Entry

CRISPR towards a Sustainable Agriculture

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Definition: Climate change and the need to feed an increasing population undermines food production and safety, representing the reasons behind the development of a new agriculture that is much more sustainable, productive and accessible worldwide. Genome editing and, in particular, clustered regularly interspaced palindromic repeats/CRISPR-associated protein (CRISPR/Cas) tools will play a major role in plant breeding to address these concerns. CRISPR/Cas includes a series of genome editing tools relying on the recognition and cleavage of target DNA/RNA sequences to introduce specific mutations.

Keywords: CRISPR; crop improvement; genetic variability; stress tolerance; food quality; synthetic biology; sustainable agriculture

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1. Introduction

The term "sustainable agriculture" encloses practices of farming addressed to the production of high-quality and safe agricultural products without compromising natural environments and the social and economic conditions of farmers. According to the Agricultural Sustainability Institute at UC Davis (https://www.nal.usda.gov/legacy/afsic/sustainable-agriculture-definitions-and-terms, accessed on 10 January 2022), the main goal of sustainable agriculture remains the preservation of the ability of future generations to meet their own needs, ensuring inclusive economic growth.

In the era of climate change, environmental threats will affect farmers at both the economic and the agronomic level, influencing crop yield and quality and with further negative effects on plant resistance to both abiotic and biotic stress. In order to counteract these environmental threats, and to have the chance to reach a sustainable production of industrial manufacture, new technologies will be used on the basis of the current knowledge in the biotechnology field.

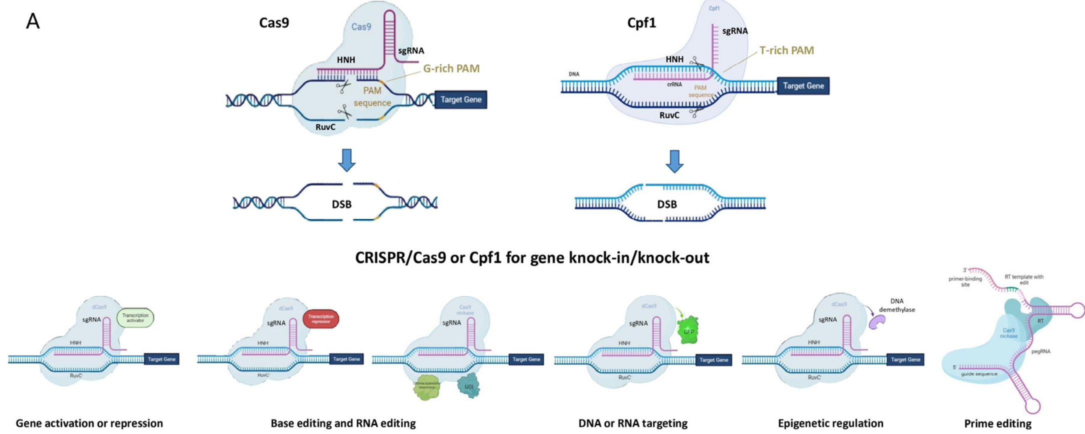
Clustered regularly interspaced palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) represent a new perspective for genetic engineering and the last frontiers of the new breeding techniques (NBTs) and genome editing (GE) tools.

1.1. CRISPR/Cas System

CRISPR/Cas systems are part of the adaptative immune system of archaea and bacteria in ensuring protection against viruses. The mechanism of action relies on the recognition and the cleavage of foreign DNA or RNA of invading viruses. One of the main characteristics is the integration of short fragments of the invading DNA (spacers) into the CRISPR locus, conferring the heritable immunity of bacteria. The CRISPR locus consists of an array of unique spacer sequences, derived from foreign invading DNA and interspaced by identical repeat sequences (crisprRNA or crRNA), along with a sequence encoding for the

trans-activating crRNA (tracrRNA) and a series of genes encoding CRISPR-associated (Cas) endonucleases, responsible for the cleavage of the genetic material. The transcription of the CRISPR locus allows for the formation of a single mRNA (pre-crRNA), which is partially complementary to tracrRNA, leading to the formation of an RNA duplex. The RNase III recognizes the RNA duplex and cleaves the double-stranded RNA (dsRNA) to form crRNA–tracrRNA complexes that activate and drive the Cas protein to the target sequence. The Cas protein is capable of introducing a double-strand break (DSB) only in the presence of a short conserved protospacer-adjacent motif (PAM) downstream of the target DNA, representing an essential prerequisite for the recognition of the target sequence [1,2].

CRISPR/Cas systems have been divided into two classes, six types and several subgroups based on Cas proteins and the nature of the interference complex [3]. To date, the most common type, used as a genome editing tool, is the Type II Cas9 from *Streptococcus pyogenes* (*Sp*Cas9). The Cas9 protein consists of a bi-lobed architecture, including a large recognition (REC) lobe and a small nuclease (NUC) lobe. The NUC lobe includes a protospacer-adjacent motif (PAM)-interacting domain (PI) and two cleavage domains known as RuvC and HNH domains, each of which cleaves one strand of the target DNA three nucleotides upstream of the PAM sequence (Figure 1A) [4,5]. The REC lobe contributes toward activating Cas proteins when combined with the tracrRNA–crRNA complex.



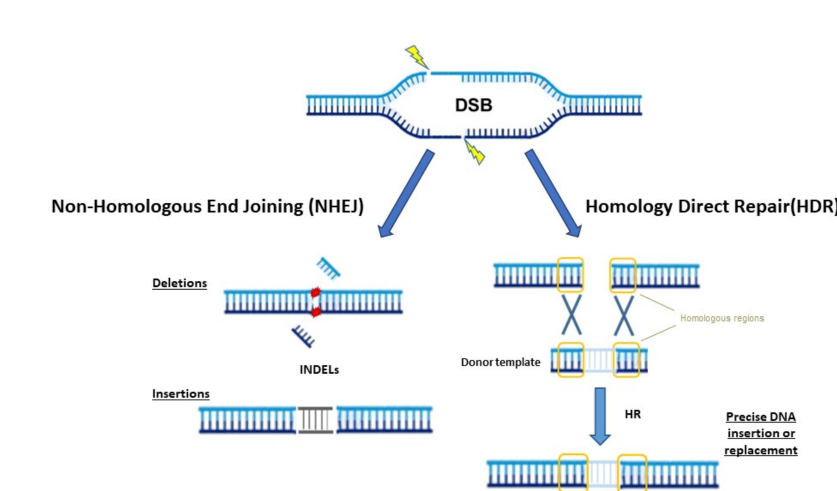


Figure 1. (**A**) Principal CRISPR/Cas9 or Cpf1 types of editing (top) and possible use for gene knock-in or knock-out (bottom), synthetic-guide RNA (sgRNA), double-strand break (DSB), protospacer-adjacent motif (PAM), reverse transcriptase (RT), uracil DNA glycosylase inhibitor (UGI), green fluorescent protein (GFP) and prime editing gRNA (pegRNA). (**B**) The two main DNA repair mechanisms and genetic mutations.

The use of the CRISPR/Cas system as a genomic engineering tool occurred when Jinek et al., 2012 [4] (Figure 2) showed that the target DNA sequence could be reprogrammed simply using a chimeric synthetic-guide RNA (sgRNA), obtained by the fusion of crRNA and tracrRNA sequences and changing the 20 nucleotides of the crRNA that confer the targeting specificity. Once the Cas9 combined with the sgRNA recognizes the complementary spacer sequence adjacent to PAMs on double-strand DNA, genetic modifications are produced through the induction of DSB (Figure 1A), followed by the activation of DNA repair mechanisms (Figure 1B) through non-homologous end-joining (NHEJ) or homologous direct repair (HDR) [6–8].

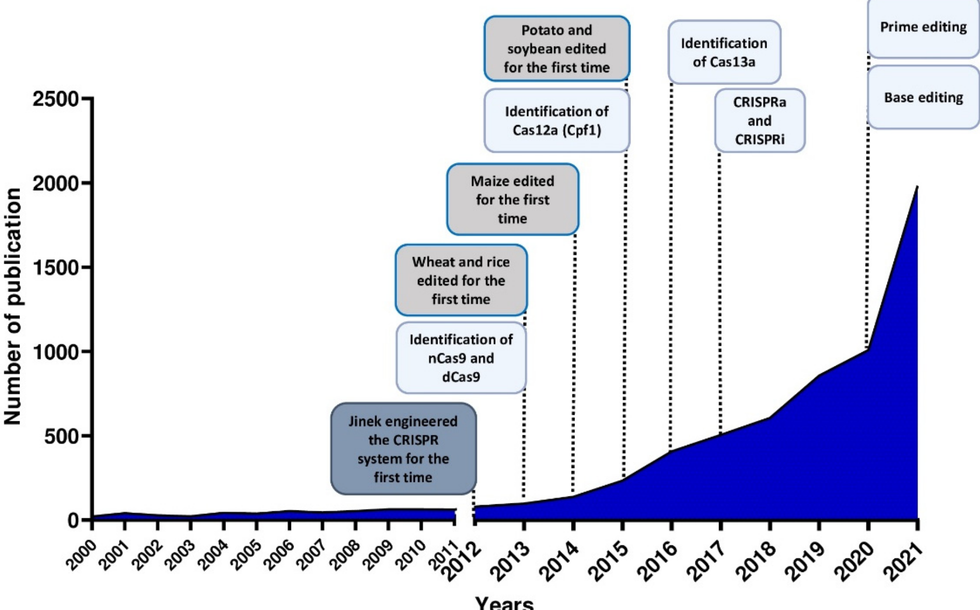


Figure 2. Number of publications in plant breeding using CRISPR systems and major achievements in the last 10 years.

1.2. CRISPR Variants, Orthologs and Engineered Systems

New types of Class II CRISPR/Cas systems are continuously found in bacteria, showing different features, and customized for use in plants (Figure 2). Several Cas9 orthologs, with different PAM specificities from other bacteria, have been discovered and some of them have already been applied for genome editing in model species [9]. Furthermore, it has been demonstrated that mutations in the PAM-interacting domains of wild-type *Sp*Cas9 could lead to *Sp*Cas9-engineered variants recognizing different PAM sequences [10], thus increasing the spectrum of editable target sites in the genome.

For example, Cas13a (C2c2) enzyme belongs to the Type VI Class II (Figure 1A). It is specialized in RNA recognition and cleavage, enabling its use in post-transcriptional repression, degradation of genetic material of RNA viruses and RNA binding [11]. Two studies have demonstrated the activity of Cas13a in rice protoplast to knock down endogenous genes and in *Nicotiana benthamiana* plants for interfering against RNA viruses [12,13]. Otherwise, the Cas12a (Cpf1) enzyme belongs to Class II Type V (Figure 1A) and differs from Cas9 in several important features: it recognizes the target region through PAM favoring AT-rich regions (5′-TTTN-3′), it cleaves the target sequence by producing DNA ends with a 5′ overhang and the crRNA directly functions as a guide RNA without the need for a complex with tracrRNA to be processed. This Cas protein has been used in many crop species [14] since its first application in rice and tobacco [15].

Furthermore, the Cas9 protein has been re-engineered through point mutations in RuvC and/or HNH nuclease domains, inactivating the catalytic activity of each domain (Figure 1A). It can be exploited to produce a nickase (nCas9) or a dead enzyme (dCas9) with complete loss of DNA cleavage activity. The nCas9 is usually used to enhance the specificity of CRISPR/Cas9, combining two nCas9 with pairs of sgRNAs that respectively cut only

one DNA strand and therefore increase the number of recognized target bases. The dCas9 protein can operate as cargo to load and deliver proteins with different functions to a specific target site. The use of inactive enzymes (nCas9 or dCas9) can also facilitate the directional introduction of DNA fragments at a specific site and the assembly of base and prime editors (discussed below). This enzyme has been employed in base editing as well as in genetic and epigenetic regulation of gene expression [16]. Otherwise, the most frequent use of dCas9 is in the activation (CRISPRa, activator) and repression (CRISPRi, interfering) of gene expression without introducing mutations in the genome (Figures 1A and 2). Following this approach, the dCas9, guided by a sgRNA to a specific regulatory region, is fused to transcriptional modulators, which are generally transcriptional factors or protein domains recruiting key regulatory elements to control gene expression. Their use has been reported in plants both as activators [17–20] and as repressors [21,22]. The dCas9, associated with an acetyltransferase or a methyltransferase, can also act at the epigenetic level as CRISPRi and CRISPRa systems [23,24].

Both nCas9 and dCas9 are involved in the assembly of base editing and prime editing systems. Base editors, including cytosine base editors (CBEs) and adenosine base editors (ABEs), catalyze C/G to T/A or A/T to G/C transitions in DNA or RNA molecules (Figures 1A and 2). They have been optimized for plant genomes, providing high efficiency and precise editing at single base resolution [25]. Base editing systems have been applied in major crops such as wheat, rice, maize, tomato, potato, soybean and rapeseed [26,27]. Prime editing enables rewriting of genetic information into a specified DNA target site using a reverse transcriptase (RT) fused to a nickase enzyme and a prime editing guide RNA (pegRNA) to copy genetic information directly into the target genomic sequence (Figures 1A and 2) [28]. Although prime editing still needs to be improved for editing efficiency in plants, it has been applied to obtain precise modifications in rice, maize, potato and tomato [27–29], especially using the second generation of prime editors (PE2), in which an engineered RT with improved features (such as increased processivity, substrate affinity and inactivated RNase H activity) is fused to an nCas9 [28,30].

1.3. New Perspectives for the Use of CRISPR/Cas System

CRISPR opens many doors for plant breeders to boost breeding programs towards ambitious targets, thanks not only to the feasibility of its application in a wide range of crop species but also to its versatility as a genetic tool that is constantly evolving. The use of CRISPR-engineered systems can foster the generation of a wide range of heritable genetic mutations such as In/Dels, targeted insertions, point mutations and nucleotide substitutions that are the most frequent modifications obtained, as well as targeted chromosomal rearrangements and genetic or epigenetic control of gene expression (Figure 1A). In addition, it offers the advantage of decreasing off-targets and pleiotropic effects, without neglecting the possibility of obtaining transgene-free edited plants.

The possibility of producing transgene-free plants has been exploited through self-pollination and segregation of exogenous DNA [31], transiently expressing a plasmid vector encoding for Cas9 and gRNAs [32,33] or through the delivery of pre-assembled CRISPR/Cas9 ribonucleoproteins (RNPs) [34]. By delivering the Cas9 protein instead of the vector, there is no transfer of specific DNA from one species to another. In addition, the Cas9 protein remains inside the cells for three/five days and then is degraded, also reducing the off-target events. All together, these approaches will make CRISPR a useful tool for a new generation of plants that potentially do not fall within the scope of the current regulation process of genetically modified (GM) products.

Moreover, the discovery and the improvement of CRISPR as a precise genome editing tool has resulted in the establishment of several CRISPR-based companies that are hoping to capitalize on this new technology. Indeed, the potential of gene editing to address the 21st century's problems has taken hold in the agricultural industry and the list of CRISPR companies is growing each day. As an example, Synthetic Genomics® (https://www.viridos.com/, accessed on 17 November 2021) uses synthetic biology solutions to produce microalgae

with higher levels of lipids to be used to address global sustainability problems. Plantedit® (https://plantedit.com/, accessed on 17 November 2021) uses genome editing to generate modified soybean with a high oil content. Pairwise Plants (https://www.pairwise.com/, accessed on 17 November 2021) is currently developing edited plants to assist farmers by providing them with new varieties of crops that require fewer resources to grow. Inari Agriculture® (https://inari.com/, accessed on 17 November 2021) is using CRISPR to enhance plant breeding, by managing specific gene expression in plants, to develop customized seeds. Hudson River Biotechnology® (https://www.hudsonriverbiotechnology.com/, accessed on 17 November 2021) employs CRISPR technology to edit plants and microorganisms through a validated molecular breeding workflow called TiGER (Target identification, Guide selection, Entry into the cell and Regeneration). Yield10® Bioscience (https://www.yield10bio.com/, accessed on 17 November 2021) aims to improve the yield of crops such as canola and soybeans, and also to increase the oil content of these and other oilseeds. Other relevant companies are Benson Hill Biosystems® (https://bensonhill.com/, accessed on 17 November 2021), Corteva® (https://www.corteva.com/, accessed on 17 November 2021) (agricultural division of DowDuPont), Syngenta® (https://www.syngentagroup.com/, accessed on 17 November 2021) and Tropic Biosciences® (https://www.tropicbioscience.com/, accessed on 17 November 2021).

Here, we focus on the recent advances in CRISPR technology for the improvement of the most cultivated crop species and its potential applications in synthetic biology (Figure 2), with particular regard to traits such as quality, yield and stress tolerance. Lastly, we report a quick focus on the global regulatory framework on GM plant legislation.

2. Applications

Since Jinek et al. (2012) [4] engineered for the first time the bacterial CRISPR system, it was not long before there was a demonstration of the application of CRISPR technology in plants [35–37], as shown by the increasing number of scientific publications that have been published during the last 10 years (Figure 2).

In 2013, as first crop species, rice was edited to disrupt the *OsPDS* and *OsBADH2* genes by Shan and colleagues, and soon after Upadhyay et al. (2013) [38,39] used CRISPR/Cas-mediated genome editing to target the *inositol oxygenase* (*inox*) and *phytoene desaturase* (*pds*) genes using a cell suspension culture of wheat. In maize, the *ZmIPK* gene was edited in protoplasts in 2014 [40]. Brooks et al. (2014) [41] produced tomato homozygous edited plants in T_0 generation targeting the *ARGONAUTE7* (*SlAGO7*) gene through CRISPR/Cas9.

Since then, many crop species have been edited by the CRISPR system. In 2015, two research groups applied CRISPR to edit potato genes [42,43] and, in the same year, many scientific works reported genome modifications in soybean with CRISPR/ Cas9 [44–47]. Consequently, CRISPR became a powerful tool in crop breeding to improve several crop traits (Table 1), including yield, quality and safety, biotic- and abiotic-stress resistance, plant pharming and other applications in the field of medical plants [48].

Even if CRISPR has been widely used for various applications during the last 10 years (i.e., in enhancing both biotic and abiotic stress, improving yield performance and quality traits through biofortification), only a few genome-edited products are currently on the market [49].

Table 1. List of CRISPR applications in crop species. Plant species, targeted genes and resulting traits are reported for each field of application.

Application	Plant Species	Target Genes	Resulting Traits	References
Genetic Variability	Rice Pea Tomato Wheat	*Zep1*	Enhanced genetic recombination frequency	[50]
		RECQ4	Increased crossover frequency	[51]
		ZIP4-B2	Enhanced recombination between homeologous chromosomes	[52]
Stress tolerance	Rice Maize Wheat Soybean Potato Tomato	*TIFY1b*	Improved adaptation to low temperature	[53]
		ERF922	Improved resistance to rice blast	[54]
		eIF4G	Improved resistance to rice tungro spherical virus	[55]
		Als2	Chlorsulfuron-resistant maize	[56]
		ARGOS8	Improved resistance to drought	[57]
		MLO	Enhanced resistance to powdery mildew	[58]
		Qsd1	Longer dormancy	[59]
		F3H1, F3H2, FNSII-1	Increased isoflavone content and resistance to soybean mosaic virus	[60]
		ALS	Development of glyphosate-resistant soybean	[61]
		RXLR effector gene *Avr 4/6*	Enhanced tolerance to *Phytophthora infestans*	[62]
		Mlo1	Reduced powdery mildew susceptibility	[31]
		ACET1a, ACET1b	Increased resistance to *Botrytis cinerea*	[63]
		MAPK3	Enhanced tolerance to heat stress	[64]
Yield	Rice Maize Wheat Soybean Tomato	*GN1a, DEP1, GS3*	Enhanced grain number and size, dense erect panicles	[65]
		GW2, GW5, TGW6	Enhanced grain weight	[66]
		PYL1, PYL4, PYL6	Improved growth and productivity	[67]
		CLE	Enhanced kernel number	[68]
		GW2	Enhanced grain size and weight	[69]
		GASR7	Enhanced grain size	[70]
		GW7	Enhanced grain size and weight	[71]
		FT2a, FT5a	Increased numbers of pods and seeds	[72]
		fas, lc	Enhanced fruit size	[73]

Table 1. *Cont.*

Application	Plant Species	Target Genes	Resulting Traits	References
Quality	Rice Maize Wheat Potato Sweet potato Tomato Barley Rapeseed	*CrtI, PSY*	High β-carotene content	[33]
		GAD3	High GABA content	[74]
		SBEIIb, SBEI	High amylose content	[75]
		GBSS	Low amylose content	[76]
		GBSS	Low amylose content	[77]
		IPK1	Low phytic acid content	[78]
		SBEIIa	High amylose content	[79]
		α-gliadin genes	Low gluten content	[80]
		CM3, CM16	Reduced amount of potential allergens	[81]
		ASN2	Reduced free asparagine	[82]
		SBEI	High amylose content	[83]
		GBSS	Low amylose content	[84]
		SBEII	High amylose content	[84]
		SGR1, LCY-E, Blc, LCY-B1, LCY-B2	High lycopene accumulation	[85]
		GAD2, GAD3	High GABA content	[86]
		GABA-Ts, SSADH	High GABA content	[87]
		GBSS	Low amylose content	[88]
		D-hordein	Reduced prolamine content and increased glutenin content	[89]
		ITPK	Low phytic acid content	[90]
Synthetic Biology	Rice Potato Tomato Barley Arabidopsis Switchgrass Rapeseed Salvia Tobacco	*CrtI, PSY*	Insertion of large DNA fragments	[33]
		>GBSS	Low amylose content	[91]
		PSY1	Inter-homologous somatic recombination	[92]
		CRTISO	Inter-homologous somatic recombination	[93]
		COMT-1	Increased bioethanol concentration of the mutant biomass	[94]
		Chromosome 1, Chromosome 2	Reciprocal translocation	[95]
		4CL	Used as lignocellulosic feedstock for bioenergy	[96]
		FAD2	Increased content of oleic acid	[97]
		CPS1	Customization of secondary metabolite profiles	[98]
		NtAn1	Increased seed lipid accumulation for biodiesel production	[99]

2.1. Broadening Genetic Variability

Crop breeding programmes generally rely on germplasm resources, the first source of genetic variation, essential to produce a progeny with suitable agronomic traits [100]. Considering that crop breeding programmes could take years due to the availability of beneficial alleles, several approaches have been developed during the last 40 years to face this problem [101]. In particular, non-naturally occurring alleles can be generated by the use of physical and chemical mutagenesis. At present, over 3000 commercial varieties of food crops have been produced by mutagenesis (IAEA Mutant Variety Database) [102]. Although the mutagenesis-based approach is important for broadening genetic variability in crops, it is time-consuming [103].

Due to their intrinsic potential, genome editing techniques, and in particular CRISPR/Cas9 systems, are currently being used in a precise and predictable way to introduce new genetic variability useful for the improvement of most cultivated crops [104,105]. In a few years, CRISPR demonstrated its ability to create variation in the gene pool, exploiting its core skills to produce gene knock-out or knock-in mutations, besides its ability to regulate gene expression at genetic and epigenetic levels. CRISPR has been used for introducing targeted single and multiple changes in plant genomes, allowing for the generation of mutants, including some genes that are difficult to access using traditional breeding.

A better understanding of the potential of the CRISPR/Cas arsenal and its potential epigenetic applications can lead to the generation of new genetic variability suitable for developing novel varieties with new allele combinations. Meiotic crossovers infrequently occur, limiting the production of new alleles and their combination into crops. In this context, the induction of crossover events between homologous or non-homologous chromosomal regions [106], manipulating meiotic recombination, can lead to broadening genetic variability [107,108]. An increase in crossover frequency has been achieved in plants through the editing of some genes that limit meiotic recombination. In particular, mutations in *RecQ Like Helicase 4* (*RECQ4*) provoked an increase in crossovers in rice, pea and tomato [51]. Moreover, mutations of *zeaxanthin epoxidase ZEP1* enhanced genetic recombination frequency in rice, as shown by Liu et al., 2021 [50]. In wheat, the *ZIP4-B2* facilitates homologous recombination (HR) but inhibits crossovers between homeologous chromosomes. Martin and colleagues (2021) [52] obtained a mutant of *ZIP4-B2* called *zip4-ph1d* that normally participates in HR but also allows recombination between homeologous chromosomes.

Indeed, these techniques represent an easier tool to introduce a novel wide range of desirable traits into cultivated crops [109–112].

2.1.1. Enhancing Stress Tolerance

Among the agricultural applications, several targets of CRISPR are related to the achievement of abiotic or biotic stress tolerance [113,114]. Generally, crop plants are able to overcome both biotic or abiotic stresses through changes that occur at the morphological, physiological, biochemical and molecular level [115]. However, the development of CRISPR may involve simple or complex mutations or the integration of specific genes in the target genome that may increase the breeding efficiency and the development of plant traits that were previously difficult to obtain [111,115]. Several crop species, including bacterial-resistant banana and rice, fungus-resistant wheat and rice, drought-tolerant soybean, rice and maize, and salt-tolerant rice, have been studied using a gene-editing approach, and some of these are near the end of the research pipeline [111,116,117].

Among cultivated crops, rice (*Oryza sativa* L.) is the major food source for more than three billion people, and several studies using CRISPR are ongoing [39,118,119]. Many studies involving the use of the CRISPR/Cas9 technique for targeting multiple genes have been carried out [120–122]. Rice productivity is harshly limited due to the high concentration of salt in the soil that negatively affects plant growth and development, with negative consequences on the yield. In addition, diseases caused by biotic agents, including bacteria, fungi, viruses and insects, also negatively affect yield loss, leading to poor product quality [119]. As an example, the *TIFY1b* transcription factor is one of

the cold tolerant genes in rice and its role was recently investigated in rice adaptation to low temperature through the CRISPR/Cas9 approach [53]. In 2019, researchers from the National Institute for Plant Biotechnology (NIPB) in New Delhi used CRISPR to study a rice able to withstand high concentrations of salt in soil [123]. In addition, Agrisea (https://www.agrisea.co.uk/, accessed on 17 November 2021), an ocean agriculture start-up, developed a salt-tolerant rice that can be grown in salty ocean water without the use of soil, fertilizer or freshwater. Agrisea expects to have multiple modular floating mini-farms in the ocean by the end of 2021 and is already engaging in talks with major rice-producing and -consuming countries such as Vietnam, China and Bangladesh. However, Agrisea is waiting for the approval of both the Food and Drug Administration (FDA) and the US Department of Agriculture (USDA) to commercialize their salt-tolerant rice seeds. Moreover, the fungus *Magnaporthe oryzae* causes the most destructive disease in rice, known as rice blast, with up to 60–100% yield losses worldwide [124]. CRISPR/Cas9-targeted knock-out of ERF transcription factor gene *OsERF922* has demonstrated enhanced resistance to rice blast [54], suggesting that these kinds of approaches are promising for future targeted improvements. In fact, in 2018, CRISPR/Cas9-mediated editing produced rice plants resistant to the rice tungro spherical virus (RTSV) responsible of the rice tungro disease (RTD), producing the novel allele *EIF4G* [55]. Lastly, Colombian scientists from the International Center for Tropical Agriculture (CIAT) used CRISPR/Cas9 to confer resistance to rice hoja blanca virus (RHBV) and *Xanthomonas oryzae* [125].

Maize represents another largely used cereal crop, providing feed, food and fuel for more than 6000 million humans [126]. Maize grain yield is closely related to plant population density and could be affected by several factors, including water availability and soil fertility [127]. The first chlorsulfuron-resistant maize plants were developed by Svitashev et al. (2015) targeting the *Als2* gene [56]. Recently, an application for a gene-edited maize that can tolerate water stress has been submitted to the Forestry and Agricultural Protection Division of the Agricultural and Livestock Service (SAG) [128]. In addition, in 2016, DuPont developed a drought-resistant maize using CRISPR [57] to edit the *ARGOS8* locus, a negative regulator of the ethylene response. The field study showed that *ARGOS8* variants had no yield loss under watered conditions [57]. Studies on maize that can withstand heat, UV radiation and drought are currently ongoing as field trials in Belgium, but only for research purposes (https://vib.be/news/permit-crispr-maize-field-trial-aims-measure-climate-stress, accessed on 17 November 2021). Furthermore, the Corteva Agri-Science and the International Maize and Wheat Improvement Center (CIMMYT) is developing maize varieties resistant to maize lethal necrosis (MLN), a viral disease that causes serious crop loss.

There are only few available studies for CRISPR techniques to address the major abiotic and biotic stresses in wheat compared to other cereal crops [129]. The first available CRISPR-mediated approach was used to knock out the *TaMLO*, *TaPDS* and *TaINOX* genes [38,39]. Subsequent studies permitted the simultaneous knock-out of *TaMLO* homeoalleles, conferring resistance to powdery mildew in bread wheat [58]. Rain-resistant wheat was developed by researchers from the National Agriculture and Food Research Organization (NARO) and Okayama University in Japan [59] targeting the homeologous *Qsd1* loci by the use of *Agrobacterium*-mediated CRISPR/Cas9.

Among cultivated crops, soybean (*Glycine max* (L.) Merr.) is a non-native and non-staple crop in sub-Saharan Africa (SSA) with potential to be a commercial crop owing to its wide range of uses as food, feed and industrial raw material. Soybean low yield can be attributed to the use of poor-performing varieties and to the limited application of fertilizers and rhizobial inoculants in soils with no history of soybean production [130]. However, multiplex CRISPR/Cas9-mediated metabolic engineering was performed to increase soybean isoflavone content and resistance to soybean mosaic virus [60]. In addition, researchers from Universidad de la República and Instituto Nacional de Investigación Agropecuaria (INIA) used CRISPR to develop glyphosate-resistant soybean [61,128], while the Brazilian company Tropical Melhoramento & Genética and the Israeli company Evogene

collaborated to develop a soybean resistant to a plant nematode that destroys crops and decreases crop yields (https://www.evogene.com/, accessed on 17 November 2021).

Among Solanaceae, potato (*Solanum tuberosum* L.) has tremendous significance due to its nutritional quality. Potato cultivation can be very challenging where there is erratic rain precipitation and water supply is scarce [131]. Due to its value, it remains worthwhile to develop biotic and abiotic stress-tolerant potatoes, considering that potato research is difficult due to the tetrasomic inheritance of cultivated potato. To date, a very limited number of studies have been reported in potato species using the CRISPR technology, and the majority were recently summarized by Dangol et al., 2019 [131]. The first edited potato was obtained in 2015 through the modification of the *StIAA2* gene, as reported by Wang et al., 2015 [42]. Attempts have been made to establish a CRISPR/Cas9-mediated editing to generate potatoes tolerant of *Phytophthora infestans*, the oomycete responsible for potato late blight. Unfortunately, no mutagenized plants were detected in this study [62]. Moreover, Russian Academy of Sciences (RAS) institutes are developing gene-edited disease-resistant varieties of potatoes [132].

Tomato (*Solanum lycopersicum* L.), as one of the most cultivated vegetables and thanks to its aptitude for transformation and regeneration, lends itself to genetic modifications in order to facilitate the characterization of gene function, precision breeding and genetic improvement for resistance to biotic and abiotic stresses [133]. For example, the CRISPR/Cas9-induced mutation of *SlMlo1* significantly reduced powdery mildew susceptibility, allowing for the generation of transgene-free powdery mildew-resistant tomato in less than 10 months [31]. Recently, CRISPR/Cas9-induced mutants of both tomato acetylenase-encoding genes *ACET1a* and *ACET1b* showed increased resistance to *Botrytis cinerea* [63]. Indeed, high temperatures are a major environmental stress that can limit plant growth and agricultural productivity. Chinese researchers focused on *SlMAPK3*, one of the genes involved in heat stress. In particular, CRISPR/Cas9-mediated *slmapk3* mutants exhibited more tolerance to heat stress than wild-type plants, suggesting that *SlMAPK3* was a negative regulator of thermotolerance [64].

Even if there is a multitude of studies regarding tomatoes, only few of them are at the end of the research pipeline. As an example, Nexgen Plants Pty Limited and University of Queensland produced gene-edited tomato allowing plants to detect and destroy the tomato spotted wilt virus and cauliflower mosaic virus. The USDA recently determined that the six tomato lines (NP-TV101-1, NP-TV101-2, NP-TV101-3, NP-TV201-1, NP-TV201-2 and NP-TV201-3) do not fall under the federal regulations for genetically engineered plants (https://www.isaaa.org/kc/cropbiotechupdate/article/default.asp?ID=17661, accessed on 17 November 2021).

2.1.2. High Yield

The impact of climate change and the need to satisfy dietary requirements due to rapid population growth are the main reasons for improving crop yield and food quality in a sustainable manner.

Crop yield is a complex trait influenced by polygenic regulation and the strong effect of environmental factors. For example, rice yield is determined by several traits such as the number of panicles per plant, the number of grains per panicle and grain weight and size. Several studies have shown that the expression of some genes negatively affects the yield and that their CRISPR/Cas9-mediated knock-out could have positive effects. To date, many genes including *GN1a*, *DEP1*, *GS3*, *GW2*, *GW5*, *TGW6* in rice have been silenced using CRISPR/Cas9 technology with a significant increase in grain weight and size, grain number and dense and erect panicles [65,66]. Similarly, the knock-out of *GW2*, *GW7* and *GASR7* genes in hexaploid wheat resulted in an increase in seed size and weight [69–71,134]. In addition, maize kernel number per ear depends on inflorescence meristem controlled by the CLV-WUS feedback signaling pathway. Genome editing of cis-regulatory regions can adjust the expression level or pattern to enhance meristem activity and grain yield [68].

Similarly, the editing of the cis-regulatory element of the CLV-WUS pathway led to a larger fruit size in tomato [73].

To date, cultivars with improved yield obtained through the CRISPR/Cas system are not yet on the market. Despite this, a few studies have reached the trial stage. As an example, the simultaneous mutation of the *PYL1*, *PYL4* and *PYL6* genes improved growth and increased grain yield in rice. Phenotypic and agronomic analyses carried out on selected mutants, grown in the paddy fields of Shanghai and Lingshui County of Hainan Island under natural conditions, showed that the triple null *pyl1/4/6* mutant had a 25–31% greater production compared to traditional varieties [67]. Furthermore, in a study conducted in China, the Chinese Academy of Sciences developed a soybean variety that can grow in warmer climates and is able to produce higher yields. Conversely, soybean crops planted in warmer climates generally have low yields due to early flowering and maturity. By silencing *GmFT2a* and *GmFT5a* through the CRISPR/Cas9 system, Cai and colleagues demonstrated that mutants flowered 31 days later and produced significantly increased numbers of pods and seeds per plant compared to the wild type [72].

In Japan, a research team from Tsukuba University used CRISPR/Cas9 to knock out two genes in rice in order to increase the grain number and size. In May 2017, the National Agriculture and Food Research Organization developed an experimental cultivation of rice with a higher yield trait obtained by CRISPR technology (Agricultural Biotechnology Annual_Tokyo_Japan_11-16-2017).

2.1.3. Quality Improvement

An important goal of genetic improvement concerns the nutritional quality of food. In fact, according to the World Health Organization (WHO, Geneva, Switzerland), malnutrition due to nutrient deficiency affects about 2 billion people worldwide, especially in underdeveloped and developing countries, leading to serious health, economic and social consequences (WHO, 2009).

In this context, vitamin A deficiency (VAD) is one of the major causes of malnutrition in the world and mainly affects children and pregnant women, causing several health complications such as vision damage, high susceptibility to infection, fetal malformations and neonatal death [135]. CRISPR/Cas9 genome editing has been successfully applied in rice and tomato using different strategies for carotenoid biofortification. In rice, the overexpression of *CrtI* and *PSY* led to marker-free mutants containing a good amount of β-carotene in dry weight [33], while in tomato the knock-out of five genes (*SGR1*, *LCY-E*, *Blc*, *LCY-B1* and *LCY-B2*) associated with the carotenoid metabolic pathway promoted the accumulation of lycopene, a bioactive component for treating chronic diseases and lowering the risk of cancer and cardiovascular diseases [85].

Another important example of biofortification concerns the increase in γ-aminobutyric acid (GABA). GABA is a non-proteinogenic amino acid inhibitory neurotransmitter effective in lowering blood pressure. The first study describing the application of the CRISPR/Cas9 system to increase GABA content in tomato fruits was carried out in Japan [86]. Through the mutation of the C-terminal region encoded by the *SlGAD2* and *SlGAD3* genes, GABA content was increased in leaves and fruits. In addition, Li and colleagues used a multiplex CRISPR/Cas9 system to knock out the *SlGABA-Ts* and *SlSSADH* genes in tomato, obtaining a twenty-fold increase in GABA content [87]. In rice, by mutating the C-terminal of the *OsGAD3* gene the GABA content increased seven-fold [74].

Phythic acid (PA) is the major phosphorus storage sink within the plant seed and, due to the lack of phytase in the digestive tract, is poorly digested by monogastric animals, including humans. PA is considered an anti-nutritional compound since it limits the bioavailability of phosphorus and minerals [136]. Low phytic acid (*lpa*) mutants were obtained in *Brassica napus* by silencing three functional paralogs of *BnITPK* gene. A 35% decrease in phytic acid and a simultaneous increase in P_i were found in the mutants [90]. Using CRISPR/Cas9 in common wheat, the disruption of *TaIPK1.A* led to an approxi-

mately 1.5-to 2.1-fold increase in the Fe concentration and to a 1.6-to-1.9-fold rise in Zn concentration due to significant reduction in phytic acid content [78].

Free asparagine is the precursor for acrylamide formation during cooking and processing [137]. Acrylamide is classified as a Group 2a carcinogen by the International Agency for Research on Cancer (IARC). Since acrylamide in food potentially increases the risk of developing cancer, the reduction of free asparagine could lead to an improvement of food quality. The accumulation of free asparagine in wheat grain is responsive to environmental and crop management factors. By silencing *TaASN2* through CRISPR/Cas9, Raffan and colleagues have demonstrated a more than 90% reduction in asparagine concentrations in the grain of edited wheat lines [82]. To date, the Department for Environment, Food & Rural Affairs (DEFRA) has given permission for the first field trials of gene-edited wheat in the UK. Furthermore, Calyxt Inc. (Roseville, MN, USA) recently announced that it has completed the first field trial in Minnesota, Wisconsin and Michigan using a cold-storable potato. This potato does not produce acrylamide, typically generated when cold-stored potatoes are cooked.

Modifying the production and composition of carbohydrates in cereal crops represents an essential approach to improve the nutritional quality of the most important source of carbohydrates in human nutrition. In particular, cereals with a high content of amylose and resistant starch are interesting for the beneficial effects on human health and on the reduction of the risk of diet-related non-infectious chronic diseases [138]. Recently, the Chinese Academy of Sciences developed a transgene-free high amylose rice through CRISPR/Cas9-mediated gene editing of *SBEIIb* and *SBEI* genes [75]. Similar results were obtained by silencing the *SBEI* and *SBEII* genes in potato and sweet potato, respectively [83,84]. In 2021, Li and colleagues [79] modified the starch composition, structure and properties through targeted mutagenesis of *TaSBEIIa* by CRISPR/Cas9 in winter and spring wheat varieties, generating transgene-free high-amylose wheat. Low amylose starch genotypes, defined as waxy genotypes producing essentially amylopectin, play an important role in the food and non-food industry. One single enzyme was found to be responsible for the synthesis of amylose, the granule-bound starch synthase (GBSS). Waxy genotypes were produced in several crops such as rice, maize, barley and potato [76,77,84,88]. Waxy potato was developed in Sweden using CRISPR/Cas9 technology, through transient transfection and regeneration from isolated protoplasts [91].

Proteins represent another important nutrient in many crop products. Their quantity and composition can affect the technological and nutritional quality of the derived foods. On the other hand, some proteins are undesirable because of their allergenicity and toxicity.

Several disorders are associated with the consumption of food products derived from wheat and other cereals. Gluten proteins, responsible for the technological properties of doughs, are also involved in triggering different pathologies such as celiac disease (CD) and wheat-dependent exercise-induced anaphylaxis (WDEIA) [139]. In 2018, the CRISPR/Cas9 technology was used to reduce α-gliadins in wheat grain in order to obtain a low-gluten transgene-free wheat [80]. Structural and metabolic proteins, such as α-amylase/trypsin inhibitors (ATI), are involved in the onset of wheat allergies and in non-celiac wheat sensitivity (NCWS) [140,141]. A CRISPR/Cas9 multiplexing strategy was used to edit the ATI subunits WTAI-CM3 and WTAI-CM16 in durum wheat with the aim of producing transgene-free wheat lines with reduced amounts of potential allergens involved in adverse reactions [81]. Lastly, in barley, the knock-out of the *D-hordein* gene leads to a considerable decrease in the prolamines and an increase in the glutenins, allowing for the modulation of gluten composition [89].

2.2. Synthetic Biology

In a broad definition, plant synthetic biology includes any re-designed plant species engineered to address specific targets by modifying, removing or introducing biological systems and components. According to this, all the achievements obtained by using CRISPR systems and discussed above could be referred to as synthetic biology approaches.

Specifically, the definition given by the Engineering Biology Research Consortium (EBRC; www.ebrc.org, accessed on 17 November 2021) states that "the element that distinguishes synthetic biology from traditional molecular and cellular biology is the focus on the design and construction of core components (parts of enzymes, genetic circuits, metabolic pathways, etc.) that can be modelled, understood, and tuned to meet specific performance criteria . . . to solve specific problems". Following this definition, synthetic biology often requires the introduction of complex DNA traits and elements for a new complete metabolic pathway or gene expression regulating system to provide the modified organism with new specific behavior.

CRISPR-mediated editing can certainly contribute to synthetic biology development. To date, not much has been done in crop species but many proofs have been reported in model plants regarding the construction of synthetic gene circuits via insertion of artificial DNA sequences or the modulation of metabolic pathways regulating gene expression. It has been shown that CRISPR systems can be used to induce chromosomal changes such as deletions, insertions and chromosome rearrangements [142–144].

Furthermore, it has been demonstrated that targeted insertion of large DNA fragments can be obtained through CRISPR editing, as was performed by Dong et al. (2020) [33] for the targeted insertion of a 5.2 kb carotenoid biosynthesis cassette at two pre-determined chromosomal regions in rice. In particular, these regions are known as genomic safe harbors (GSHs) that can accommodate transgenes without adverse effects on the host organism.

In addition, CRISPR can also mediate chromosome-engineering inducing crossovers and chromosomal rearrangements mediated by DSBs that favor HR between alleles of target genes [107,108]. Here, inter-homologous somatic recombination was induced by targeted induction of DSBs in the *PHYTOENE SYNTHASE* (*PSY1*) gene and *Carotenoid isomerase* (*CRTISO*) locus in tomato plants using the CRISPR/Cas9 system [92,93].

Frequently, parts of chromosomes do not participate in crossover due to the suppression of meiotic recombination caused by chromosome rearrangements, such as inversions and translocations. CRISPR can induce chromosomal rearrangements, including the induction or reversion of chromosomal inversions, useful for breaking or strengthening genetic linkages and inducing reciprocal translocations. Thanks to the introduction of multiple DSBs on the same chromosome or on heterologous chromosomes, CRISPR facilitates the possibility of inducing deletion and inversions or reciprocal translocation. Recently, Schwartz and colleagues [145] demonstrated the capability of CRISPR/Cas9 to mediate targeted 75.5-Mb chromosomal inversion in maize, producing two DSBs, one on each side of the inversion. In 2020, Beying et al. [95] induced reciprocal translocation of about 1 Mb in *Arabidopsis thaliana* between Chromosome 1 and 2. The target sites were located in intergenic regions, 0.5 Mb from the end of the long arms of both chromosomes.

Furthermore, synthetic promoters and transcription factor engineering is becoming more and more useful in gene circuit design. These approaches lead to the production of specific compounds that could be used for industrial or medical purposes. In this context, CRISPR variants and orthologs represent the best way to act at the transcriptional or epigenetic level [146] and to fine-tune gene expression. To date, transcriptional regulation and epigenetic manipulation of plant genes have been reported in model plant species [147], demonstrating the wide potential of their application in crops. In addition, translation regulation mediated by the modification of upstream open reading frames (uORFs) is often used to regulate the translation of a specific protein [148]. Nucleotide insertions and deletions into the uORFs of genes involved in development and biosynthesis of antioxidants in *Arabidopsis thaliana*, lettuce and tomato have been reported by Zhang et al. (2018) [149].

All these achievements show the ability of CRISPR systems to easily overcome some limitations of plant synthetic biology such as the introduction of large DNA fragments, the manipulation of chromosome structure or the regulation of gene expression via genetic and epigenetic approaches.

Following plant biotechnology development, the use of plants as a bio-factory to produce biopolymers, biofuels and compounds for medical or industrial purposes cannot

be separated from synthetic biology. One interesting application of CRISPR for the paper industry is the knock-out of the *GBSS* gene in potato to obtain amylopectin-enriched starch that is more suitable compared to amylase-enriched starch for paper manufacture [91].

Although climate change correlates with the consequence of arable land losses and the urgent need for sustainable production of foods, the constant fuel consumption severely affects biofuel demand. Oil seed crops with higher oil content and optimal fatty acid composition are required in order to breed new crops for biodiesel production. The modification of a *fatty acid desaturase 2* (*FAD2*) gene, which encodes an enzyme that catalyzes the desaturation of oleic acid in *Brassica napus*, has been obtained using CRISPR/Cas9, determining an increase in the content of oleic acid of mutant plant seeds compared with oleic acid content of wild-type seeds [97]. Seed lipid accumulation increased in *Nicotiana tabacum* seeds through the knock-out of the *NtAn1* transcription factor [99]. These tobacco plants are suitable for biodiesel production.

Moreover, the reduction of lignin content in biomass crops could be useful for a wide range of applications spanning from the paper and textile manufacture to the production of more digestible forage or biofuel production. In 2017, Park et al. [96] reported the reduction of lignin content obtained through the knock-out of the *4-coumarate: coenzyme A ligase* (*4CL*) gene in perennial grass species switchgrass (*Panicum virgatum*), used as lignocellulosic feedstock for bioenergy. The mutant switchgrass plants showed reduced lignin and increased sugar release. In the 2020, Lee et al. [94] reduced the lignin content in barley through CRISPR editing of the *caffeic acid O-methyltransferase 1* (*COMT-1*) gene, increasing significantly the bioethanol concentration of the mutant biomass.

Lastly, CRISPR-editing can also be used for the customization of secondary metabolite profiles in medical plants, as reported by Li et al., 2017 [98] in *Salvia miltiorrhiza*, a medicinal herb with significant pharmacological activities. The knock-out of the *diterpene synthase* (*SmCPS1*) gene, which is involved in the tanshinone biosynthesis pathway, blocks the metabolic flux to tanshinones, re-directing it towards other diterpene compounds that have the same precursor. In the field of plant-made pharmaceuticals, nicotine-free tobacco plants could be interesting for their use as platforms for the production of medical compounds, as reported by Schachtsiek in 2019 [150].

3. Legislation Limits

Since the release of the first genetically modified (GM) crops were established in the mid-1990s, a political and public debate has taken place. This debate has resulted in several regulations with the aim of ensuring the safety of the GMOs in order to achieve a high level of protection to humans, animals and environment. In general, GM crops are regulated at the country or regional level, generating a fragmented regulatory framework worldwide.

In particular, the United States has adopted the principle of substantial equivalence, establishing that GM products similar to commercialized ones should be considered conventional products.

Conversely, the GMO legislation of the European Union regulatory framework introduced the precautionary approach that was adopted as a guide in Directive 2001/18/EC.

Russia, in accordance with the amendments in Federal Law No. 358-FZ-2016, prohibited the cultivation of GM plants but not the imports of approved GM food and feed.

In addition, Canadian legislation distinguishes products on the basis of the novel traits considered and not on the basis of the technological approach.

A different approach was adopted in India, where the legislation is made on a case-by-case basis, while in Japan only products that do not contain inserted DNA or RNA are not considered as GMOs.

Lastly, Australia, New Zealand, Venezuela, Ecuador and Peru do not permit cultivation of GM crops while Brazil, Argentina and China are listed among the top five GM cultivating countries.

After more than 20 years of consumption of GM foods, no adverse effects on health or the environment have been found, and at present there is common agreement in the scien-

tific community that an alternative regulatory system should be adopted, focusing on risk assessment of the trait/product rather than the technology used to produce it [112,151–153].

During the last years, with the development of the NBTs, the GMOs debate has been rekindled. Indeed, there are many concerns regarding regulation of these new techniques (Hartung and Schiemann, 2014). In particular, genome-editing approaches should overcome obstacles such as public acceptance of the technology and government regulatory policies [100,154], and some progress is already underway. As an example, in 2016 the USDA ruled that gene-edited plants without foreign DNA would not be considered GMOs and authorized the commercialization of DuPont Wx1 corn, which was produced to redirect the starch metabolic pathway towards amylopectin biosynthesis [34].

As recently recommended by the European Academies for Sciences applied to Agriculture, Food and Nature (UEAA, Union Européenne des Académies d'Agriculture, Paris, France) the GMO Directive approved in 2001 by the European Union is not appropriate because it was drafted before the discovery of NBTs. Thus, a new regulation frame taking into account the use of these methodologies to modify plant genomes in a precise manner is required.

Even if the European Court of Justice (ECJ) ruled that crops generated using genome-editing technologies be subjected to the above mentioned 2001/18/EC, the European Commission is expected to publish in the near future about the regulatory certainty of genome editing.

4. Conclusions

Crop breeding has been revolutionized by the development of CRISPR systems. Given their efficiency, simplicity and high specificity, genome editing approaches provided many advantages compared to traditional breeding. In contrast to transgenic approaches and mutagenesis, which lead to random insertions and to the production of unwanted phenotypes, genome-editing methods produce precise mutants, reducing off-targets and pleiotropic effects. Furthermore, interesting traits useful for boosting resilience to biotic and abiotic stress or affecting plant performance and the quality of derived foods have been introduced in plant genotypes, obtaining homozygous mutants in one or few generations; by contrast, classical breeding approaches require about 10 years to fix a mutation. CRISPR/Cas technology also represents an appropriate tool to produce transgene-free modified organisms. The transient expression of sgRNAs and Cas9, the segregation of sgRNA/Cas9 transgenes later in generations, or the use of ribonucleotide protein (RNP) complexes allow for obtaining transgene-free progeny with desired modifications.

Genome editing tools perfectly fit into the development of more sustainable agricultural systems. Through the development of edited crops with improved yield or with enhanced water use efficiency, they offer new ways to reach a greater production of foods with a high level of non-renewable resource saving (such as soil, energy and water). Moreover, many successful attempts have been reported to boost the resilience to heat and drought stresses that reduce crop productivity and undermine food security. As regards biotic stresses, GE crops could represent an opportunity to decrease the use of phytochemicals to both safeguard the environment and produce cost savings for farmers.

CRISPR-mediated editing is also a great opportunity for the improvement of plant synthetic biology. Some applications spanning from the performance improvement of crops and quality of food products to plant pharming can benefit from the versatility of CRISPR systems, which allows for introducing substantial modifications in chromosomes and modulating or producing synthetic gene circuits.

Hence, it is necessary to revise current legislation, especially in those countries with more restrictive rules, in order to avoid limiting the applications of genome editing techniques to the support of sustainable agriculture. In the near future, together with developing technologies, a science-based regulatory framework designed for edited crops and GMOs will need contributions from both scientists and policymakers to devise comprehensive plans for CRISPR-based approaches.

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