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Sustainable Tree Farming: The Role of New Breeding Techniques

For thousands of years, the development of agricultural crops has been facilitated by extensive genome modification through plant crossing and offspring selection. Traditional breeding has progressively improved agricultural productivity. Conventional breeding has however not been able to guarantee stability and food security to farmers and society in face of disease outbreaks and climate instability **(1)**. The past century, following the Green Revolution, which enabled crop genetic adaptation for mechanical operations **(2)**, saw the introduction of modern agro-techniques, and genetic modification tools, such as mutagenesis (utilizing chemicals and radiation), transgenesis (inserting specific genes into genomes), and recently, precise genetic modification tools known as New Breeding Techniques (NBTs) or New Genomic Techniques (NGTs **(3).** These tools for plant genetic modification have significantly enhanced productivity, improved protection, and promoted sustainable food production **(4)**. In this White Paper, we will review the most recent advancements of NBTs, emphasizing DNA genome editing (GEd) tools (such as CRISPR) and examining their potential applications with an emphasis on the global regulatory status guiding their use.

What are NBTs?

NBTs refer to a set of genetic modification techniques that employ modern biotechnological tools used in plant and animal breeding to introduce desirable traits more precisely and efficiently than traditional breeding methods. These techniques offer several advantages, such as reducing the time required to develop new varieties and enabling the introduction of traits that are challenging to achieve through conventional methods **(5).** They hold significant potential for enhancing crop yield, disease resistance, herbicide tolerance, drought and climate resilience, nutritional quality and safety, biomolecule refining, and environmental sustainability. Common recent NBTs are described in Table 1.

Table 1: NBT tools and its description:

NBT tools	Description
GEd using site-directed nucleases (SDNs) (6)	Nucleases are proteins that act like scissors and can cut DNA in specific places. This enables the addition, removal, or alteration (mutation) of specific genes. Notably, CRISPR, a prominent example, utilizes enzymes such as Cas-9, Cas-12, and similar. Other notable SDNs include Meganucleases, Zinc Finger Nucleases (ZFNs), and TALENs.
GEd using Oligonucleotide-Directed Mutagenesis (ODM) (7)	A method that uses synthetic DNA fragments to introduce specific mutations at targeted locations in the genome.
Cisgenesis and Intragenesis (8)	Transferring genes within the same species or between closely related species, using only genes from the species' own gene pool.
Epigenetic Methods (9)	Techniques that control changes in DNA and the proteins around it, which can regulate gene expression (turn ON or OFF) without altering the DNA sequence.
Grafting (5)	The rootstock is genetically engineered to possess specific beneficial traits that protect and/or enhance the scion shoot (upper part of the plant), which by itself has no genetic modification.

What is CRISPR?

CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. It is a sophisticated biological system which evolved in bacteria that helps these bacteria to fight viruses known as bacteriophages. The system comprises two key components: a guide RNA (gRNA) and an enzyme called Cas nuclease. The gRNA is a short RNA sequence that precisely matches the target bacteriophage DNA sequence while Cas nuclease executes a DNA cleavage (cut) at the designated location identified by the gRNA, effectively eliminating the bacteriophage and safeguarding the bacteria **(10).**

Scientists have engineered this system to cut any DNA in any living organism, adopting several CRISPR systems (likeCas-9 and Cas-12).

How is CRISPR-Cas different from other GEd SDN tools?

Prior to the CRISPR-Cas discovery, genome editing employed tools such as Meganucleases, ZFNs, and TALENS. These SDN enzymes are huge molecular-size proteins that are expensive to synthesize and recognize the target DNA sequence based on highly complex structures. . Consequently, the sequences that these enzymes can identify are limited and a specific SDN enzyme needs to be produced for each desired sequence.. In contrast, Cas nuclease is relatively small, is universally applicable to cutting the DNA and is separately targeted to the desired DNA sequence by the gRNA, a simple and inexpensive

biomolecule to produce, that can be synthesized to match any DNA sequence. The low cost, ease of use, and endless versatility of DNA sequences that can be targeted have led to the rapid adoption of the CRISPR-Cas tool by scientists worldwide, making it the primary tool for genome editing **(6).**

How can a DNA break made by a nuclease result in an edited genome?

A double-stranded DNA break is induced when the CRISPR-Cas nuclease, guided by the guide RNA (gRNA), targets a matching DNA sequence. Alternatively, Meganucleases, ZFNs, and TALENS recognize and bind specific genomic DNA targets. Upon DNA cleavage, damage occurs, triggering a cellular alarm that initiates a series of DNA repair events. This situation is so critical to the cell that the emergency DNA repair often results in inaccuracies, prioritizing the preservation of genomic DNA integrity over maintaining the original sequence, which can lead to changes in the DNA sequence. This type of DNA repair is known as Non-Homologous End Joining (NHEJ). If a break occurs in a coding or regulatory gene sequence, these genes are repaired and edited with modifications and potential mutations. Additionally, cells possess an alternative repair mechanism that is more accurate, called Homology-Directed Repair (HDR). In HDR, a homologous DNA sequence serves as a template to precisely repair the break. Researchers can provide this DNA template to introduce specific targeted changes to the DNA **(11).**

What are the potential genomic outcomes of modified plants? (12, 13)

1. **Gene Knockouts** - The rapid and inaccurate DNA repair process often results in insertions or deletions (InDels) of one to a few nucleotides, leading to significant alterations in the coding sequence. Consequently, the edited gene becomes dysfunctional and inactive (turned off), a phenomenon called “gene knockout”.
2. **Mutations** - In rare instances, inaccurate DNA repair can simply replace one nucleotide with another, resulting in an active gene but with a mutation. If this mutation is positive, it can enhance the gene’s activity and lead to improved plant traits. Researchers have developed specialized modified Cas enzymes that enhance the likelihood of mutations over InDels and knockouts. These enzymes are known as “Base Editors” and are designed to enable precise single-base modifications in DNA without causing double-strand breaks. They are created by fusing a Cas enzyme with a deaminase enzyme.
3. **Regulatory sequences editing** – When InDels or mutations in the gene regulatory elements, such as the promoter, terminator, and introns, alter the timing, tissue specificity, or expression level of a gene.
4. **Gene/fragment replacement** – Utilizing a process called HDR, scientists can replace a gene with a new one, or make other changes, including multiple mutations and knockout events. The resulting outcomes are usually akin to genetic transformation.
5. **Epigenetic editing** – Modifying the chemicals that bind DNA and histones, such as methylation or acetylation, without altering the DNA sequence can substantially affect gene expression levels and timing **(9).**

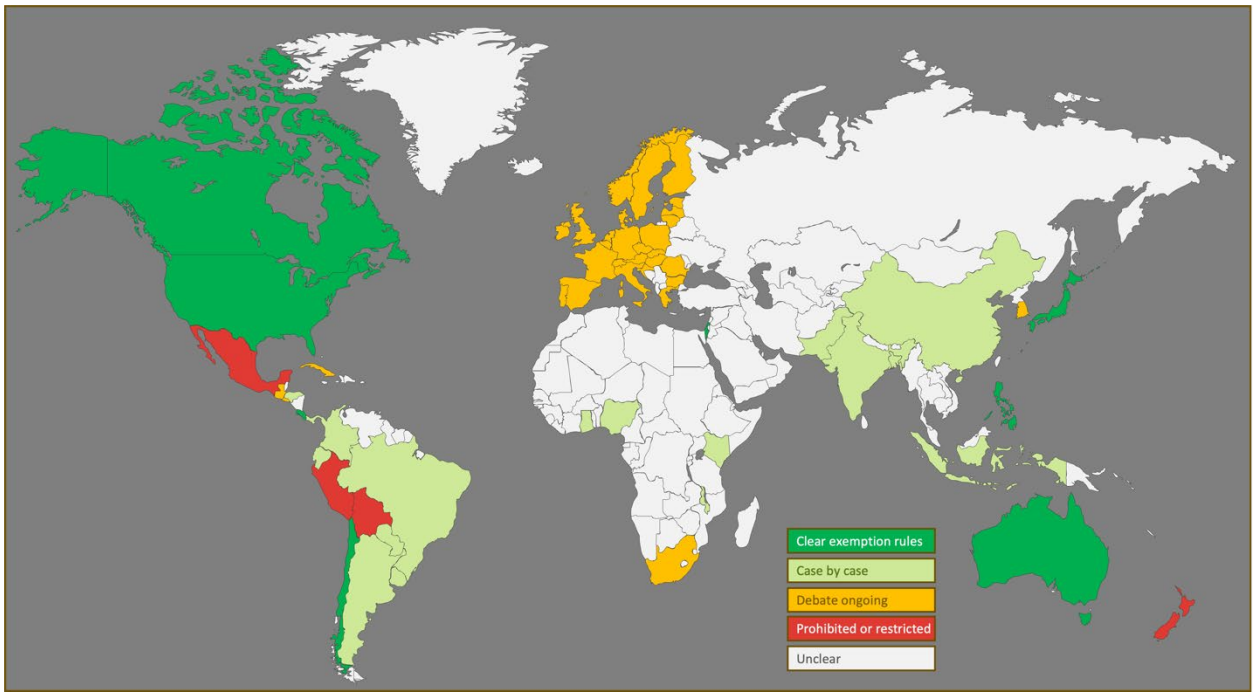
The regulatory framework for each tool and outcome.

NBTs have been rapidly advancing, with regulatory bodies monitoring recent developments and exchanging information on techniques, organisms, applications, and regulatory implications. The regulation of products derived from NBTs has been a topic of considerable global debate. A key issue is whether products obtained using NBTs should be regulated as GMOs or be exempt, similar to other genetic modifications achieved through breeding, chemical and radiation mutagenesis **(14).**

Debates on the regulation of NBTs, particularly concerning genome editing, involve possible risk and environmental impacts, as well as their coexistence with organic and GM-free agriculture and the consumer's right to information. Other concerns often relate to the possibility of unintended changes called "off-target effects". Arguments against the extensive regulation of some NBT products highlight the difficulty in distinguishing them from products derived through conventional breeding or mutagenesis. This could lead to disproportionate risk assessments for identical products. Additionally, such regulation may hinder the trade and exchange of traditional breeding materials by parties seeking to protect their edited equivalents **(15).**

To simplify the comprehension of the global regulatory framework, we can categorize NBT products into two primary categories. The first category encompasses simple mutations, such as substitutions and InDels, which yield outcomes comparable to random genomic modifications achieved through conventional breeding or antiquated biotechnology methods, including chemical or radiation-induced mutagenesis (which are exempt from regulation). The second category involves the insertion of substantial DNA fragments encompassing entire genes at specific genomic locations or multiple alterations in various locations within the DNA, resulting in outcomes akin to genetic transformation.

In certain jurisdictions, such as the United States, Japan, and Canada, distinct regulations are established for the classification of NBT products that are exempt from regulation and those that must adhere to the comprehensive regulatory process **(16).** Other jurisdictions, including Argentina, Brazil, and China, adopt a case-by-case approach to approving NTB products and determine the regulatory pathway based on scientific discussions that assess the genomic alterations induced. Countries like the United Kingdom and the European Union are currently engaged in a discussion process regarding whether to approve NBTs on a case-by-case basis or adopt alternative regulatory frameworks. Only a few countries prohibit or restrict NBTs completely, like GMOs. Currently, the regulatory frameworks in most of Africa and Russia remain ambiguous (Map 1).



Map 1: Worldwide regulation of NBTs by country and regions as of February 2025.

Adopted from “Regulation Tracker”. (https://crispr-gene-editing-regs-tracker.geneticliteracyproject.org/).

The regulatory framework is undergoing a substantial transformation as global agencies revise their policies in response to scientific evidence and geopolitical considerations. For instance, the European Union (EU) is currently contemplating transitioning from the restriction policy that mandates a full regulatory pathway to a policy that permits exemptions for some NBT products. The subsequent section provides a comprehensive analysis of the regulatory status of NBTs in Brazil, the United States, and the EU.

Brazil

Brazil has adopted a progressive approach to regulating NBTs for crops. In 2018, Brazil's National Technical Commission for Biosafety (CTNBio) declared that NBTs without foreign genes would not be deregulated or considered GMOs. CTNBio evaluates these techniques on a case-by-case basis, focusing on the characteristics of the final product rather than the process used to create it. Consequently, gene-edited crops that do not contain foreign DNA (such as SDN-1) are regulated similarly to conventional plants **(17).**

USA

The United States regulatory framework for NBTs is scientifically based and risk-proportionate. Three federal agencies primarily oversee the regulation of both GMOs and NBTs for crops: The Animal and Plant Health Inspection Service (APHIS) within the U.S. Department of Agriculture (USDA), the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA)(18, 19).

- **APHIS** grants cultivation approvals for genetically modified plants that pose no risk to plant health, including NBTs. They issue permits for field trials and, through a petition process, can waive the regulation process.
  APHIS is currently revising its regulatory framework to establish a system that simplifies the decision-making regarding the regulation and exemptions of NBTs and transgenic plants. The framework, named SECURE, was implemented in May 2020 with the goal of streamlining the regulatory process, reducing the burden on developers, and facilitating the development of innovative agricultural technologies. SECURE focused on the final product trait rather than the methods used to produce it. However, the framework was recently suspended by a district court in California due to technical governance concerns **(20).**
- **The EPA** regulates the environmental impact and safety of pesticide and herbicide use, including *in-plant* applications.
- **The FDA** oversees the safety of food and feed derived from genetically modified plants. Each agency has a specific role in ensuring the safety and efficacy of these crops on a case-by-case basis. NBT crops, such as SDN-1, which are similar to conventional breeding, are exempt from regulations.

EU

The regulation of NBTs for crops in the European Union follows the 2018 ruling by the Court of Justice of the European Union (CJEU) that organisms obtained through NBTs (such as CRISPR) are subject to the same regulations as genetically modified organisms **(21).** In July 2023, the European Commission proposed a new regulation for plants produced by certain NBTs **(22).** The proposal aims to create a more enabling environment for research and innovation while maintaining high levels of health and environmental protection. It introduces two categories for NBT/NGT plants:

Category 1 NGT Plants: These are plants that could also occur naturally or through conventional breeding. They would be subject to a verification procedure and, if they meet specific criteria, would be treated like conventional plants and exempt from GMO legislation

Category 2 NGT Plants: These include plants that do not meet the criteria for Category 1 and would be subject to the current GMO legislation, including risk assessment and authorization before being placed on the market.

Category 1 imposes very strict definitions and instructions, such as limiting the number of edited genes and nucleotide substitutions, ensuring no changes to regulatory sequences or introns, and preventing chimeric outcomes **(23).** This could establish a distinct standard for regulatory agencies worldwide and significantly impact NBT products globally.

The matter is still under discussion within the European Commission.

Conclusions.

Debates on the regulation of NBTs, particularly genome editing, delve into potential risks and environmental impacts. These concerns are intertwined with the coexistence of organic and GM-free agriculture, as well as the consumer's right to information. The spectrum of regulatory approaches ranges from granting exemptions to conducting thorough regulatory analyses akin to those for GMOs.

Other arguments in favor of regulation highlight the possibility of unintended changes, such as "off-target effects" and unintended DNA insertions. These concerns underscore the need for comprehensive regulatory frameworks to ensure the safety and responsible use of NBTs.

On the other hand, arguments against extensive regulation emphasize the challenges in distinguishing NBT products from those derived through conventional breeding or mutagenesis. This distinction can lead to disproportionate risk assessments for products that are essentially identical. Furthermore, such regulation may hinder the trade and exchange of traditional breeding materials, by parties seeking to protect their NBTs equivalents.

Potential products derived from NBT have the potential to enhance conventional breeding by accelerating the development of desired traits. This approach offers an advantage over traditional breeding methods and GMO in some cases, when complex regulation is not required. However, many cases involve complex traits such as herbicide tolerance, insect resistance, and climate resilience. These traits can only be achieved through NBTs that introduce intricate genetic modifications (akin to Category 2 in the EU proposals) or in combination with genetic transformation and GMOs, necessitating a comprehensive regulatory process.

Although in some jurisdictions, certain NTB products can be labeled as "Non-GMO," the majority of plant crop traits with the potential to revolutionize agriculture, such as mitigating the impacts of climate change, addressing food security, and bringing social and environmental benefits **(24),** will be *per-force* be categorized as complex traits (Category 2 in the EU proposals), forcing their regulation as GMOs.

Key Takeaways:

- NBTs, particularly CRISPR-based genome editing, are powerful tools for advancing plant breeding and addressing many agriculture challenges.
- These technologies offer unprecedented precision, speed, and versatility in modifying plant genomes and serve as complementary tools for genetic transformation.
- Regulatory frameworks are evolving, and there is significant ongoing debate about how best to manage the risks and benefits of NBTs. The central issue influencing regulatory decisions is the distinction between products created by NBTs that are similar to those obtained through natural means and those that are not.
- Many high-end-potential crop traits will involve a combination of complex NBTs, along with GMOs, resulting in a comprehensive regulatory framework.

Potential NBT traits in Eucalyptus tree farming:

Eucalyptus trees are cultivated in Brazil similar to other row crops, with the exception of lower plant density per hectare and a longer rotation from planting to harvest. Like row crops, eucalyptus tree farms face challenges such as weed competition, pest and disease attacks, and environmental stress influenced by climate instability. Genome editing and other NBTs provide promising tools for enhancing eucalyptus traits, addressing these challenges and others such as long breeding cycles and regulatory constraints. NBTs enable precise modifications and serve as an additional tool for the genetic modification of eucalyptus. When combined with genetic transformation, they facilitate faster and more sustainable advancements in eucalyptus cultivation.

We present below potential traits that could be introduced into eucalyptus using NBTs such as CRISPR:

Gene knockouts and mutations that probably will be exempt from regulation in most jurisdictions:

- **Improved Wood Quality:** Knocking out genes involved in cell wall polymer biosynthesis, such as those regulating lignin and hemicellulose composition, can enhance wood quality and processing efficiency in pulp mills. A notable example is the **CAD-null variants** found naturally in pines, which modify the lignin composition and enhance wood quality. Using genome editing, similar mutations can be replicated in other tree species to produce high-performing varieties optimized for industrial applications (under development).
- **Enhanced Growth and Yield:** Eliminating growth-inhibitor genes can accelerate biomass accumulation, thereby improving sustainable wood production (under development).
- **Herbicide Tolerance:** Point mutations in target genes can confer resistance to specific herbicides by altering binding sites without disrupting enzyme functionality. For example, mutations in the **ALS (Acetolactate Synthase)** gene can provide tolerance to ALS-inhibiting herbicides, while edits in the **PPO (Protoporphyrinogen Oxidase)** gene can confer resistance to PPO inhibitors. These precise modifications enable more effective and sustainable weed management.
- **Enhanced Recombination:** Knocking out genes that suppress genetic recombination can increase crossover rates during meiosis, enabling faster introgression of traits into breeding populations. This approach boosts genetic diversity and shortens breeding timelines, which is especially beneficial for species like eucalyptus with long breeding cycles.

Complex editing and other NBTs that probably fall under the full regulatory pathway:

- **Regulatory sequences editing:** by fine-tuning the expression levels and timing of specific gene, or sets of genes, we can better control the development of the trees, flowering time, growth patterns, cell wall biosynthesis and the responses to changing environments and stresses (such as drought, heat and salinity).
- **Targeted insertion:** Combining novel gene introductions with **targeted integration** into genomic hotspots unlocks nearly unlimited opportunities for trait development

while ensuring high trait stability and effectiveness. By aligning gene insertion with the optimal genomic locations, both breeding priorities and molecular biology characteristics are optimized. This includes considerations such as expression levels, methylation/demethylation, acetylation, and chromatin accessibility.

- **Grafting with inducible Rootstocks:** Induction via GM rootstocks provides a platform for accelerated breeding by inducing early flowering, which causes the scion to produce pollen and seed within a few months. Additionally, gene editing enables precise modifications in scions, offering a clean pathway for genetic improvements without directly modifying the scion's genome.
- **Epigenetic editing:** Chemical modifications of DNA and histones that can alter gene expression levels and timing.

Glossary

APHIS: The Animal and Plant Health Inspection Service (APHIS) is an agency within the USDA. It is responsible for protecting and promoting U.S. agricultural health, regulating genetically engineered organisms, administering the Animal Welfare Act, and managing wildlife damage.

Cisgenesis: Genetic modification of a plant with genes from the same species or a closely related one. Cisgenesis involves the transfer of genes that could naturally occur in the plant's gene pool.

CRISPR/Cas9: A genome editing tool that allows for precise, directed changes to genomic DNA. CRISPR/Cas9 uses a guide RNA to direct the Cas9 enzyme to a specific location in the genome, where it makes a cut, allowing for the addition, deletion, or alteration of genetic material.

CTNBio: The Brazilian National Technical Biosafety Commission responsible for regulating GMOs. CTNBio evaluates the safety and environmental impact of genetically modified organisms and oversees their use in Brazil.

DNA Double-Strand Break (DSB): A type of DNA damage involving the breaking of both strands of the DNA molecule. DSBs can be caused by environmental factors, such as radiation, or by cellular processes, and require repair to maintain genomic stability.

EPA (Environmental Protection Agency): The U.S. agency responsible for protecting human health and the environment. The EPA enforces regulations to reduce pollution, manage hazardous waste, and ensure clean air and water and regulated the use of herbicides and pesticides.

European Parliament: The legislative branch of the European Union. The European Parliament is responsible for passing laws, approving budgets, and overseeing other EU institutions.

FDA (Food and Drug Administration): The U.S. agency responsible for protecting public health by ensuring the safety and efficacy of drugs, biological products, and medical devices. The FDA also regulates food safety, cosmetics, and tobacco products.

GM (Genetic Modification): The direct manipulation of an organism's genes using biotechnology. Genetic modification involves techniques like gene cloning, gene transfer, and genome editing to alter the genetic makeup of an organism.

GMOs (Genetically Modified Organisms): Organisms whose genetic material has been altered using genetic engineering techniques. GMOs are created to introduce new traits or enhance existing ones, such as increased resistance to pests or improved nutritional content.

Homology Directed Repair (HDR): A mechanism in cells to repair double-strand DNA lesions using a homologous sequence as a template. HDR is a precise repair process that can be harnessed for genome editing to introduce specific genetic changes.

Intragenesis: Genetic modification involving the insertion of a reorganized DNA sequence from the same species. Intragenesis allows for the introduction of new traits while maintaining the genetic integrity of the species.

Meganucleases (MN): Enzymes that recognize and cut long DNA sequences, used in genome editing. Meganucleases are highly specific and can be engineered to target unique DNA sequences for precise genetic alterations.

New Breeding Technologies (NBTs): Advanced methods used to alter the genetic makeup of organisms more precisely and quickly than traditional breeding techniques. These technologies include genome editing tools like CRISPR/Cas9, TALENs, and Zinc Finger Nucleases, which allow for targeted modifications at specific sites in the DNA.

New Genomic Techniques (NGT): Advanced methods for altering the genetic material of organisms. NGTs include genome editing tools like CRISPR/Cas9, which allow for precise and targeted modifications.

Non-Homologous End-Joining (NHEJ): A pathway that repairs double-strand breaks in DNA by directly joining the broken ends. NHEJ is a quick and efficient repair mechanism but can introduce small insertions or deletions at the break site.

SECURE: “Sustainable, Ecological, Consistent, Uniform, Responsible and Efficient” and isa set of revised biotechnology regulations implemented by the USDA. These regulations, which were fully implemented in October 2021, focus on the properties of genetically engineered organisms rather than the methods used to produce them. SECURE framework was vacated by the U.S. District Court for the Northern District of California on December 2, 2024. The court ruled that the USDA did not adequately consider certain procedural and statutory requirements when implementing the rule. As a result, the regulatory process for genetically engineered organisms has reverted to the pre-2020 framework.

Site Directed Nucleases (SDNs): Enzymes used to cut DNA at specific sites to introduce genetic changes. SDNs include tools like CRISPR/Cas9, TALENs, and Zinc Finger Nucleases, which enable precise genome editing by creating double-strand breaks at targeted locations.

TALENs (Transcription Activator-Like Effector Nucleases): Engineered proteins that cut DNA at specific sites to enable genome editing. TALENs are designed to recognize and bind to specific DNA sequences, allowing for targeted modifications.

USDA (United States Department of Agriculture): The U.S. federal department responsible for developing and executing federal laws related to farming, forestry, and food. The USDA promotes agricultural production, ensures food safety, and supports rural communities.

Zinc Finger Nucleases (ZFN): Engineered DNA-binding proteins that facilitate targeted editing of the genome. ZFNs use zinc finger domains to recognize specific DNA sequences and create double-strand breaks, enabling precise genetic modifications.

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