Genome editing

Overview of the New Genomic Techniques and their applications.

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Abstract

The New Genomic Techniques (NGTs) have spread widely in the last 20 years, finding application in various sectors ranging from medical, pharmaceutical, industrial and agri-food. Applied to agri-food these tools can contribute to the transition to a more equitable and environmentally friendly food system.
Several agro-industrial giants are invested in genome editing, prompting countries worldwide, concerned about climate changes, to revise their polices.
This white paper provides an overview of NGTs and their applications and outlines the changing regulatory frame-work in the various countries, and finally offering a traceability method for this new OGM.

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TÜV SÜD's expert

Monica Filippini

Head Service Line Life Science - pH s.r.l. – TÜV SÜD Goup

Monica Filippini has a degree in Biology and a Ph.D. in clinical pathophysiology.
She has been in charge of the biomolecular laboratory since 2000, the year the first GMO food analyses began. Monica has developed analytical methods for the detection and quantification of GMOs, allergens, pathogens and animal species. In 2014 she became head of TÜV SÜD "pH Labs" microbiological laboratory, and since 2020 she has been responsible for testing the biocompatibility of medical devices.

Introduction

Since ancient times, farmers have sought to improve the yield and quality of their crops through crossbreeding and selection. Thanks to Mendel discoveries on hereditability, the breeding process became science based. In the 1900s, when the Mendel theory was further understood, various breeding technologies, such as chemical and physical mutagenesis, were developed.
Discoveries in molecular biology tools such as restriction enzymes, DNA sequencing, DNA cloning, molecular marker-assisted trait selection, led to the birth of "genetic engineering", using recombinant DNA technology to enlarge the gene pool available to breeders (Perez de Castro et al, 2012, Lanigan et al, 2020). These technologies are widely used to develop biofortified crops and plants varieties with high yield and resistance to insect pests and diseases: around 40 billion hectares worldwide have been allocated to the cultivation of transgenic plants, which have been commercialized since the biosafety assessment in 1999 (Ahmar et al, 2020).

In the last 20 years, the development of breeding techniques has progressed rapidly, leading to the establishment of new methods to create organisms with novel traits (Broothaerts et al, 2021). Therefore, together with conventional genetically modified organisms, other improved, new organisms have appeared on the scene: the Genome Edited Organisms, organisms that were created through the development of new platforms that, unlike the established genomic techniques, have the common features of being able to induce targeted mutation without leaving any imprint in the host genome: transgene is used only in intermediate breeding, and then selected for removal (Lusser et al, 2012). The combination of these techniques is called Genome Editing and is part of the new breeding techniques (NBT).
Conventional genomic techniques lead to the production of a random mutation in the host organisms, which requires a lengthy process of selecting the desired mutation.
New platforms have been developed that can act like molecular scissors, inducing the required mutation in a chosen genomic region: the NGTs. Among the different platforms available, CRISPR is by far the preferred one, thanks to the ease of use and because it is time saving and inexpensive. CRISPR has been likened to a multifunctional Swiss army knife, equipped with a compass to locate the right spot, a vice for gripping DNA and shears for cutting it.

Genome Edited Organisms were created through the development of new platforms that have the common features of being able to induce targeted mutation and that do not leave any imprint in the host genome

The Genome Editing regulations

With this in mind, how should Genetically Edited Organisms be defined? Could they be considered as conventional GMOs or not? Most of the countries in the world have based their GMO legal definition on the Cartagena Protocol on Biosafety and its definition of a Living Modified Organism (LMO) as "any living organism that possesses a novel combination of genetic material obtained using modern biotechnology'' (Secretariat of the Convention on Biological Diversity 2000).
As regarding the definition of Genome Edited Organisms, we should understand that Genome Editing encompasses several distinct types of alterations generating different products, introducing new challenges in regulatory distinctions and traceability.
The search for a legislation definition for these genetically edited organisms, should consider that the induced mutation could be similar to a mutation generated by conventional mutagenesis, or also found in nature; some of the tools involved do not lead to the introduction of genetic material that is foreign to the organism (SDN-1, SDN-2, ODM) or that is, in any case, eliminated (Aglawe et al. 2017, Metje-Sprink et al. 2019,). On the other hand, the various genomic editing techniques are not free from the presence of off-targets, which can lead to unknown nucleotide changes, which could be translated in unknown phenotypes.
Again, if donor sequences are used as template; the cell introduces a foreign gene or DNA sequence that may not arise by natural recombination or inbreeding, therefore, this new sequence may have unexpected negative consequences for the environment or human health (Aglawe et al. 2017, Metje-Sprink et al. 2019).
The increasingly common use of these tools by breeders has prompted countries around the world to revise their regulations, so that they can be applied to these new products improved by genome editing.
The regulatory approaches of Genome edited organisms differ across the world: some countries have focused on the product obtained and the risks it may pose, while other countries have focused on the process used to obtain that product, with the sole common aim of protecting human and animal health and the environment.
The United States policy is product-based, as the process used for the genetic improvement of an organism is not considered harmful per se, as set out in the 1986 Coordinated Framework for Regulation of Biotechnology and its subsequent update, published in 2017 (Sprink et al.2016, Entine et al. 2021,).
In the USA, the revision of their biotechnology regulations, now called SECURE (Sustainable, Ecological, Consistent, Uniform, Responsible, Efficient) stipulates that certain class of GEOs do not fall under the regulation for GMOs. Exempt GEOs must meet certain criteria: the induced mutation must be the result of an internal cell repair mechanism, without any repair templates being introduced; it must be achieved by targeted single base substitution or the introduction of

a gene from the plant's gene pool, or it must make modifications in the targeted sequence to correspond to a known allele of such a gene or to a known structural variation present in the gene pool (Hoffman 2021).
The US approach is also found in Australia, which gave notice in 2019 of the "Gene Technology Amendment", stating that organisms obtained from SDN-1, i.e. in which no novel combination of genetic materials was introduced, are excluded from the definition of GMOs provided by the Gene Technology Act 2000, effectively making the regulation of genome editing product-based, despite the traditional regulation of GMOs being process-based (Jenkins et al. 2021, Tsuda et al. 2019, Metje-Sprink et al. 2020).
Argentina's approach is also product-based: in 2015 this Country issued its own regulation, stating that GEOs must be defined GMO or not according to a product-by-product analysis.
Products that are not in the scope of GMO regulations remain subject to the same laws and regulations as for plant-developing through conventional breeding (Whelan and Lema, 2015).
Brazil and Chile have also followed Argentina's footsteps, stating that GEOs not containing foreign DNA are not considered as GMOs (Tsuda et al 2019, Entine et al. 2021).
Canada has presented a case-by-case risk assessment taking into account only the new trait obtained in the organism, by assessing "novelty" in plants and derived food, without taking into account how this novelty was pursued: the Plant with a Novel Traits regulations (PNTs) (Smyth and McHughen, 2008), where a Plant with a Novel Trait is ''a plant that contains a trait which is both new to the Canadian environment and has the potential to affect the specific use and safety of the plant with respect to the environment and human health.
These traits can be introduced using biotechnology, mutagenesis, or conventional breeding techniques" (Canadian Food Inspection Agency). With this product-based focus, Canadian agencies have been challenged to contemplate how to address novelty in the context of techniques like genome editing that may not create novel genetic combinations (Jenkins et al. 2021).
In February 2019, Japan ruled that certain GEOs must be subject to the "Act on Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms" (known as Cartagena Act), while others should be exempt, more specifically those organisms obtained through SDN-1, as they are considered similar to those produced by conventional breeding technologies and through SDN-2, if they do not possess inserted extracellularly-processed nucleic acid (Tsuda et al. 2019).

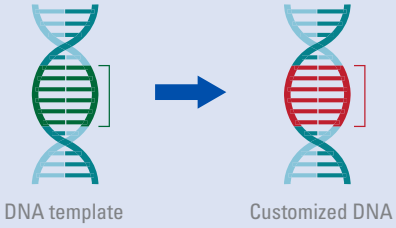
Genome Editing encompasses several distinct types of alterations generating different products, introducing new challenges in regulatory distinctions and to traceability

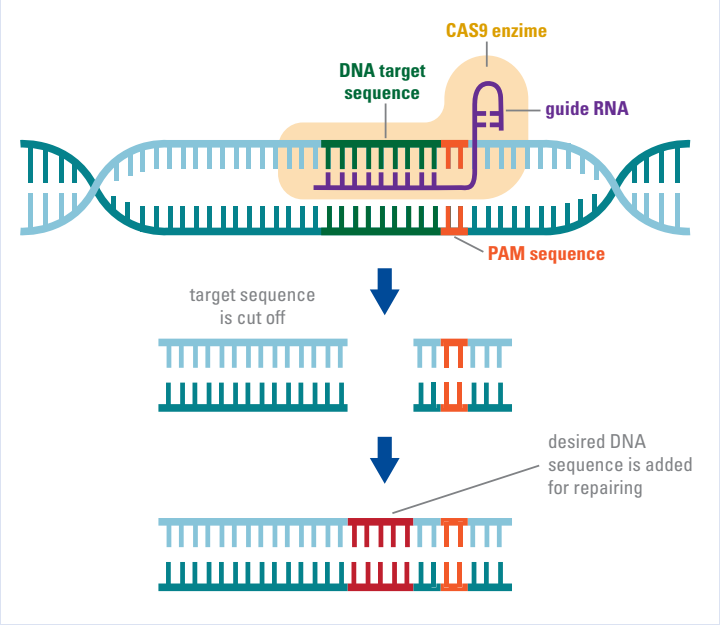
A regulation that focuses on the process is found in New Zealand, which initially excluded GEOs from the Hazardous Substances and New Organisms (HSNO) Act 1996, which represents the regulatory framework for GMOs. Here a GMO is defined as any organism in which the genes or genetic material have been modified by in vitro techniques. In fact, in 2013, the Environmental Protection Authority (EPA) concluded that the non- transgenic gene editing approach was more like chemical mutagenesis, which is why the organisms thus obtained had to be included within the HSNO regulations exception.
The EPA decision was appealed by the Sustainability Council of New Zealand in the High Court and the case ended up with GEOs being considered as GMOs, because the Court decided that Genome Editing tools were excluded from the list of techniques listed in the HSNO (Organisms Not Genetically Modified) Regulations of 1998, that was considered as a closed list; so, adding them to the exceptions list was a political decision, not an administrative one (Fritsche et al. 2019).
In Russia, a legislative plan for genome editing has yet to be determined; as for traditional GMOs, the regulation in Russia is process-based, (as it is almost throughout Europe). What was hoped was that GEOs would be excluded from the restrictive regulation in force for GMOs (Bogatyreva et al. 2021), also because a federal program aimed at creating 10 new varieties of gene-edited crops and animals by 2020 and other 20 by 2027, with a view to their commercialization (Dobrovidova 2019).

Together with Russia, also other countries, including some important trading countries such as China, are discussing what path to take in the area of gene-editing regulation. (Entine et al. 2021, Jenkins et al. 2021).

How does CRSPR-Cas9 work?

- Adapted from defense mechanism against virus of bacteria
- Cas9 is an RNA-guided enzyme that can cut DNA sequences in a target way
- A desired genetic sequence could be added for repairing the system to customize DNA





The European case

In Europe, the issue of regulating genetically engineered organisms was raised in 2014, when the Finnish competent authority asked the Commission's view on the regulatory status of the oligonucleotide mutagenesis (ODM) techniques (Ministry of social affairs and health, Board for Gene Technology 2014). The opening of the debate led to the emergence of two factions. On one hand, there was the German Federal Agency for Consumer Protection and Food Safety (BVL) and other competent authorities pushing for a product-based reading of Directive 2001/18/EC (BVL, 2015). An immediate response to this notification was issued in 2015 by Non-Governmental Organizations, which in a letter to the Commissioner for Health & Food Safety demanded that European laws continue to operate according to the precautionary principle of transparency and traceability, by reading directive 2001/18/EC under a process point of view, and that "All non-traditional breeding processes that change the structure of DNA using genetic engineering technologies, or interfere with gene regulation, fall within the scope of these GM regulations" (NGO coalition, 2015). It is in this divided scenario that the European Court of Justice (ECJ) decided that organisms obtained using new breeding techniques should also be considered as GMOs (European Court of Justice, 2018).

In 2019, the Council of European Union asked the Commission to conduct a study regarding the status of new genomic techniques under the Union law, following the ruling issued by the European Court of Justice in the Case C-528/16 (Council Decision EU, 2019/1904). The study covers various consultations that highlight the views of Member States, stakeholders and expert opinions, such as the EFSA and the Commission's Joint Research Center (EFSA et al, 2021, Broothaerts et al, 2021). The study ended on 29 April 2021, and what emerges from it is that NGTs are valuable weapons that can contribute to the sustainability of food systems, in accordance with the objectives of the "European Green Deal" and "Farm to Fork" Strategy, as well as to a more competitive economy. But along with these advantages, concerns were raised regarding above all safety and environmental impacts, e.g. on biodiversity, coexistence with organic and GMO-free agriculture, and labelling, thus affecting consumers' freedom of choice and their right to be informed (European Commission, 2021). The NGT need a new regulatory system that has to be adapted to scientific and technological progress, so that the full potential of these new tools can be utilized.

The New Genomic Techniques (NGTs)

The New Genomic Techniques (NGTs) are defined as techniques capable to change the genetic material of an organism and that have emerged or have been developed since 2001, when the EU Directive 2001/18/EC on GMO was adopted (van der Vlugt, 2021, Broothaerts et al, 2021).
The main feature of NGTs tools is that they succeed in making directed genetic manipulation. This means that they can surgically modify a specific and chosen target sequence. Organisms produced via NGTs lack of foreign DNA integration: the transgenes are used in intermediate breeding and then removed via subsequent backcrossing and selection steps (Lusser et al, 2012).
The conventional GMO, instead, contain foreign promoters such as the commonly used cauliflower mosaic virus (CaMV), and terminators (P-35S or T-35S) (Grohmann et al, 2019).
In the past decades, these new types of genomic techniques have been largely used in agri-food and medical, pharmaceutical, and industrial sectors. In agri-food, the NGTs are used to improve traits such as biotic and abiotic stress tolerance, plant yield and architecture, modified content of substances, and herbicide tolerance (Fig.4) (Parisi et Rodríguez-Cerezo, 2021).

Main traits modified via NGT platforms in plants

Biotic stress tolerance Resistance to biotic stressors such as nematodes, fungi, bacteria, viruses and other pests, pathogens or parasites	**Abiotic stress tolerance** Resistance to abiotic stressors such as drought, heat salt, rain or ultraviolet radiation	**Herbicide tolerance** Tolerance to various types of herbicides
Modified colour or flavour Modified colour/flavour	**Modified composition** Modified content of substances such as starch, oil, proteins, vitamins, fibres, toxic substances, allergens etc. to improve food/feed quality or for a better industrial use (includes seedless fruits as a quality characteristic)	**Plant yield and architecture** Yield increase (or yield stability) related to higher number of flowers/ seeds/fruits, to fruit size/weight or to photosynthetic efficiency. Includes other changes in plant architecture like plant height and shape, fruit shape and growth pattern
Storage performance Improvement of characteristics such as shelf life and tolerance of storage conditions (e.g. cold storage), including non-browning and reduced black spot	**Breeding tools** Reproductive/flowering characteristic inducing induction of sterility, early flowering and haploid techniques	**Other traits** Trait not classified in the above categories, including production of molecules of industrial interest, flowering time for agronomic purposes and nitrogen use

Parisi et Rodríguez-Cerezo, 2021

NGTs can be divided into four groups based on the way the active components of the tools act on the host genome.
The more widespread tools are the ones belonging to the first group, and the most used platform is CRISPR, which that can produce a point mutation
or a deletion by performing double DNA strands breaks and triggering the inner cell mechanism repairs. When a homologous template is available, also a correction or the introduction of a new gene can be performed.
The accuracy of these systems must deal with off-target effects, as a results of the capacity to perform a cleavage in a non-target region, which can lead to lethal genetic mutation in animals and undesirable phenotype in plants.
From a study conducted by the European Union regarding the status of NGTs under the Union law, several doubts have been raised about the labelling and traceability of these products. In fact, one of the many arguments concerning the regulatory status of genome-edited products is the difficulty or impossibility of developing a detection method, since NGTs can induce mutations that are similar to the ones obtained through mutagenesis or in nature.
In addition, these new organisms lack of foreign DNA integration, since the transgene is used in an intermediary stage and then removed via backcrossing and selection.
So, genome-edited organisms do not show the presence of regulatory sequences as the conventional GMOs.

The four groups of NGTs

1 "Site-Directed Nuclease (SDN)" techniques

The SDN induces a site-specific double strand cleavage in the DNA molecule, in this way triggering the cell's DNA repair mechanisms.

2 NGTs that can induce a single break or no break at all in the host genome

In this second group, nucleases derived from the first group are used, but with mutations at their catalytic site.
An example is the dCas9, used in Base Editing, achieved by fusing it with an engineered base converter enzyme, which mediates the conversion of one base to another under the guidance of a sgRNA (Li et al 2021).

3 NGTs capable to specifically change the epigenetic set-up of an endogenous genomic site

Epigenetic editing is based on the ability to create fusion proteins comprising a domain given by epigenetic enzymes and another domain given by programmable DNA-binding platforms.

4 NGTs that act specifically on RNA, at post-transcriptional level

An example of RNA base editing, is performed thanks to the chimeric protein obtained through the fusion of Deactivated Cas13 with Adenosine Deaminases Acting on RNA (ADAR), thus performing the conversion of adenosine into inosine (Matsoukas 2018).

A tailored traceability method

When we talk about genome-edited organisms, of course we cannot perform the conventional GMO detection methods, such as the Screening for the regulatory sequences, for the reasons we have said before: no foreign promoters or terminators are present in the new organism.
We tried to develop a method that allows us to discriminate the genome-edited tomato from the wild types (tomato varieties commonly found on the market). The method is based on Real Time-PCR and a subsequent HRM assay, that are commonly used in food labs.
As regarding the organism, we choose the Sicilian Rouge High GABA tomato, produced through CRISPR/ Cas9, developed by the University of Tsukuba and produced and sold in Japan by SANATECH-SEED company. This tomato shows high GABA content, a proteinogenic amino acid that plays an important role in the plant growth and development, and in responses to different stresses.
In humans, GABA seems to have a positive effect on some life-style related diseases such as hypertension and diabetes; when its content is significantly low, it also seems to lead to insomnia and depression.
In drawing our assay, we started from collected information about the mutation induced in the new organism: from Euginius GMO database and different studies conducted by the University of Tsukuba, we learnt that the mutation is localized in a gene codifying for an enzyme involved in GABA production. This enzyme shows an autoinhibitory domain, that folds the active site leading to a reduction of enzyme activity. The insertion of a single nucleotide upstream the autoinhibitory domain, triggers the production of a truncated enzyme that leads to an increase in GABA content.
The identification of the edited tomato became less challenging when we learned information about the technique used for the development of the genomic and the phenotypic characteristics: we designed specific primers that allowed “seeing” the modified gene region: the mutation turned out in a difference in the melting temperatures between edited and wild type tomatoes. We have found that even in samples obtained by blending different ratios of Sicilian rouge High GABA tomato and wild type variety, it is possible to detect the presence of the edited organism.

European market study

To date several companies have started using genome editing. An example is the agro-industrial giant Syngenta that is investing in genome editing technology in countries such as the US and China to modify nutrient values, increase yields and improve pest and disease resistance in a number of crops.
As reported in a European study of 2021, several projects worldwide are in pre-marketing phase, while others are already on the market.
As far as plants are concerned, we see the use of NGTs in as many as 427 applications, of which 3 are already on the market: the Herbicide-resistant SU Canola produced by CIBUS, the high-oleic soybean produced by Calyxt and the Sicilian Rouge High GABA tomato, produced by SANATECH SEED.
As far as animals for food purpose are concerned, no genome-edited animal is on the market, but 4 are in pre-commercial stage and 59 are in R&D stage NGTs have spread rapidly in recent years, and some products can already be found placed on the market outside the EU.

Regulatory framework

Genome editing organisms encompass several distinct types of alterations generating different products, introducing new challenges in regulatory distinctions and traceability.
In the search for a legislation definition for these genetically edited organisms we can distinguish two main trends: on one hand there are countries that have based the regulation on the product obtain and the risks it may pose, while on the other hand countries in regulating these products look at the process used to obtain it, with the common aim of protecting human and animal health and the environment.

USA

The US Policy is product-based. The revision of its biotechnology regulations stipulates that certain classes of edited organisms do not fall under the regulation for GMOs. In the USA the revision of their biotechnology regulations, now called SECURE (Sustainable, Ecological, Consistent, Uniform, Responsible, Efficient) states that certain classes of GEOs do not fall under the regulation for GMOs. Exempt GEOs must meet certain criteria: the induced mutation must be the result of internal cell repair mechanism, without any repair templates being introduced; it must be achieved by targeted single-base substitution, or introducing a gene from the plant's gene pool, or modifying the targeted sequence to correspond to a known allele of such a gene, or to a known structural variation in the gene pool (Hoffman 2021).

Australia

The US approach is shared by Australia, which in 2019 gave notice of a "Gene Technology Amendment", stating that the organisms obtained from SDN-1 in which a novel combination of genetic material is not introduced, are excluded from the definition of GMOs provided by the Gene Technology Act 2000, making effective the product-based regulation of genome editing, despite the traditional regulation of GMOs being process-based (Jenkins et al. 2021, Tsuda et al. 2019, Metje-Sprink et al. 2020).

Argentina

Argentina has also a product-based approach: in 2015, it issued its own regulation, stipulating that GEOs must be considered as GMO or not by doing a product-by-product analysis, and product that are not in the scope of the GMO regulations remain subject to the same laws and regulations for plant developing through conventional breeding (Whelan and Lema, 2015).

Canada

The Canadian approach is based on a case-by-case risk assessment, taking into account only the new trait obtained in the organism, by assessing "novelty" in plants and derived food and feed without determining how this novelty was pursued. This is stated in The Plant with a Novel Traits regulations (PNTs) (Smyth and McHughen, 2008), in which a Plant with a Novel Trait is ''a plant that contains a trait which is both new to the Canadian environment and has the potential to affect the specific use and safety of the plant with respect to the environment and human health. With this product-based focus Canadian agencies have been challenged to contemplate how to address novelty in the context of techniques like genome editing that may not create novel genetic combinations (Jenkins et al. 2021).

Japan

In February 2019, Japan ruled that certain GEOs must be subject to the "Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms" (known as Cartagena Act), while others should be exempt. More specifically, those organisms obtained through SDN-1 as they are considered similar to those produced by conventional breeding technologies and through SDN-2, if they do not possess inserted extracellularly processed nucleic acid (Tsuda et al. 2019).

New Zealand

New Zealand regulation focuses on the process. The Environmental Protection Authority (EPA) concluded that the non-transgenic gene editing approach is more similar to chemical mutagenesis, which is why the organisms thus obtained was included within the HSNO regulations exceptions. the EPA decision was appealed by the Sustainability Council of New Zealand in the High Court and the case ended up with GEOs being considered as GMOs.

Others

Together with Russia, other countries are also discussing what path to take in the area of gene editing regulation, including some important trading countries such as China (Entine et al. 2021, Jenkins et al. 2021).

Conclusion

To date, several biotech companies have launched the use of genome editing. These genome-edited organisms will be more and more present on the market. To date, we see the use of NGT in as many as 427 applications, of which 3 are already on the market; as far as animals for food purposes are concerned, no animal product is on the market, but 4 are in the pre-commercial stage and 59 are in the R&D stage, above all with an increasing use of the CRISPR technique (Parisi et Rodríguez- Cerezo, 2021). From the study conducted by the European Commission regarding the status of NGT under Union law emerged that the GMO legislation, currently applied also to edited organisms, is not suitable for them. Despite this, doubts have been raised about the labeling and traceability of these products.

In fact, one of the many arguments concerning the regulatory status of genome-edited organisms is the difficulty and impossibility of developing a detection method, since genome editing can produce a mutation similar to the one obtained in nature, or through random mutagenesis (Directorate-General for Research and Innovation, 2019).

Our study highlighted that by knowing the locus and the gene modification, it is possible to design a "tailor-made" simple assay such as Real Time-PCR and HRM analysis.

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Genome editing

www.tuvsud.com/it-it/ph-labs
phlabs@tuvsud.com

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TÜV Italia srl
Via Bramante, 10/12
50028 Barberino Tavarnelle (FI) Italy
+39 055 80677
www.tuvsud.com/it-it/ph