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Towards social acceptance of plant breeding by genome-editing

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Although genome-editing technologies facilitate efficient plant breeding without introducing a transgene, it is creating indistinct boundaries in the regulation of genetically modified organisms (GMOs). Rapid advances in plant breeding by genome-editing require the establishment of a new global policy for the new biotechnology, while filling the gap between process-based and product-based GMO regulations. In this opinion article we review the recent developments for producing major crops using genome-editing and propose a regulatory model that takes into account the various methodologies to achieve genetic modifications as well as the resulting types of mutation. Moreover, we discuss the future integration of genome-edited crops into society, specifically a possible response to the Right-to-Know movement which demands labelling of food that contains genetically-engineered ingredients.

The need for regulatory models

Genome-editing via technologies such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas systems (e.g. Cas9) offers the ability to perform robust genetic engineering in many species [1-3]. For example, by utilizing plant genomic information, genome-editing is expected to generate many new crop varieties with traits that can satisfy the various demands for commercialisation. However, one of the new plant breeding techniques, genome-editing allows plant breeding without introducing a transgene which has led to new challenges for the regulation and social acceptance of genetically modified organisms [http://ipts.jrc.ec.europa.eu/publications/pub.cfm?id=4100] [4–8]. This modern genome-editing can produce novel plants that are similar or identical to plants generated by conventional breeding techniques, thus creating indistinct boundaries with regards to GMO regulations [4-8]. Therefore an appropriate regulatory response is urgently

required towards the social acceptance of genome-edited crops. Here, we review the recent development of genome-edited major crops and propose a concept of appropriate regulatory models by unraveling the indistinct boundaries. In addition, we discuss how breeders should respond to the Right-to-Know movement which demands labelling of genome-edited crops that are released to the consumers.

Genome-editing-mediated plant breeding

Conventional genetic engineering begins with extracellular DNA manipulation to construct a plasmid vector harboring a gene or a specific DNA sequence to be transferred into the chosen organism. The entire plasmid or only the DNA fragment is then shot into plant cells by using particle bombardment or delivered into the cells by polyethylene glycol or agrobacterium-mediated transformation. The modified plant cells are then used to generate a GM plant. When the gene is derived from an unrelated, cross-incompatible species, the process is referred to as transgenesis. When an identical copy of a gene from a cross-compatible species (cisgene) is transferred to a related species, the process is called cisgenesis [9]. In intragenesis, transferring a DNA sequence creates a new combination of gene elements (promoter, coding region and terminator,) derived from different genes within the cross-compatible species [9]. However, because homologous recombination rarely occurs in plants, exogenously-delivered DNA molecules, even if they are designed to induce homologous recombination in a target gene, frequently integrate into nonspecific sites in the plant cell genome [10, 11] via non-homologous end joining (NHEJ) [12]. Thus, conventional genetic engineering is labor intensive and requires time-consuming screens to identify the desired plant mutants. By contrast, genome editing is an advanced genetic engineering tool that can more directly modify a gene within a plant genome [1-3]. The desired genetic modification is initiated by inducing double-stranded breaks (DSBs) in a target sequence by using nucleases, and is subsequently attained by DNA repair through NHEJ or homology-directed repair (HDR) [13]. The NHEJ pathway efficiently yields a small insertion or deletion (referred to as indel) in a specific locus without the use of exogenous DNA. By contrast, the HDR pathway can introduce a desired DNA sequence or gene into a targeted site, depending on the length of the exogenous DNA that is delivered to the plant cells together with the nucleases. Recent reports regarding genome-edited major crops, including barley (*Hordeum vulgare*), maize (*Zea mays)*, rice (*Oryza sativa*), soybean (*Glycine max*), sweet orange (*Citrus sinensis*), tomato (*Solanum lycopersicum*), and wheat (*Triticum*), have demonstrated a high efficiency of indels [14-25] in addition to the introduction of exogenous DNA in a targeted locus [17,

26][Table 1]. Some reports have demonstrated that NHEJ-mediated indels can lead to disease resistance, without the need to use a transgene [16-18, 25]. Most notably, three homoeoalleles of *TaMLO* were simultaneously edited in hexaploid bread wheat, resulting in heritable resistance to powdery mildew [25]. Moreover, maize which has indels in *ZmIPK1 or ZmIPK* is expected to have improved nutritional value due to a decrease in the phosphorus content [15, 26]. Furthermore, rice with indels in *OsBADH2* [17, 19] may appeal to consumers due to its improved fragrance [27, 28]. Such results show that genome-editing dramatically simplifies plant breeding even in major crops with potential impact on the future of agriculture and human nutrition. However, the modification efficiency appears to vary based on the locus selected [17, 19], although the selection of genome-editing systems [15] and crop species [17, 23] has no significant effect on the efficiency [Table 1]. Moreover, although Cas9-treated rice showed off-target mutations in *OsMPK2* [17] [Table 1] in most cases no off-target mutations were observed [17, 20, 22, 23, 26]. However, most of these reports did not address potential off-target mutations [17, 20, 22, 23, 26][Table 1]. The occurrence of off-target mutations is one of the crucial issues in the agricultural use of genome-editing. Some off-target mutations are likely to result in silent or loss-of-function mutations, others might lead to immunogenicity or toxicity in the food products by changing amino acids within a protein, although there has been no documented report regarding any adverse effect resulting from foods produced from GM plants [29]. It has also been speculated that the cultivation of crops with off-target mutations might affect an ecosystem as a result of crossbreeding. Notably, a plant with an entirely new trait, the resistance to two different herbicides, was recently found in North Dakota, USA [30]. It was reported that the herbicide resistance developed in the field owing to the crossbreeding of wild-type canola with herbicide-resistant genetically modified canola.

Although it is difficult to identify off-target mutations in the plant genome, breeders should demonstrate that there are no significant off-target mutations that are associated with potential health or environmental risks. Otherwise, the imprudent use of genome-editing may lead to its rejection in agricultural and environmental applications.

Regulatory controversies

According to the Cartagena Protocol on Biosafety, a ‘living modified organism’ (the technical legal term that is close to GMO) is stipulated as ‘any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology’[http://bch.cbd.int/protocol/text/]. This definition suggests that some of the plants modified by genome-editing may be outside the scope of current GMO

regulations, because genome-editing can produce a null segregant (lines that lack the transgenic insert), thus blurring the boundaries in the GMO regulations [4-8].

The regulatory response to genome-editing of plants has been considered or a decision has already been made in Argentina, Australia, the EU, New Zealand, and the USA [4, 8]. We have analyzed such regulatory responses and summarized them in two categories regarding the presumed treatment of genome-edited organisms under the product-based or process-based GMO regulations, respectively [Box 1][4]. It has been suggested that HDR-mediated gene addition can be regulated under these two regulatory approaches. However, this analysis also suggested that the regulatory boundary in the process-based regulations is more indistinct than that in product-based regulations, reflecting the cautious regulatory attitude toward NHEJ-mediated indels in the EU [31]. In addition, there was recently a significant lawsuit in New Zealand. The high court overruled an Environmental Protection Authority (EPA) decision that plants modified via NHEJ do not need to be regulated under the GMO regulations [http://www.nzherald.co.nz/nz/news/article.cfm?c_id=1&objectid=11268377]. This is a telling episode that reflects the difficulty in formulating general regulations for genome-edited plants. Meanwhile, random mutagenesis induced by using X-rays or chemicals has been a standard tool for conventional plant breeding. At least 2543 plant varieties in 175 plant species, including barley, rice, wheat and other species have been developed using these strategies [32]. However, random mutagenesis frequently produces plants with multiple and unspecific genetic changes and screening mutants is very time-consuming. For instance, a decade has been spent in developing a rice variety with a low amylose content, Milky Queen, which underwent random mutagenesis using *N*-methyl-*N*-nitrosourea [https://www.naro.affrc.go.jp/publicity_report/publication/files/2-2.pdf]. Because the agricultural use of genome-editing can produce NHEJ-mediated indels similar to random mutagenesis with substantially shorter development time of the desired crops, regulatory controversy is likely to increase worldwide. Therefore, a new global policy regarding plant breeding by genome-editing should be established, with the aim to fill the gap between process-based and product-based GMO regulations.

In our opinion, the time is right to gradually transition from process-based GMO regulations to product-based GMO regulations, because many countries have had sufficient regulatory experience regarding conventional transgenesis since the early 1990s [30, 33]. Likewise, genome-edited crops should be regulated based on the end product including a comprehensive survey of off-target mutations.

A regulatory concept for genome-edited crops

To enhance the likelihood of the future acceptance of genome-edited crops as new crop varieties that differ from conventional GMOs, we unraveled the indistinct boundaries in GMO regulations, which are associated with mutants generated via NHEJ, or HDR provided with exogenous DNA.

Some of the current genome-editing techniques are likely to cause off-target mutations other than the intentional mutation [Table 1][17]. This technical hurdle is likely to be overcome, due to genome-editing rapidly advancing. For example, enhanced modification specificity can be achieved via the use of more sophisticated nucleases and meticulously designed homing molecules [1-3]. Moreover, whole genome sequencing can be used to identify plant varieties produced without off-target mutations during breeding. Although some technical challenges in the genome sequencing will remain due to the unclear distinction between an off-target mutation and a reading error or single nucleotide polymorphism, as well as whole genome shotgun assemblies of plant species with a larger and more complicated genome structure, such as bread wheat (genome size approx. 17Gbp, 2n=6×=42, allopolyploid) [34]. In such cases, an alternative method can be used to survey comprehensively potential off-target sites in the plant genome, deduced from the target sequences of homing molecules (ZFNs and TALENs) or guide RNAs (Cas9) [17, 20, 22]. In the following paragraphs we present a regulatory concept in which only desired mutations induced by genome-editing are considered on the product-basis.

After taking into account mutation types, as well as gene modification pathways, a new regulatory concept for genome-edited crops was developed [Figure 1]. This diagram shows the regulatory analysis of genome-edited crops on a product-basis, while taking into account the method of gene modification. This chart indicates that NHEJ as well as HDR potentially cause gain-of-function mutations that might be subject to regulations in a given country, such as New Zealand’s high court overruling of the EPA decision [GM guardian's error a grave failing. *New Zealand Herald.*. http://www.nzherald.co.nz/nz/news/article.cfm?c_id=1&objectid=11268377]. For instance, it has been demonstrated that TALEN-mediated disruption in the rice bacterial blight susceptibility gene *OsSWEET14* led to the acquisition of disease-resistance [16][Table 1]. Moreover, we suggest that four regulatory lines can be drawn for various genome-edited crops. Line 1 denotes the most permissive regulations. In this case, most of the genome-edited crops are considered to be outside the regulations, although transgenesis achieved by HDR still falls under regulations. By contrast, Line 4 indicates the most stringent regulations, in which only a portion of indels that lead to leaky or null

mutations, fall outside the regulations. Recently, following case-by-case considerations according to the definition of 'Novel Foods' in the Food and Drug Regulations [http://www.hc-sc.gc.ca/fn-an/gmf-agm/appro/index-eng.php; http://www.inspection.gc.ca/active/scripts/database/pntvcn_submitdb.asp?lang=e&crops=1&company=26&trait=herbicide&events=all; http://laws-lois.justice.gc.ca/eng/regulations/c.r.c.,_c._870/FullText.html], two Canadian authorities have approved a variety of canola in which a short repair oligonucleotide was introduced to direct the innate DNA repair system to induce a gene modification for sulfonylurea tolerance [http://cibus.com/press/press031814.php]. Although this was attained by oligonucleotide-directed site-specific mutagenesis [http://ipts.jrc.ec.europa.eu/publications/pub.cfm?id=4100], rather than genome-editing, the regulatory approval could correspond to the yellow area labelled as 'Gain-of-function mutations'.

The diagram can serve discussions at domestic as well as international level in order to determine where regulatory lines should be drawn for genome-edited crops. In so doing, some important future questions would be addressed [Box 2]. With a broad international cooperative effort, a consensus can emerge where a sole regulatory line is used for all crops developed by conventional genetic engineering, as well as genome-editing.

Response to "Right-to-Know"

Even if a genome-edited crop is approved by a regulatory authority, released into the environment for cultivation, and commercialized as a food product, another concern may emerge. Recently, the "Right-to-Know" movement against GMOs has been increasing, even in the USA. For example, the governor of Vermont signed a bill of law that will require the labeling of GM foods [http://governor.vermont.gov/newsroom-gmo-bill-signing-release)]. Some people will demand to know which food products are produced from genome-edited plants, regardless of the degree of genetic modification.

For inspectors, validating food labelling is challenging due to the difficulties in distinguishing between small indels via NHEJ and spontaneous mutations that can naturally occur in plant genomes. In addition, the detection of such small mutations in a plant genome by PCR, which is one of the major testing strategies used for the detection of a transgene by inspection bodies [35], is likely to increase the difficulties associated with validating food labelling. However, a possible solution is the introduction of a DNA tag [36] into the crop genome to readily confirm a variety of genome-edited crops

by PCR. For example the HDR pathway can efficiently attain DNA tagging in crop genomes [17][Table1]. Safe DNA tagging in plants would become feasible if a useful locus, similar to mouse *Rosa26*, the disruption of which shows no overt phenotype [37], is available in each variety of plant. Despite the fact that this solution requires additional gene modification, it is likely to be considered favourably as an option in countries where researchers have an ongoing open communication with the public.

Concluding remarks

We propose that each country or international body, such as the Convention on Biological Diversity, should consider introducing regulation standards according to Line 4 in Figure 1 initially, because health and environmental risks not anticipated might result from plant breeding with genome-editing. We can reconsider mitigating the regulations towards Line 1 when sufficient regulatory experience has been gained regarding genome-edited crops. Such a cautious approach would contribute to harmonizing countries that regulate GMOs on a process-basis, with those that regulate on a product-basis. The emergence of genome-editing should encourage us to reconsider the worldwide regulatory gaps regarding GMOs.

In the genome editing era, the dissemination of plants developed by advanced genetic engineering is not hampered by technological aspects, but by the understanding and acceptance of such technologies in society. Researchers, the public, and regulatory bodies should proactively discuss the socially acceptable integration of genome-edited crops if they recognize that the agricultural use of genome-editing can satisfy the needs of breeders and consumers alike and improve global food security.

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Box 1. The process-based and product-based GMO regulations

In general, the process-based GMO regulations are stricter than the product-based GMO regulations, thus requiring more time to gain regulatory approval [4, 8, 33, 38, 39].

The process-based GMO regulations

GMOs are subject to regulatory review involving a detailed procedure based on a scientific assessment of the risks to human health and the environment. Notably, the EU adopted this regulatory approach to GM crops under Regulation (EC)1829/2003. Other countries which have adopted this regulatory approach include Australia (the Gene Technology Act 2000) and New Zealand (the Hazardous Substances and New Organisms Act 1996).

The product-based GMO regulations

Some countries, including Argentina (the National Biosafety Framework), Canada (the Food and Drugs Act) and the USA (7 CFR Part 340) assess health and environmental risks which are associated with a GMO, based on the final product rather than the processes. For instance, the US regulation defines GMOs as organisms and products altered or produced through genetic engineering that are plant pests or for which there is reason to believe are plant pests. The Canadian Act stipulates that a GMO is a food that is derived from a plant, animal or microorganism that has been modified through genetic engineering to have altered characteristics

Box 2. Outstanding questions

・Although the regulatory lines in Figure 1 are all linear, can polygonal lines be drawn?
・What are the significant differences between random mutagenesis and NHEJ-mediated indels in plant breeding?

・How should we assess the environmental risk of genome-edited crops if some varieties of such engineered crops coexist in cultivated fields?

Table 1. Examples of reported genome-editing-mediated gene modifications in major crops

Species	Target locus	Genome editing	Modification type	Efficiency of modification	Off-target mutation	Genotyped subject	Refs
Barley	*HvPAPhy_a*	TALEN	Indel	16-31%	N.D.	Plantlets	14
Maize	*ZmIPK1*	ZFN	Inserting PAT	3.4-22.1% (autonomous)[a] 16.7-100% (non-autnonomous)[a]	No	Calli	26
	ZmIPK	TALEN	Indel	39.1%	N.D.	Plants	15
	ZmIPK	Cas9	Indel	13.1%	N.D.	Protoplasts	15
Rice	*OsSWEET14*	TALEN	Biallelic indel	6.7% - 27%	N.D.	Plants	16
	OsPDS-SP1, OsBADH2, OsMPK2	Cas9	Biallelic indel	3.1% (*OsPDS-SP1*), 0% (*OsBADH2*), 0% (*OsMPK2*)	No(*OsPDS, OsBADH2*), Yes(*OsMPK2*)	Plants	17
	OsSWEET11, OsSWEET14	Cas9	Indel	91% (*OsSWEET11*)[b] 90% (*OsSWEET14*)[b]	N.D.	Protoplasts	18
	OsBADH2, OsCKX2	TALEN	Biallelic indel	12.5% (*OsBADH2*), 3.4% (*OsCKX2*)	N.D.	Calli	19
	OsBEL	Cas9	Biallelic indel	2.2%	No	Plants	20
	OsPDS	Cas9	Introducing KpnI + EcoRI sites	6.9%	No	Protoplasts	17
Soybean	*DCL4b*	ZFN	Biallelic indel	25%	N.D.	Plants	21
	FAD2	TALEN	Biallelic indel	33.3%	N.D.	Plants	22
Sweet orange	*CsPDS*	Cas9	Indel	3.2-3.9%	No	Leaf	23
Tomato	*PROCERA*	TALEN	Biallelic indel	2.5%	No	Tissue culture (explants)	24
Wheat	*TaMLO*	Cas9	Indel	28.5%	N.D.	Protoplasts	17
	TaMLO	TALEN	Heterozygous indel for all three homoeoalleles	3.70%	N.D.	Plants	25

Abbreviations: N.D., not determined.

[a]Two different donor constructs containing short homology arms were used: one with an autonomous herbicide tolerance gene expression cassette (PAT), the other with a non-autonomous donor that relied on precise trapping of the endogenous *ZmIPK1* promoter for expression of the marker.

[b]Indicates the results of sequencing after the enrichment of mutated alleles.

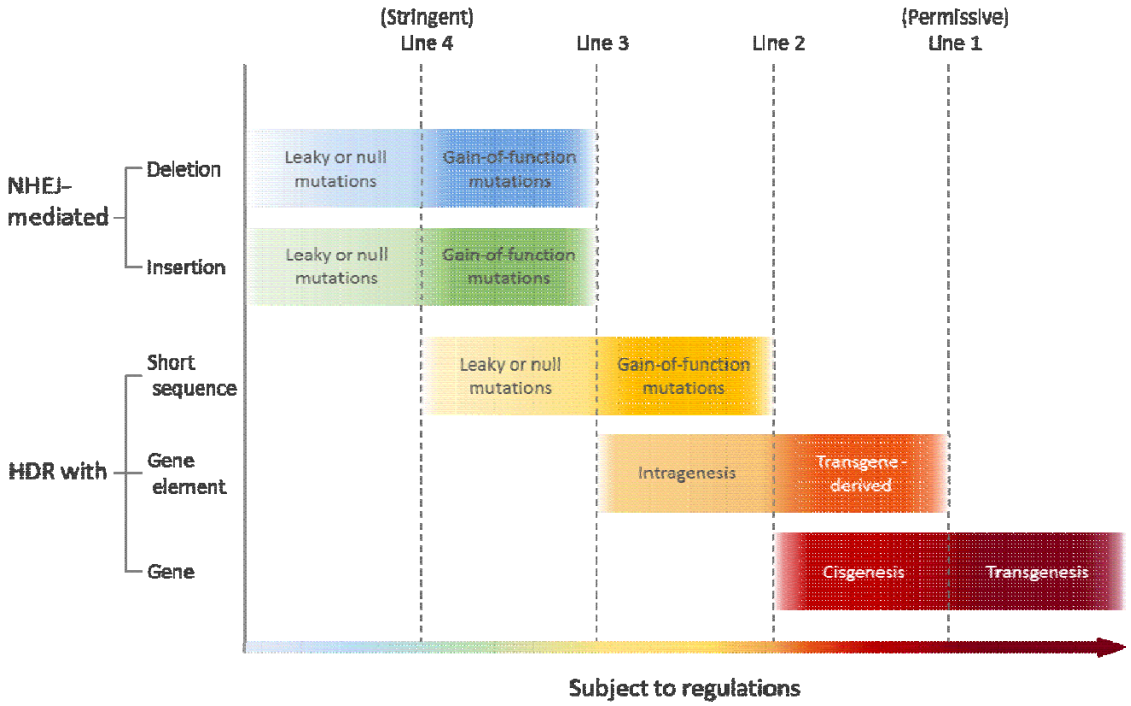

Figure 1. The presumed regulatory relevance of crop mutants generated with genome-editing. This analysis assumed that genome editing enzymes are introduced in the form of protein or RNA, not DNA. Firstly, the genome-editing pathways were categorized as NHEJ and HDR because HDR requires exogenous DNA that may potentially increase the regulatory relevance in light of the definition of a 'living modified organism' in the Cartagena Protocol on Biosafety. HDR was further segmented into three pathways according to the length of exogenous DNA. Although NHEJ can cause gene modification similar to HDR with a short DNA sequence, such pathways should be treated differently from a regulatory viewpoint. Therefore, NHEJ-mediated indel was further subdivided into deletion and insertion, with insertion being placed near the HDR with a short sequence due to the higher similarity of these two gene modifications . Secondly, genome-edited crops were subdivided based on types of mutation, in order to map these mutations according to their regulatory relevance. NHEJ-mediated deletion and insertion were categorized into gain-of-function and leaky or null mutations. HDR using a gene were categorized into transgenesis and cisgenesis. Cisgenesis was considered to be less subject to the regulations than transgenesis because a cisgene is derived from a cross-compatible species. Likewise, crops resulting from HDR with a gene element were subdivided and placed according to the origin of the element. Moreover, they were considered to be less subject to the regulations than crops derived from HDR with a gene, since only a gene element was

used. Similarly, HDR with a short sequence were considered to be less relevant to the regulations than HDR using a gene element because the short sequence is only a portion of the gene element. Because crops generated via HDR with a short sequence are more likely to resemble crops generated via NHEJ on a product-basis, HDR with a short sequence was subdivided into gain-of-function mutations and loss-of-function mutations. Crops generated via HDR with a short sequence were considered to be more relevant to the regulations than crops produced via NHEJ owing to the use of exogenous DNA. Four potential regulatory lines are vertically indicated from the most stringent (Line 4) to the least stringent (Line 1). Mutants which are mapped beyond the regulatory line are subject to the regulations. Leaky mutations denote a type of mutation that may leave some function, but not at the level of the wild type allele. Abbreviations: HDR, homology-directed repair; NHEJ, non-homologous end joining.