determine the status of the genetic modification on the basis of the traceability document and the state of the art will be able to orient the assessment of the plant in line with the framework shown.

As a second step, for plants/products not coming under assessment procedures laid down by existing regulations, in-depth appraisal through phenotypic testing (Figure 5) would be designed to assess expected direct and indirect risks (to health and the environment) and (costs and) benefits associated with products obtained from the transformed plant and identified in the course of appraisal.

In the case of a *Novel Trait*[97] in a product for food use, the criteria concerning toxicity and nutrient production may be similar to those laid down in the European Commission's novel food regulation[98] (relating to a product's toxicity and any nutritional imbalances resulting from its inclusion in a general diet), which are currently assessed by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES). This health assessment could be complemented by an environmental assessment.

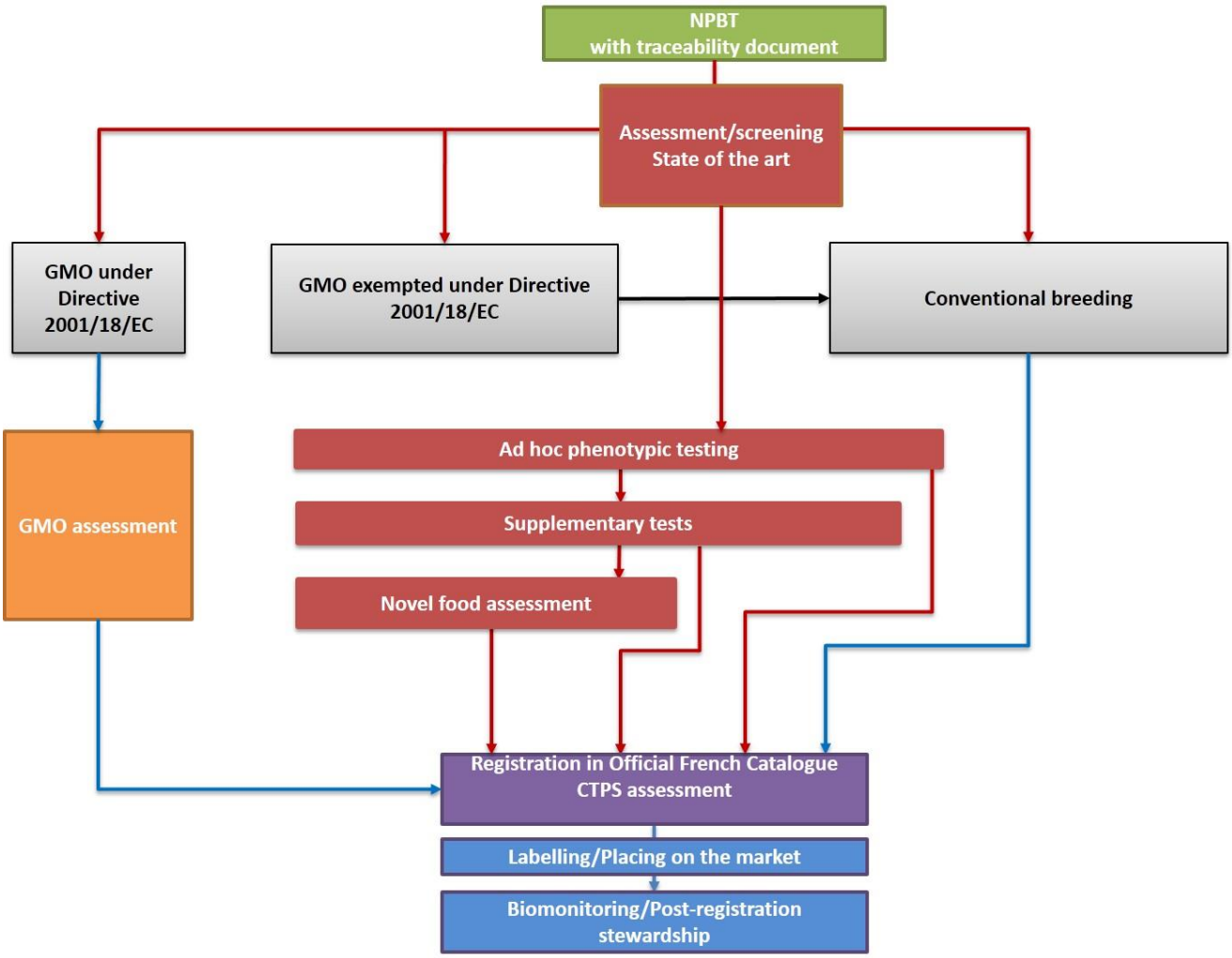

Figure 5: Proposed intermediate options (in red) between the assessment laid down by Directive 2001/18/EC (in grey) and registration in the catalogue (in purple) for assessment and placing on the market of plants obtained by NPBTs.

In some cases, as a third stage, the assessment body might ask for supplementary tests to clarify certain points or for post-registration stewardship measures.

In a spirit of transparency such testing would also be designed to clarify the trait's novelty in order to provide information for professionals and civil society. The novelty might not be associated with a risk,

[97] See Section 1.3 for definition.

[98] http://ec.europa.eu/food/safety/novel_food/index_en.htm

but knowledge of it could affect choices by professionals and civil society as well as the conditions in which the innovation is used.

In every case, registration in the Official French Catalogue would be necessary prior to placing on the market, followed by post-market biomonitoring in certain cases.

7.3. Proposed assessment methods

Novel traits

The Scientific Committee observes that a number of potential risks listed for NPBTs appear to be no different from those associated with what is known as conventional breeding, which would support use of the same assessment process in both cases. A history of safe use should be taken into account to adjust assessments.

Use of mesocosms for assessments prior to field release might be considered where relevant.

Direct risks to human health

These concern, firstly, the potential toxicity of novel components that might be present in the plant and its by-products and their impact in terms of nutritional balance, in combination with dietary exposure, and, secondly, possible allergic phenomena following airborne exposure (pollen), skin exposure (among workers in particular) or food exposure. These risks are common to all new varieties.

The food-safety assessment strategy for NPBTs must take account of the specific features of each of these techniques.

In this respect, the summary table of risks, their distinctive features and associated mitigation methods (Table V.1) provides pointers to two possible sets of standards:

- “Conventional breeding”, for which no particular requirements for food-safety assessment are contemplated, other than the health regulations applying to food in general.
- “Transgenesis”, for which specific provisions are planned, covering the three strands referred to above.

Ten categories of potential risks arising out of unintended effects have been identified:

Effector persistence, vector persistence, breeding method, unintended genome modification, off-target genome modifications due to delivery and regeneration, combination of targeted modifications, pleiotropy, expression of short interfering RNA, dissemination of short interfering RNA, and unintended systemic and multigenerational effects.

Two of these categories (effector persistence and combination of targeted modifications) are confined to SDNs, which must therefore demonstrate absence of effectors.

For two others (vector persistence and breeding method), risks have also been identified concerning transgenesis, and the relevant provisions apply to these NPBTs.

For the risks of short interfering RNA expression and dissemination, the provisions for transgenesis apply to cisgenesis/SDN-3 techniques.

As for risks also identified for conventional breeding or random mutants (off-target genome modifications due to delivery and regeneration; pleiotropy; unintended systemic and multigenerational effects), and for non-specific risks, there would be no need in principle to consider special provisions.

In conclusion, food safety provisions for NPBTs must be based on those for the two ends of the spectrum, which are conventional breeding on the one hand and transgenesis on the other.

Unintended modifications

The HCB Scientific Committee suggests that when using site-directed nucleases, regions identified in silico as having sequences similar to that of the region targeted for modification should be sequenced whenever possible.

Combination of targeted modifications

Biomonitoring and specific testing systems could be used for the varieties concerned. The Scientific Committee recommends studying such applications on a case-by-case basis as and when they occur, since there are no examples of this type of modification to date.

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Annexe I Saisine

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

La ministre de l'Environnement, de l'Energie et de la Mer, chargée des relations internationales sur le climat

Le ministre de l'Agriculture, de l'Agroalimentaire et de la Forêt Porte-parole du Gouvernement

Paris, le 22 février 2016

Madame la Présidente,

Les nouvelles techniques de sélection ou NBT (New Breeding Techniques) comprennent un ensemble de techniques récentes de modification du génome qui mettent en œuvre des processus tels que mutation, réplication, activation ou extinction de gènes, etc. Ces techniques visent à modifier de façon précise et ciblée une séquence génétique.

Certaines techniques ont d'ores et déjà conduit à la mise sur le marché de variétés végétales.

Le développement des NBT soulève plusieurs questions en raison des caractéristiques suivantes :

- certaines ne sont pas détectables dans le produit final obtenu, ce qui pose des interrogations quant à leur traçabilité et au contrôle de leur utilisation, notamment de leur dissémination dans l'environnement,
- elles sont généralement protégées par des brevets,
- elles ne sont pas mentionnées dans les listes de techniques qui définissent le champ de la réglementation sur les OGM et dont la rédaction est antérieure à l'apparition de ces techniques.

Madame Christine NOIVILLE
Haut Conseil des Biotechnologies
244 boulevard Saint-Germain
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De ce fait, il n'est pas aujourd'hui juridiquement établi si l'utilisation de ces techniques de sélection doit ou non respecter le cadre réglementaire tel qu'il a été conçu pour les OGM : évaluation, autorisation, traçabilité et contrôle.

La Commission européenne a, depuis 2007, commandé une série de travaux d'expertise : groupe d'experts ad hoc sur les questions techniques de définition des OGM, avis de l'AESA (Agence Européenne de Sécurité des Aliments) sur l'identification des risques sanitaires et environnementaux et la faisabilité de leur évaluation, rapport du JRC (Joint Research Center) sur les enjeux plus globaux du développement de ces techniques, et enfin une expertise encore en cours sur le plan juridique pour proposer une interprétation de la réglementation. La représentation néerlandaise a annoncé que le sujet figurerait à l'ordre du jour des travaux du Conseil dont elle assure la présidence pour le premier semestre 2016.

Le HCB a déjà rendu un rapport provisoire sur les sujets liés à l'apparition et au développement des NTB le 4 février dernier.

Suite à ce rapport, le Gouvernement dispose d'une première analyse sur les éléments suivants :

- Une synthèse des travaux déjà entrepris au sein du HCB, comprenant une description de ces techniques et des risques éventuels qu'elles présentent pour a santé et l'environnement, ainsi qu'une identification des positions des parties prenantes et des enjeux qui y sont liés.

- Un éclairage du HCB sur les caractéristiques variétales obtenues par ces techniques ;

- L'expertise du HCB sur le statut réglementaire des nouvelles techniques ;

Nous souhaitons désormais que le HCB élargisse son expertise aux éléments suivants, pour les techniques qu'il n'a pas identifiées comme susceptibles d'entrer dans le champ de la directive 2001/18/CE :

1. Méthodes d'analyse et de traçabilité des produits et plantes issus des techniques étudiées ;

2. En lien avec le point précédent, les enjeux pour la coexistence des filières ;

3. Les risques directs pour la santé et l'environnement liés aux caractéristiques nouvelles des produits obtenus ;

4. En lien avec le point 3, les mesures de gestion à mettre en place pour prévenir et limiter les risques pour la santé et l'environnement liés à l'utilisation de produits issus de ces nouvelles techniques, si de tels risques sont mis en évidence ;

5. Les impacts de ces nouvelles techniques sur les capacités d'innovation des acteurs économiques ;

6. Enjeux pour l'accès aux ressources génétiques liés à la propriété industrielle, en lien notamment avec le point 1 ;

7. L'analyse de l'interprétation juridique de la Commission européenne sur le statut réglementaire des nouvelles techniques dès lors qu'elle sera disponible ;

8. En lien avec le point 7 proposer des pistes intermédiaires entre les dispositions du catalogue européen et celles de la directive 2001/18/CE, qui vous paraitraient utiles pour encadrer l'usage de ces nouvelles techniques sur le territoire européen, intégrant votre analyse des enjeux socio-économiques.

Ces travaux seront menés en priorité sur l'année 2016.

Nous vous prions de croire, Madame la Présidente, à l'assurance de notre considération distinguée.

Ségolène ROYAL

Stéphane LE FOLL

Annexe II Lettre de cadrage

Mandat des GT NPBT 2

Le HCB a été saisi le 22 février 2016 par Ségolène Royal et Stéphane le Foll de la question des nouvelles techniques de sélection végétale (NPBT). Il lui est demandé d'élargir son expertise à 8 points, et ce « pour les techniques qu'il n'a pas identifiées comme susceptibles d'entrer dans le champ de la directive 2001/18/CE ».

En préalable, le Bureau du HCB rappelle que la directive 2001/18/CE vise les techniques qui produisent des produits classés comme OGM selon une définition qui n'a pas été actualisée depuis sa rédaction. Cette directive soumet certains de ces produits à différentes règles : évaluations, traçabilité, etc. Elle en exempte d'autres (annexe IB). La directive ayant été rédigée à une époque où les nouvelles techniques de sélection végétale (NPBT) n'existaient pas, un certain nombre de questions se posent :

- les nouvelles techniques produisent-t-elles des produits qualifiables « OGM » selon la définition de la directive 2001/18/CE ?
- dans l'affirmative, ces OGM doivent-ils être soumis à des exigences réglementaires (notamment d'évaluation préalable à leur culture et commercialisation) ou exemptés ?
- si des exigences réglementaires s'imposent, celles prévues par la directive 2001/18/CE sont-elles adaptées aux caractéristiques des différentes NPBT ou de leurs produits ?
- à défaut, quelles autres modalités d'évaluation seraient pertinentes ?

Si la plupart de ces questions relèvent de choix politiques qui n'incombent pas au HCB, il lui revient en revanche de fournir aux autorités compétentes les éléments scientifiques, techniques, économiques, éthiques et sociaux de nature à éclairer leurs choix.

Le Bureau du HCB souhaite rappeler ce que les deux comités ont jusqu'ici produit dans cette perspective et les modalités selon quelles ils devront travailler.

I.

Le Bureau du HCB a demandé au comité scientifique (CS) d'éclairer deux points :

- la qualification des produits qui sont issus de ces techniques : doivent-ils ou non être considérés comme des OGM au sens de la directive 2001/18/CE ?

- les éventuels risques environnementaux et sanitaires liés aux techniques utilisées.

Sur la première question, le CS s'est accordé sur le fait que, à l'exception de certains types de RDdM et de l'agroinfiltration utilisée pour des productions transitoires, ces techniques pourraient entrer dans le champ des techniques donnant lieu à la production d'OGM au sens de la définition de la directive 2001/18/CE.

Sur la deuxième question, le CS du HCB a précisé qu'il ne pouvait identifier de risque intrinsèque aux nouvelles techniques considérées. Les risques éventuels découleraient de l'utilisation qui pourrait être faite de ces techniques et des produits obtenus ou des pratiques associées à leurs caractéristiques. Dans cette logique, le CS a comparé, d'un point de vue biologique, les produits des NPBT avec ceux obtenus par des techniques soumises à la directive 2001/18/CE et avec ceux obtenus par des techniques qui en sont

exemptées (annexe IB). Le CS a conclu que certains produits des NPBT sont biologiquement comparables à des produits exclus du champ d'application de cette directive.

Du côté du CEES, les organisations et personnalités qualifiées ont été invitées à faire état de leurs points de vue sur la question des NPBT et de leurs impacts potentiels aux plan économique, éthique et social. Ces contributions ont été compilées et ont fait l'objet d'une synthèse. Par ailleurs, un travail d'analyse juridique a été conduit par le Secrétariat du HCB, résumant les nombreuses analyses juridiques existantes (en Europe notamment) relatives au statut des NPBT et les deux personnalités du CEES qualifiées au titre de leurs compétences en droit ont, toutes deux, produit des éléments d'analyse détaillés sur cette question.

II.

La saisine ministérielle en date du 22 février 2016 invite le HCB à élargir son expertise à une série de questions. Elle précise que ces questions concernent « les techniques qui n'ont pas été identifiées par le HCB comme susceptibles d'entrer dans le champ de la directive 2001/18 CE ». Comme rappelé plus haut, dans sa note du 4 février 2016, le CS du HCB a énoncé qu'en majorité[1], les produits issus de l'utilisation de ces nouvelles techniques répondent à la définition d'OGM au sens de la directive 2001/18/CE et que certains d'entre eux sont biologiquement comparables aux produits exemptés du champ d'application de cette directive. On rappellera, par ailleurs, que c'est aux pouvoirs publics qu'il revient de trancher la question de savoir s'il faut réglementer ou non les produits issus de ces techniques et, si oui, sur le fondement de quel dispositif juridique. La saisine demande au HCB qu'il fournisse les éléments nécessaires à la construction de cette décision.

Afin de proposer la réponse la plus complète possible, le Bureau du HCB a décidé d'ouvrir cette saisine à l'ensemble des techniques qui ont déjà été traitées lors de la première phase de réflexion. De plus le Bureau souhaite que les groupes de travail discutent aussi de techniques émergentes telles que la biologie de synthèse et d'autres techniques exploitant l'épigénétique.

Cela posé, les groupes de travail du HCB s'interrogeront sur les points suivants (dont le Bureau précise ci-dessous lesquels relèvent de quel(s) comité(s)) :

1. Pour le CS et le CEES : Les méthodes d'analyse et de traçabilité des produits issus des techniques étudiées ;

2. Pour le CS et le CEES : les enjeux pour la coexistence des filières ;

3. Pour le CS : les risques directs pour la santé et l'environnement liés aux caractéristiques nouvelles des produits obtenus ;

4. Pour le CS : si de tels risques sont mis en évidence, les mesures de gestion à mettre en place pour prévenir et limiter les risques pour la santé et l'environnement liés à l'utilisation de produits issus de ces nouvelles techniques ;

[1] Sauf pour le RdDM dans certains cas ainsi que certaines utilisations de l'agroinfiltration.

5. Pour le CEES : les impacts de ces nouvelles techniques sur les capacités d'innovation des acteurs économiques ;

6. Pour le CEES : les enjeux pour l'accès aux ressources génétiques liés à la propriété industrielle, en lien notamment avec le point 1 ;

7. Le point 7 sera traité ultérieurement ;

8. Pour le CS et le CEES : proposer des pistes intermédiaires entre les dispositions du catalogue européen d'inscription des variétés et celles de la directive 2001/18/CE qui paraîtraient utiles pour encadrer l'usage de ces nouvelles techniques sur le territoire européen, intégrant l'analyse des enjeux socio-économiques.

Annexe III Liste des membres du Groupe de Travail

Le rapport du groupe de travail a été élaboré à partir des discussions qui ont eu lieu au sein du groupe de travail du CS du HCB entre le 29 mars et le 15 juin 2016 sous la responsabilité du rapporteur du CS Marie-Bérengère Troadec.

Le groupe de travail du CS du HCB est un groupe pluridisciplinaire composé de personnalités scientifiques choisies pour leur expertise afin de répondre aux questions posées dans la saisine. Par ordre alphabétique des noms de famille, le GT du CS du HCB est compose de :

Frédérique Angevin, Claude Bagnis, Cécile Collonnier, Marie-Anne Félix, Jeanne Garric, Philippe Guerche, Valérie Le Corre, Thierry Orsière, Michel Renard et Marie-Bérengère Troadec.

Le groupe de travail a auditionné deux experts externes ayant une expertise dans le domaine de l'épigénétique et des effets hors-cibles des CRISPR/Cas9 :

Vincent Colot et Jean-Paul Concordet.

Ces deux experts ont été entendus dans le cadre des travaux du groupe de travail mais n'ont pas pris part à la rédaction du rapport ni aux discussions qui ont eu lieu au sein du groupe de travail.

Annexe IV Liste des membres du comité scientifique

Séance plénière du 13 juillet (matin)

présents :

Jean-Christophe Pagès, Président, Claudine Franche, Vice-présidente, Pascal Boireau, Vice-président,

et par ordre alphabétique des noms de famille :

Claude Bagnis, Bruno Chauvel, Denis Couvet, Elie Dassa, Hubert de Verneuil, Joël Guillemain, Guillermina Hernandez-Raquet, Bernard Klonjkowski, Olivier Lemaire, Didier Lereclus, Rémi Maximilien, Nadia Naffakh, Didier Nègre, Sergio Ochatt, Catherine Regnault-Roger, Michel Renard, Patrick Saindrenan, Pascal Simonet, Marie-Bérengère Troadec, Bernard Vaissière, Jean-Luc Vilotte.

- absents, représentés :

Philippe Guerche, Eliane Meurs.

- absents, excusés :

Avner Bar Hen, Marie-Anne Barny, Philippe Berny, Yves Bertheau (démissionnaire), Thierry Brévault, Nathalie Eychenne, André Jestin, Marc Lavielle, Valérie Le Corre, Jean-Louis Noyer, Daniel Parzy.

Séance plénière du 13 juillet (après-midi)

présents :

Jean-Christophe Pagès, Président, Pascal Boireau, Vice-président,

et par ordre alphabétique des noms de famille :

Claude Bagnis, Bruno Chauvel, Denis Couvet, Elie Dassa, Hubert de Verneuil, Joël Guillemain, Guillermina Hernandez-Raquet, Didier Lereclus, Rémi Maximilien, Didier Nègre, Sergio Ochatt, Catherine Regnault-Roger, Michel Renard, Patrick Saindrenan, Pascal Simonet, Marie-Bérengère Troadec, Bernard Vaissière, Jean-Luc Vilotte.

- absents, représentés :

Claudine Franche, Philippe Guerche, Eliane Meurs.

- absents, excusés :

Avner Bar Hen, Marie-Anne Barny, Philippe Berny, Yves Bertheau (démissionnaire), Thierry Brévault, Nathalie Eychenne, André Jestin, Bernard Klonjkowski, Marc Lavielle, Valérie Le Corre, Nadia Naffakh Jean-Louis Noyer, Daniel Parzy.

Séance plénière du 21 septembre (matin)

présents :

Jean-Christophe Pagès, Président, Claudine Franche, Vice-présidente, Pascal Boireau, Vice-président,

et par ordre alphabétique des noms de famille :

Claude Bagnis, Avner Bar Hen, Marie-Anne Barny, Bruno Chauvel, Denis Couvet, Elie Dassa, Hubert de Verneuil, Joël Guillemain, Guillermina Hernandez-Raquet, Bernard Klonjkowski, Marc Lavielle, Valérie

Le Corre, Didier Lereclus, Rémi Maximilien, Eliane Meurs, Nadia Naffakh, Didier Nègre, Jean- Louis Noyer, Sergio Ochatt, Catherine Regnault-Roger, Michel Renard, Patrick Saindrenan, Pascal Simonet, Marie-Bérengère Troadec, Jean-Luc Vilotte.

- absents, représentés :

Thierry Brévault, Philippe Guerche, Olivier Lemaire.

- absents, excusés :

Philippe Berny, Yves Bertheau (démissionnaire), Nathalie Eychenne, André Jestin, Daniel Parzy, Bernard Vaissière.

Séance plénière du 21 septembre (après midi)

- présents :

Jean-Christophe Pagès, Président, Claudine Franche, Vice-présidente,

et par ordre alphabétique des noms de famille :

Claude Bagnis, Marie-Anne Barny, Bruno Chauvel, Denis Couvet, Joël Guillemain, Guillermina Hernandez-Raquet, Bernard Klonjkowski, Olivier Lemaire, Didier Lereclus, Rémi Maximilien, Nadia Naffakh, Jean-Louis Noyer, Sergio Ochatt, Michel Renard, Patrick Saindrenan, Pascal Simonet, Marie-Bérengère Troadec, Jean-Luc Vilotte.

- absents, représentés :

Pascal Boireau, Elie Dassa, Hubert de Verneuil, Philippe Guerche, Eliane Meurs, Catherine Regnault-Roger, Bernard Vaissière.

- absents, excusés :

Avner Bar Hen, Philippe Berny, Yves Bertheau (démissionnaire), Thierry Brévault, Nathalie Eychenne, André Jestin, Marc Lavielle, Valérie Le Corre, Didier Nègre, Daniel Parzy.

Séance plénière du 23 novembre (matin)

- présents :

Jean-Christophe Pagès, Président, Claudine Franche, Vice-présidente, Pascal Boireau, Vice-président et par ordre alphabétique des noms de famille :

Claude Bagnis, Marie-Anne Barny, Thierry Brévault, Denis Couvet, Hubert de Verneuil, Philippe Guerche, Joël Guillemain, Guillermina Hernandez-Raquet, Bernard Klonjkowski, Marc Lavielle, Valérie Le Corre, Olivier Lemaire, Didier Lereclus, Rémi Maximilien, Eliane Meurs, Didier Nègre, Sergio Ochatt, Catherine Regnault-Roger, Patrick Saindrenan, Pascal Simonet, Marie-Bérengère Troadec, Bernard Vaissière, Jean-Luc Vilotte.

- absents, représentés :

Elie Dassa, Nadia Naffakh , Michel Renard.

- absents, excusés :

Avner Bar Hen, Philippe Berny, Yves Bertheau (démissionnaire), Bruno Chauvel, Nathalie Eychenne, André Jestin, Jean-Louis Noyer, Daniel Parzy.

Séance plénière du 23 novembre (après-midi)

- présents :

Jean-Christophe Pagès, Président, Claudine Franche, Vice-présidente

et par ordre alphabétique des noms de famille :

Claude Bagnis, Marie-Anne Barny, Thierry Brévault, Denis Couvet, Hubert de Verneuil, Philippe Guerche, Joël Guillemain, Guillermina Hernandez-Raquet, Bernard Klonjkowski, Marc Lavielle, Valérie Le Corre, Olivier Lemaire, Didier Lereclus, Rémi Maximilien, Eliane Meurs, Didier Nègre, Sergio Ochatt, Catherine Regnault-Roger, Patrick Saindrenan, Pascal Simonet, Marie-Bérengère Troadec, Bernard Vaissière, Jean-Luc Vilotte.

- absents, représentés :

Pascal Boireau, Elie Dassa, Nadia Naffakh, Michel Renard.

- absents, excusés :

Avner Bar Hen, Philippe Berny, Yves Bertheau (démissionnaire), Bruno Chauvel, Nathalie Eychenne, André Jestin, Jean-Louis Noyer, Daniel Parzy.

Séance plénière du 15 décembre (matin)

- présents :

Jean-Christophe Pagès, Président, Claudine Franche, Vice-présidente, Pascal Boireau, Vice-président

et par ordre alphabétique des noms de famille :

Claude Bagnis, Marie-Anne Barny, Thierry Brévault, Bruno Chauvel, Denis Couvet, Elie Dassa, Hubert de Verneuil, Guillermina Hernandez-Raquet, Valérie Le Corre, Olivier Lemaire, Didier Lereclus, Didier Nègre, Sergio Ochatt, Catherine Regnault-Roger, Michel Renard, Pascal Simonet, Marie-Bérengère Troadec, Bernard Vaissière, Jean-Luc Vilotte.

- absents, représentés :

Philippe Guerche, Joël Guillemain, Bernard Klonjkowski, Eliane Meurs, Nadia Naffakh, Jean-Louis Noyer, Patrick Saindrenan.

- absents, excusés :

Avner Bar Hen, Philippe Berny, Yves Bertheau (démissionnaire), Nathalie Eychenne, André Jestin, Marc Lavielle, Rémi Maximilien, Daniel Parzy.

Appendix V Indirect and non-specific risks

Risks associated with traits already obtained by regulated or unregulated techniques

If the modifications induced remain within the limits of variation for the species and its relatives, the risks will be the same as those resulting from conventional breeding.[99]

Among the impacts on agricultural ecosystems already observed for plants obtained by both regulated and unregulated techniques, some were foreseeable and have already been widely recorded: they include circumvention of resistance genes following evolution in the target pathogens (Burdon et al., 2014; Jones et al., 2014). Similarly, a number of populations of target species for varieties expressing a Bt protein are showing resistance (Devos et al., 2013; Tabashnik et al., 2013). Unexpected effects have sometimes been found: a frequently cited example is that of maize varieties carrying the Texas sterility cytoplasm, which have been susceptible to corn leaf blight (Smith et al., 1971; Tatum, 1971).

- Direct risks[100] to human health

These concern, firstly, the **potential toxicity** of novel components that might be present in the plant and its by-products and their impact in terms of **nutritional balance**, in combination with dietary exposure, and, secondly, possible **allergic** phenomena following airborne exposure (pollen), skin exposure (among workers in particular) or food exposure. These risks are common to all new varieties.

- Direct risks to ecosystem health

The first type of risk concerns dispersal and the risk of **invasion** of natural plant communities if a variety's new phenotypic characteristics are likely to increase its ability to persist in fields by forming volunteer populations and to disperse outside cultivated areas (feral populations) (Garnier and Lecomte, 2006; Garnier et al., 2006, 2008), possibly invading natural environments (never recorded for a crop plant to date). This first type of risk may also occur through gene flow by pollen to wild forms of the same species or related wild species (Chèvre et al., 1997) if hybrids have increased persistence and/or invasion capability (here again not recorded to date).

The second type of risk concerns impacts on biodiversity through **modification of ecological and particularly trophic interactions** between the plant and pathogenic, parasitic or consumer organisms, as well as mutualists or symbionts, since these organisms can come from the whole range of biodiversity (bacteria, fungi, plants, insects, other animals, etc.). In the case of traits directly aimed at making a variety pest-resistant or pest-tolerant or intended to improve interaction with symbionts, it is useful to distinguish effects on target organisms from effects on non-target organisms. Direct risks can cover toxic effects on non-target animal consumers, the effect on communities of limiting a target

[99] Although insertion of a sequence encoding a single desired gene using an NPBT may be considered to entail less risk than traditional introgression, through hybridisation and backcrossing, of a region encoding this same gene.

[100] Within the meaning of Directive 2001/18/EC.

species (species replacement), and resistance evolution in the target species in response to evolutionary pressure from a variety.

- Indirect risks[101]

These may arise from changes in farming practice (including those associated with new varieties, such as use of certain active ingredients (Bohan et al., 2005; Bonny, 2016; Lamichhane et al., 2016)) and modification of habitats for wanted or unwanted species owing to the proliferation of "modified" varieties (ease of growth, evolutionary pressure).

Specific case of herbicide tolerance: a collective scientific assessment on breeding of herbicide-tolerant plants is available from the French National Institute for Agronomic Research (INRA).[102] This issue could be considered by any organisation, including HCB, in a dedicated working group**.**

Management of these risks for both regulated techniques (GMOs) and unregulated techniques (conventional breeding, random mutagenesis) necessitates biomonitoring systems that generally make it possible to identify and limit the onset of these types of risk or prevent them from developing.

The potential risks arising out of desired traits are different for each individual trait and are not affected by the technique used to obtain it.

Risks associated with human impact on the pace of change of crop organisms

The power to modify crop species may lead to a faster, human-controlled pace of phenotypic evolution. Site-directed modifications have broken with the rules of biological evolution based on random mutation and recombination events and are followed by natural selection (Vigouroux et al., 2011). Thus human beings could further increase their impact on the living organisms that they grow by comparison with the impact of domestication and selection of random variations. Human beings thus have a not inconsiderable effect on their environment.

However, ever since they were domesticated, crop plants have followed an evolutionary pattern dependent on selection by farmers and breeders (Vigouroux et al., 2011). Thus, until recently, artificial selection of plants by man was not based on any special knowledge of the genes involved. This has changed over past years: random mutagenesis techniques made their appearance some twenty years ago, followed by molecular selection of the desired modification, usually a gene deletion (Mba, 2013; McCallum et al., 2000; Parry et al., 2009; Suprasanna et al., 2015; Tadele et al., 2010).

The past twenty years have also seen the option of high-throughput laboratory screening for a wide combination of random modifications at multiple gene sites or in multiple genes at once, an approach often called "directed evolution" (even though it uses laboratory selection and randomness). An alternative approach is rational design (Castle, 2004; Dixon et al., 2003; Rao, 2008; Yuan et al., 2005). In both cases, laboratory improvement of a protein or metabolic pathway is followed by its introduction into the organism of interest by site-directed mutagenesis or transgenesis.

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[101] Within the meaning of Directive 2001/18/EC.

[102] https://www6.paris.inra.fr/depe/Projets/Varietes-Vegetales-Tolerantes-aux-Herbicides (summary available in English).

Red Queen effect on biodiversity

Change in evolutionary parameters, such as the rate of phenotypic change, could lead to qualitative changes in interaction between species and have far-reaching implications for ecosystems, depending on the extent to which these plants are adopted.

The overall effects on biodiversity are hard to predict, since they depend in large measure on the degree of environmental exposure (adoption, and changes in cropping patterns, as is already the case). However, mention may be made of:

- Greater selection pressure on target pests (pathogens, insects) with faster widespread deployment of resistance genes in crop varieties. The outcome of such an increase in selection pressure is uncertain. In theory, while NPBTs allow simultaneous accumulation of multiple major and/or quantitative resistance genes in plant varieties, pests' ability to evolve in response to this new selection pressure should decline (Burdon et al., 2014; Jones et al., 2014). However, inappropriate deployment of resistance gene combinations could have the opposite effect. Other evolutionary responses may also occur, such as changes in host range, with pests becoming harmful for new species, a development also found in non-GM crops (Stukenbrock and McDonald, 2008).
- Extension of farming to new environments with introduction of plant varieties tolerant to extreme conditions such as drought and salinity and with more land under cultivation if plant traits allow new uses such as chemurgy (biofuels, polymers, therapeutic molecules, etc.).[103] An increase in farmland is the primary cause of biodiversity decline (Newbold et al., 2015), since uncultivated ecosystems are being replaced by agricultural ecosystems. It should, however, be noted that positive impacts may also be expected (for example, more carbon storage in soil, leading to lower emissions of some greenhouse gases, or reduced use of fossil fuels).

The HCB Scientific Committee notes that most breeding methods, whether NPBT or conventional, may show bias owing to the sampling used for reseeding (Vigouroux et al., 2011), which would require corrective action.

The HCB Scientific Committee further notes that issues relating to deliberate modification of non-crop plants (such as ragweed to make it less allergenic) should be discussed in a different context.

Risks associated with any persistence of delivery tools

For use of SDNs, RdDM, negative segregants, cisgenesis and intragenesis, a number of methods for introducing reactants into a cell (delivery) are possible (see Section 2.3). Up to now, these methods have concerned only "conventional" GMOs. Some countries such as the United States authorise

[103] http://institut.inra.fr/en/Objectives/Informing-public-policy/Foresight/All-the-news/Agrimonde-Terra-foresight-study

genetically modified plants on the basis of delivery method,[104] for example regulating use of bacteria or viruses in a specific way.

For *Agrobacterium* delivery and agro-infiltration there is a risk that the bacteria carrying modified T-DNA may propagate in the environment with sequences of other bacteria or eukaryotic sequences. Such release is itself subject to regulation and must therefore be verified in contained use for SDNs, RdDM, negative segregants, cisgenesis and intragenesis. The bacteria can be eliminated by appropriate antibiotic treatment, but it must be ensured that they are actually absent before any uncontained use of these plants.[105] This requires the type of delivery and the absence of bacteria release during the process and in the plants then marketed to be documented.

For delivery through an autonomously replicating episome (virus sequences, for example), this autonomous replication may persist. Most of the virus sequences used do not usually enter the gametes (see Section 2.6.1), but in the absence of sufficient information, this must be verified.

In the case of biolistics, use of protoplasts and plant regeneration from a cell, a number of unwanted genetic and epigenetic modifications are inevitable (see off-target effects).

Risks associated with modification selection methods

For cisgenesis, intragenesis, transgenesis, SDN and RdDM techniques there are additional risks relating to persistence of selectable markers (often transgenes from other species; see Section 2.4). It must be ensured that these selectable markers have been removed and their removal has not caused damage. If the selectable markers remain, the plants are then *de facto* GMOs according to the regulations and must be assessed as such.

Risks associated with short interfering RNA expression in the plant

One subcategory of cisgenesis (cisgenesis of a micro-RNA gene, for example) and of intragenesis consists in expression of a micro-RNA, an antisense RNA or a double-stranded RNA interfering with gene expression through various mechanisms (Auer and Frederick, 2009). In this case, short interfering RNA can cause off-target effects for modification of genetic expression (Molesini et al., 2012; Xu et al., 2006). Since plants have amplification enzymes for short RNA, the latter can be amplified in the plant and propagate in different cells.

The specificity of short RNA measuring around 20-25 base pairs is low (Xu et al., 2006), especially in the case of micro-RNA, which can suppress targets with mismatches of several base pairs (Molesini et al., 2012). This can affect expression of a number of genes and therefore could in theory affect the plant's phenotype, such as its dispersal capacity or metabolite/toxin production, if the plant contains such genes. Plants express a number of micro-RNAs that might have this type of effect through

[104] https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/SA_Regulations

[105] However, the Scientific Committee notes that at present most of these plants are for contained use.

mutation. To date there have been no cases of toxin induction through mutation of a micro-RNA as far as the Scientific committee of the HCB is aware.

Risks associated with release of short interfering RNA into the environment

In the case of intragenesis consisting in expression of short interfering RNAs, double-stranded RNAs may be released into the environment and affect other organisms, since some of the latter (nematodes, arthropods, eukaryotes, single-cell organisms, etc.) may import double-stranded RNAs from the environment.[106] Double-stranded RNAs could thus reach and affect other non-target organisms (Ramesh, 2013).

These off-target effects are, however, hard to predict, and it would be impossible to test all the organisms that might be found in agricultural conditions.

Risks associated with unintended systematic and multigenerational effects

RdDM aims to produce a multigenerational effect that is not strictly genetic (DNA methylation), but effects on other loci can also occur unintentionally. As above, this can affect expression of a number of genes.

In the case of grafts, these unintended effects may occur through systemic propagation of short interfering RNA in the plant.

In the case of negative segregants, unintended multigenerational effects may occur, particularly with DNA methylation. This type of effect is also to be found in interspecific hybridisation, and even in conventional hybridisation within the same species.

The Scientific Committee notes that a risk of the same nature theoretically exists with hybridisation in general, although it has not been recorded.

Genome- and environment-dependent risks

In the same way that a combination of two genetic modifications can result in unforeseen phenotypes, each modification interacts with the genetic background and the environment. Modification of trait expression under the influence of environmental conditions is taken into account when testing new varieties in different environmental conditions, but this cannot cover the whole range of possible conditions. For the time being, use of models to complement or replace testing is not possible, since statistical models for “genotype x environment” interaction or including genomic covariates are more explanatory than predictive (Gauffreteau et al., 2016; Gauffreteau, A. et al., 2015; Heslot et al., 2014).

[106] This phenomenon is used in particular to target a gene in a pathogen outside the plant, such as an insect (Younis et al., 2014). This targeting of another organism employs a foreign transgene (conventional GMO) and therefore does not come within the category of intragenesis.

Modification as a result of the genomic context would be relevant if a genetic modification was authorised irrespective of its genetic background (the rest of the genome). Introduction of the same modification into a different genetic background in the same species can have different effects on the phenotype and its variability, and all the more so in another species (Chandler et al., 2013; Hall et al., 2007; Roles et al., 2016).

Risks associated with off-target genome modifications due to delivery and regeneration

- Unintended genome modifications due to use of protoplasts, *Agrobacterium* for transfection, and/or regeneration

In the case of biolistics, use of protoplasts and plant regeneration from a cell, unwanted genetic and epigenetic modifications (some inheritable) are frequent, including alterations in chromosome dosage (Bairu et al., 2011; van den Bulk et al., 1990; Jiang et al., 2011; Kaeppler et al., 1990; Machczyńska et al., 2015; Ong-Abdullah et al., 2015; Rhee et al., 2010; Stelpflug et al., 2014). Moreover, the whole plant is regenerated from undifferentiated cells through clonal propagation of varieties obtained by conventional breeding. How a genotype can result in a variety of phenotypic outcomes under the same culture conditions is still not properly understood (Krishna et al., 2016), but since some causes are known, action can be taken to reduce their impact on regenerated plants (Bairu et al., 2011). This includes i) choice of a genotype free of variations or *in vivo* mutations and one that is associated with regeneration by embryogenesis rather than organogenesis (if available), ii) fewer passages between culturing of explants and regeneration, iii) use of the lowest possible growth-regulator concentrations to shorten the growth phase of undifferentiated tissues prior to regeneration as much as possible and promote better cell-cycle control, and iv) prevention of action of known effectors of oxidative stress responsible for breaks in DNA and risks of DNA hyper- and hypomethylation. *Agrobacterium* transformation may also occasionally produce unintended modifications, for example through insertion of bacterial chromosomal DNA (Ülker et al., 2008) or breaks in T-DNA (Schouten and Jacobsen, 2007).

A paper has shown that when plant protoplasts were transfected with a plasmid encoding both the nuclease and guide RNA, in 0.06% to 0.14% of cases, small insertions of DNA from the plasmid would occur at the cleavage site (Kim and Kim, 2016).

In the case of **agro-infiltration**, unwanted integration of transgenes may cause off-target effects of the same nature.

The Scientific Committee would nevertheless point out that generation of transgenic plants using these delivery methods has been taking place for twenty years, and **it is not aware that any plants placed on the market have shown a problem associated with these methods.**

- Unintended genome modifications due to integration site in the case of SDN-3, cisgenesis and intragenesis (together with conventional GMOs)

In the case of **cisgenesis** and **intragenesis**, insertion of the transgene into the genome may have uncontrolled effects, such as interruption of an endogenous gene at the locus, formation of new coding sequences or deregulation of gene expression at the integration site or other loci. In the case of

cisgenesis, although the transgene is usually expected to be transcribed intact, insertion may produce knock-out or combination with the endogenous locus where the insertion occurs, which may lead to abnormal transgene expression with unforeseen regulatory sequences and production of an abnormal fusion transcript or even an antisense transcript, which would then amount to intragenesis.

As for SDN-3, advances in plant genomics will probably make it possible to identify “safe harbour loci”, i.e. sites that are in non-coding regions and possess high, stable gene expression, which could be targeted for transgene insertion (Cantos et al., 2014).

Risks associated with pleiotropy

Gene modification (SDNs, ODM and RdDM) and transgene insertion (intragenesis and cisgenesis) can affect traits other than those associated with the organism’s desired phenotype (pleiotropy). Pleiotropic effects (i.e. affecting plant traits other than the desired trait, although only the target gene has been modified) can result from targeted genetic modifications. These effects exist whatever the plant breeding method used (conventional breeding, random mutagenesis, etc.). It is difficult to test all these methods and anticipate them in field conditions. Some may be identified by the breeder in the course of the breeding process or when the crop is grown. Such modifications might not have been detected in the laboratory during phenotypic testing of varieties if they altered the level of certain components, for example, or susceptibility to particular pathogens. In some cases it is conceivable that gene control or silencing through SDNs, ODM or grafting may activate synthesis of more or less undesirable or even toxic products that were not synthesised in the crop variety originally. However, there are no known examples.

Risks associated with new ecological interactions

These new ecological interactions cover plants, animals and microorganisms. They may lead to a decline in, and even extinction of, some species, and conversely a proliferation of others, and consequently to changes in the composition of the communities to which these species belong. However, these species and communities have already been exposed to the effects of natural mutations and have had the opportunity to adapt. Natural mutations give rise to ecological interactions that are part of the evolution of ecosystems.

The new risks that may result concern health and the environment. Various types of new ecological interactions must be considered:

- Interactions with different human microbiota, particularly in the gut, in relation to proper functioning of the immune system (O’Doherty et al., 2014), and the various dysbioses that may arise.
- Interactions with the different microbiota of plants and the microbiota of the animals that eat these plants.
- Interactions with soil microorganisms. The latter play a major part in the functioning of ecosystems – soil fertility, carbon storage and water and air purification – as well as in the health of living organisms, particularly human beings.
- Interactions with other species, particularly those playing a key role in the functioning of ecosystems, such as pollinators.

These “new ecological interactions” can have a direct impact on human beings without the intermediary of other species. New proteins produced may, for example, result in allergenicity (Traidl-Hoffmann et al., 2009) or a reduction or increase in components beneficial to human health (vitamins, antioxidants, polyunsaturated fatty acids, etc.).

The *Novel Traits* obtained necessitate a risk assessment for human beings and/or ecosystems on a case-by-case basis, with assessment systems that include the impact on human and non-human microbiota.

Appendix VI Glossary

For reasons of consistency the HCB Scientific Committee has chosen to give certain terms that might be used in other contexts a specific meaning that is explained in this glossary. To avoid any confusion, the Scientific Committee would like to clarify that this glossary applies only to this opinion.

Effectors: Molecules (proteins or nucleic acids (RNA or DNA)) used to obtain the desired modification in the plant.

Vectors: In this definition a vector is the tool for transferring a gene. Vectors are needed because genetic information is not easily transferable into a cell.

A vector consists of two types of molecule:

- The molecule to be transferred: this carries the genetic information, whether as RNA or DNA. The same vector can transfer a number of molecules, including different types of molecule.
- The complex of molecules that makes the transfer possible. For plants, this complex is usually viral or bacterial. It contains microorganisms that have been modified to transfer genetic information. The modification of the parent organism that becomes the vector is intended to eliminate the pathogenicity of the original microorganism. The vector is easy to handle *in vitro* and will be the vehicle for transfer.

During or after transfer the second vector component is usually removed from the cell in which the genetic information has been inserted.

Some vectors are called "inert" because they consist of synthetic molecules whose physicochemical capacities allow the genetic material to be inserted into the cell. Methods of transfer also exist that are mainly physical (electroporation, ultrasound, etc.).

Choice of vector depends on the cell into which the genetic information is to be transferred, taking account of its efficiency and persistency.

Proteins can also be delivered in vector form, usually by physical methods. Use of a vector, whatever its nature, results in a transient modification in the target cell.

Novel trait: A new characteristic arising out of the insertion of one or more genes or from modification of the expression of one or more genes in the organism in question. The Scientific Committee draws a distinction between two types of novelty:

- Introduction into a variety of a trait identified in another variety or another related or sexually compatible species: the idea is to enhance existing genetic diversity by introducing allelic states of interest. Consequently, there is no addition[107] of genetic sequences or any modification of the function of the genes already present in the plant.

[107] It is important to clarify the concept of addition for cisgenesis and intragenesis. Genetic material may be introduced, but the genes inserted already exist in the species in a different allelic state or are present in certain varieties of the same species.

- Introduction of an entirely new trait into a variety and/or related species:[108] the novel trait stems from the fact that the gene is not naturally present in the species in question or the modification of an existing gene introduces a new metabolic pathway or new function into the species.

Biomonitoring introduced by the 1999 Outline Farming Act: The purpose of the latter was to identify and monitor any emergence of unintended effects of new GM varieties on:

- Pest populations,
- Wild flora and fauna,
- Aquatic environments,
- Microbial populations (including viruses).

In fact, some unintended effects might emerge only after large-scale cultivation and over a number of years (e.g. onset of resistance in target insects, emergence of resistant weeds). But these are issues affecting any varietal innovation and are not specific to products obtained by NPBTs. Post-market monitoring procedures were set out by EFSA in 2006 and updated in 2011 (EFSA, 2011). They were tested for assessment during the EU AMIGA project.[109] The results of this project will support thinking on how to establish large-scale monitoring. This could also be organised around existing monitoring and surveillance networks (EFSA, 2014; Reboud et al., 2013). Moreover, there already exists a national biological monitoring network for plants, responsible for monitoring unintended effects of plant protection practices (coordinated by the Plant Protection and Quality Subdirectorate of the Directorate General for Food, cf. Theme 3 of the Ecophyto II plan published in October 2015), and a national network for epidemiological crop surveillance.

Directive 2001/18/EC: Directive 2001/18/EC regulates cultivation and placing on the market of GM plants (if not excluded under Annex I B). It covers the transgene insertion event and allows authorisation of import or cultivation of plants containing such events whatever their genetic background.

An application for authorisation (import for food or feed uses or for cultivation) of a genetically modified plant must include a full description (laid down in Annex III B of Directive 2001/18/EC) of the plant and its transformation together with information about its cultivation (taxonomic identification, reproduction, compatibility with other cultivated or wild plant species, distribution in Europe of compatible species, survivability, dissemination associated with pollen quality and viability,

[108] This distinction is highlighted in Canada's GMO rules, which provide for assessment only in the case of varieties having a new trait not previously present in the variety or related species (http://www.hc-sc.gc.ca/fn-an/gmf-agm/index-eng.php).

[109] AMIGA, **A**ssessing and **M**onitoring the **I**mpacts of **G**enetically Modified Plants on **A**gro-ecosystems (FP7, 2011 - 2016): http://www.amigaproject.eu/

mechanisms of interaction with target and non-target organisms, site management after cultivation, etc.).

An assessment of risks to health (allergenicity, toxicity and nutrient composition) and to the environment (direct and indirect risks, immediate or delayed effects, as well as cumulative long-term effects) must be carried out. Environmental risks are assessed by comparing the genetically modified plant (GMP) with an untransformed organism. Annex II of the Directive specifies the adverse effects that must be considered. Applications have to contain information on: (i) likelihood of persistence and invasiveness, including gene flow between plants; (ii) potential plant-to-microorganism gene transfer; (iii) interactions between the GMP and target organisms, and their consequences; (iv) interactions between the GMP and non-target organisms; (v) effects on biogeochemical processes. Routes of exposure must also be considered. Last but not least, a risk management strategy must be proposed. The application must outline post-market monitoring built around the environmental monitoring plan with scenarios for various critical situations. This monitoring will have two strands: case-specific monitoring for foreseeable effects (e.g. onset of resistance in target insects) and general surveillance, i.e. with no prior assumptions (Regnault-Roger, 2014).

Mesocosm: A mesocosm is a controlled or semi-controlled contained environment where an investigator can vary some or all of its parameters over a significant period of time and thus reveal a large number of ecological effects on interacting species, particularly microorganisms, that would otherwise have gone unnoticed. The trial must last long enough for ecological interactions and feedback to develop. Use of mesocosms should be considered whenever relevant to assessment prior to field release,. This type of study, preceding field trials, could help uncover changes in the functioning of microbial and animal soil communities and analyse their causes (De Vries et al., 2015; Wolfarth et al., 2016) as well as analyse the effects of global change at small spatial scales (Stewart et al., 2013).

Mesocosm studies would make it possible, for example, to assess the impact of a novel trait on microbiota diversity in the light of current knowledge concerning microbial ecology.