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Genome Editing Technologies to Improve Health-Related Phytocompounds in Crops

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ABSTRACT

Due to rapid global population growth and the resulting significant increase in food demand, the world is facing an epidemic of malnutrition. Although yield improvement remains one of the main targets of breeding programs, much attention is being paid to the nutritional aspects of crops, including nutrients and bioactive compounds that are often important for general human health and disease prevention. Phytocompounds such as allergens, antinutrients, antioxidants, vitamins, and fatty acids are among the most important classes of chemical substances that affect human health and thus contribute to the nutritional value of crops. Conventional breeding for these traits consists of laborious and time-consuming methods, but recent advances in new genome editing (GE) technologies offer a valuable, time-saving, and cost-effective alternative. The article reports on the extensive use of GE tools to modify the content of health-relevant bio-compounds and to obtain crops with higher nutritional quality.

1 | Introduction

Until three decades ago, more than 60% of people worldwide lived in rural areas (FAO 2017). Today, we are experiencing a shift to urban areas, resulting in about 54% of all people living in cities. The world population is projected to reach 9.1 billion people by 2050 (FAO 2017), so an increase in food production is inevitable. The demand for agricultural products will be greater than the available resources. As a result, agriculture will face many challenges: producing more food using more efficient and sustainable methods to feed an ever-growing population, increasing the production of raw materials to respond to the bioenergy market, adapting to climate change, conserving natural habitats, and preserving biodiversity.

Improving crop yields has been the main goal of breeding programs in recent decades (Liu et al. 2021). With increasing urbanization and people's rising living standards, consumers are paying more attention to crop quality and the impact of food on health and disease prevention (Yang et al. 2022). Growing more

Maria Dellino, Cinzia Montemurro, Domenica Nigro, and Claudio de Giovanni contributed equally to this work.

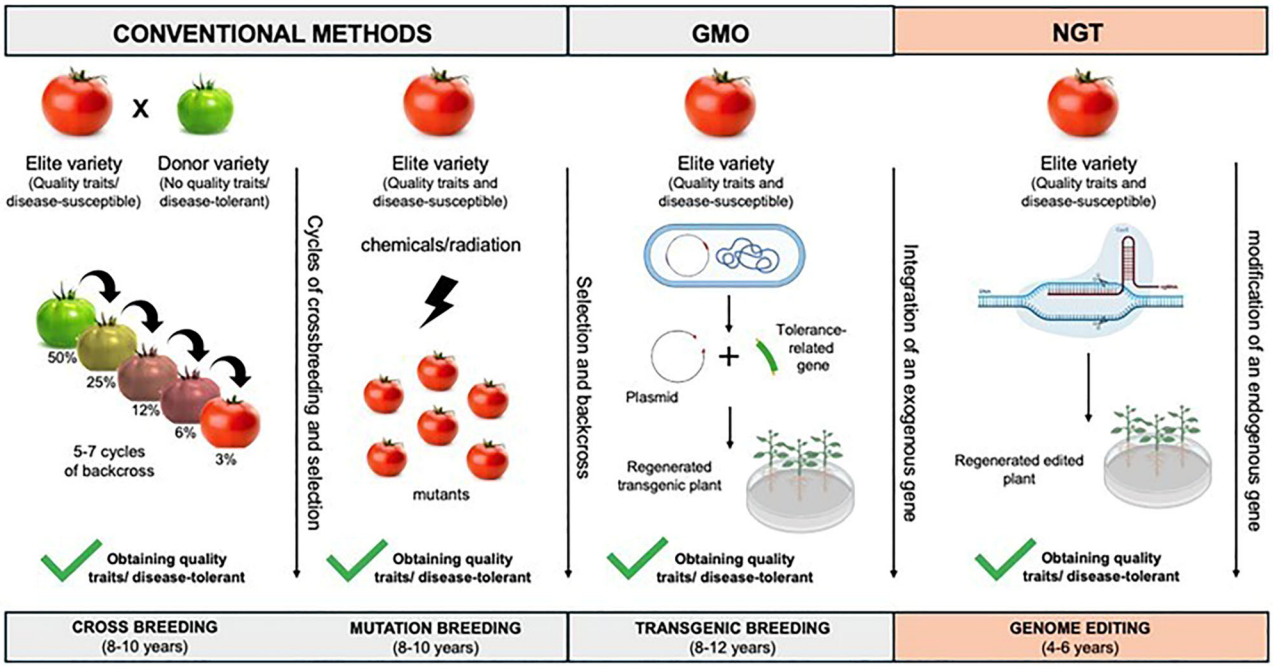

FIGURE 1 | Comparison among the different methods used in crop breeding to improve traits (e.g., disease resistance, higher yield, and better quality). Conventional methods include crossbreeding and random mutagenesis. In crossbreeding, an elite recipient line is crossed with a donor line, and after several cycles of backcrossing, the line with the desired traits is selected. In mutation breeding, an elite line (e.g., seed) is treated with chemical or physical mutagens to produce mutants by random mutagenesis. Both methods are laborious and take a long time, around 8–10 years, to produce a new improved variety. The transgenic breeding method makes it possible to obtain varieties with the desired characteristic by transferring exogenous genes into the elite variety. This method is also laborious and time-consuming, taking around 8–12 years. With the help of genome editing techniques, improved varieties can be obtained in a short time, approximately 4–6 years, by precisely modifying the target genes of an elite variety.

nutritious, tastier, and healthier crops has become a target of modern agriculture as they are a source of substances beneficial to human health, for example, phytocompounds such as antioxidants, vitamins, and fatty acids (FAs) (Yang et al. 2022). To this end, various strategies have already been successfully applied; however, conventional breeding methods such as crossbreeding, mutation breeding, and transgenic breeding are laborious and time-consuming, especially for polyploid crops (Figure 1). In addition, quality-related traits are controlled by an extremely complex genetic network and are influenced by environmental factors (fertilizers, climate, biotic, and abiotic stress) that hinder their genetic improvement.

Genome editing (GE) techniques using artificial sequence-specific nucleases (SSNs) have enabled precise, efficient, and targeted modifications of the plant genome and have shown clear advantages in plant selection (Figure 2). These techniques use artificial nucleases to generate targeted DNA double-strand breaks (DSBs) that trigger cellular DNA repair mechanisms, which can be either nonhomologous end-joining (NHEJ) or homologous recombination (HR) (Figure 3. The NHEJ mechanism involves direct ligation of broken ends without a template strand, causing random insertions or deletions (indels) that can lead to frameshift mutations in the gene coding region, thus resulting in a gene knockout. In contrast, the HR mechanism requires an undamaged homologous sequence to serve as a template for the repair of both broken strands, leading to precise modifications or gene insertions (Figure 3) (Symington and Gautier 2011; Bortesi and Fischer 2015).

The first GE technique on plant relied on the utilization of the zinc-finger nucleases (ZFNs) (Lloyd et al. 2005; Kumar et al. 2015), followed by the use of the transcription activator-like effector nucleases (TALENs) adapted from *Xanthomonas* bacteria (Boch et al. 2009; Christian et al. 2010; Khan et al. 2017). However, the design and manipulation of both ZFNs and TALENs constructs suitable for targeted genome modifications are very complex and costly, which hinders their application in plants and organisms.

A breakthrough technology for GE is represented by CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats associated with Cas9), which was adapted from *Streptococcus pyogenes* (Jinek et al. 2012). This system is much easier to manipulate than ZFNs and TALENs, and it is currently the most widely used tool for GE in many organisms, including plants (Figure 4). CRISPR arrays were first identified in the genome of *Escherichia coli* in 1987 (Ishino et al. 1987), but their biological functions were not understood until 2005, when the finding that the spacers were homologous to viral sequences and plasmids suggested a role in adaptive immunity (Mojica et al. 2005). The discovery that a target DNA sequence can be reprogrammed by modifying 20 nucleotides in the crRNA to combine it with the tracrRNA to form a chimeric single-guide RNA (gRNA) (Jinek et al. 2012) led to the CRISPR/Cas system evolving from a biological phenomenon to a genomic engineering tool. The system is based on the use of a DNA-targeting class 2 endonuclease Cas9, which ensures precise and efficient editing (Knott and Doudna 2018) by the gRNA. Stable binding to the target DNA is promoted by a specific protospacer adjacent

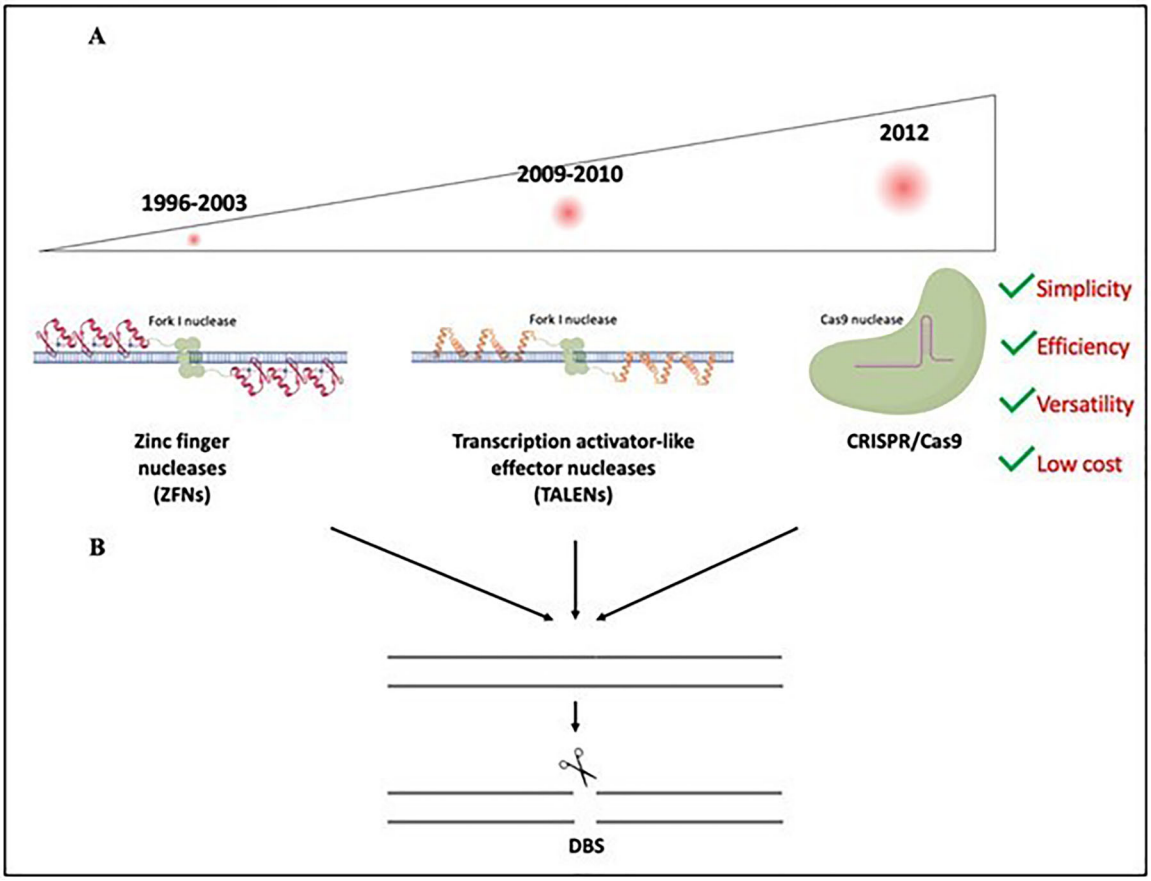

FIGURE 2 | (A) Timeline reporting the development of editing techniques. (B) Illustration of the most important genome-editing technologies genome editing tools are able to induce DNA double-strand breaks (DSB) that can be repaired by nonhomologous end-joining (NHEJ) and homologous recombination (HR). The discovery and development of the CRISPR/Cas system have made it possible to perform extremely simple, efficient, versatile, and cost-effective experiments thanks to the use of the Cas9 nuclease and specific gRNA-DNA recognition.

motif (PAM) (Mojica et al. 2009; Marraffini and Sontheimer 2010) which triggers Cas9 to generate a dsDNA break at ~3 bp upstream of the PAM motif (Jinek et al. 2012). In this way, the dsDNA break can be repaired by NHEJ mechanism which leads to the elimination of the gene (knock-out), or by HR, to the insertion of precise modifications or genes (knock-in).

In many successful examples of the use of the CRISPR/Cas9 system, the changes introduced are usually knock-out mutations at the loci of interest, but the technology is rapidly evolving to improve the efficiency and precision of targeting. In particular, base editing (BE) and prime editing (PE) approaches are being developed. The former induces transitions from C to T or from A to G through a deaminase fused to a Cas nickase or a dead Cas (dCas), whereas the latter mediates targeted insertions, deletions, and all base-to-base conversions by exploiting a reverse-engineered transcriptase enzyme and a PE gRNA (pegRNA) fused to a Cas nickase (Cardi et al. 2023). These approaches do not rely on repairing a DSB in the DNA but can generate single nucleotide substitutions, in-frame insertions, and large deletions by using specific proteins that make precise changes in the DNA, increasing the efficiency and specificity of the CRISPR system.

The first applications of the CRISPR/Cas9 system on plants were carried out on the model species *Arabidopsis thaliana*, *Nicotiana benthamiana*, and the crop plant rice (*Oryza sativa* L.) (Feng et al. 2013; Miao et al. 2013; Xie and Yang 2013) and have since been extended to 65 crop plants with 287 target genes being modified to increase yield and quality as well as resistance to biotic and abiotic stress factors (Ukhatova et al. 2023; Nigro et al. 2023).

In this review, we systematically analyze the applications of the CRISPR/Cas9 GE tool to improve the nutritional and functional quality of crops, the content of carbohydrates, proteins, Fas, and secondary metabolites, and the reduction of anti-nutritional compounds. The prospects for the future application of GE technology and the problems that have emerged so far related to their application are also critically examined.

2 | CRISPR/Cas9 System Applications to Reduce Allergens and Anti-Nutritional Compounds

Plants and their products are the most important sources of nutrients for human and animal nutrition. However, they also contain secondary metabolites and bioactive compounds that can have harmful effects on human and animal health, such as allergens and anti-nutritional factors. Allergens are substances that can cause allergic reactions in some people by prompting the immune system to produce antibodies to protect the organism.

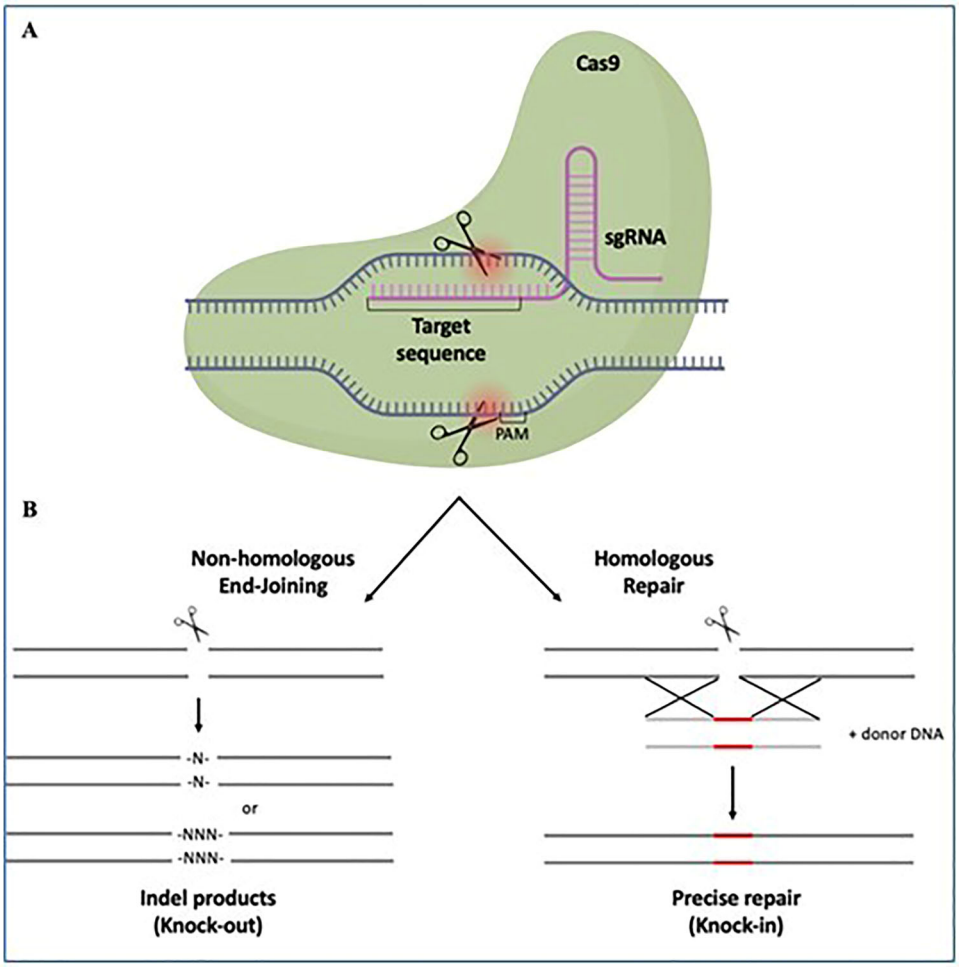

FIGURE 3 | Illustration of the (A) Class 2 type II CRISPR-Cas system formed by the effector nuclease Cas9, the target sequence, the sgRNA, and the PAM motif. (B) The two main repair mechanisms in genome editing: nonhomologous end-joining (NHEJ) and homologous recombination (HR) which, respectively, cause a knock-out and a knock-in of the gene of interest. sgRNA, single-guide RNA; PAM, protospacer adjacent motif.

Antinutrients are food compounds that can impair digestion and reduce the bioavailability of key nutrients through chelation and enzyme inhibition (Duraiswamy et al. 2023) and can cause serious illness and even death if ingested in excess (Frick et al. 2018). To make the intake of plant foods safe and effective, it is important to reduce allergens and anti-nutritional compounds in the main food crops. In the next sections, we report on the successful application of CRISPR/Cas technology to reduce or eliminate some allergens and anti-nutritional compounds.

2.1 | Allergens

Increasing urbanization and the resulting changes in 21st-century working lifestyles have led to poor dietary habits that rely on packaged and processed foods containing additives, allergens, and contaminants. Today's consumers are increasingly concerned about food safety and the negative effects of allergies, which are estimated to have increased in 3%–4% of the adult population and 5% of children in recent decades (Sicherer and Sampson 2010). The most important allergenic plants are peanuts, tree nuts, wheat, soya, beans, and tomatoes (Lokya et al. 2023). To date, 963 allergenic plant proteins have been identified and characterized, and all information on their sequence, structure, and nomenclature is available in various databases such as WHO/IUS (http://www.allergen.org), Allergome (http://allergome.org), and AllerBase (bioinfo.unipune.ac.in/AllerBase/Home.html).

Prevention of food allergies can be achieved by removing allergens or reducing their antigenic properties during industrial processes or by selecting cultivars with low allergen content. New genome engineering techniques such as CRISPR/Cas9 has proven to be an effective in developing low-allergen food crops. The main allergens in plant foods are proteins involved in seed storage and plant defense. Seed storage proteins include cupin (vicilin and legumin types) and the prolamin superfamily (found in various types of legumes, nuts, cereals, and fruits), the 2S albumin proteins, the nonspecific lipid transfer proteins, and the cereal α-amylase and protease inhibitors (Breiteneder and Radauer 2004). Plant defense proteins include pathogenesis-related (PR) proteins, nsLTPs, and profilins, which are involved in defense mechanisms against pathogenic microorganisms, insects, and herbivores (Breiteneder and Radauer 2004).

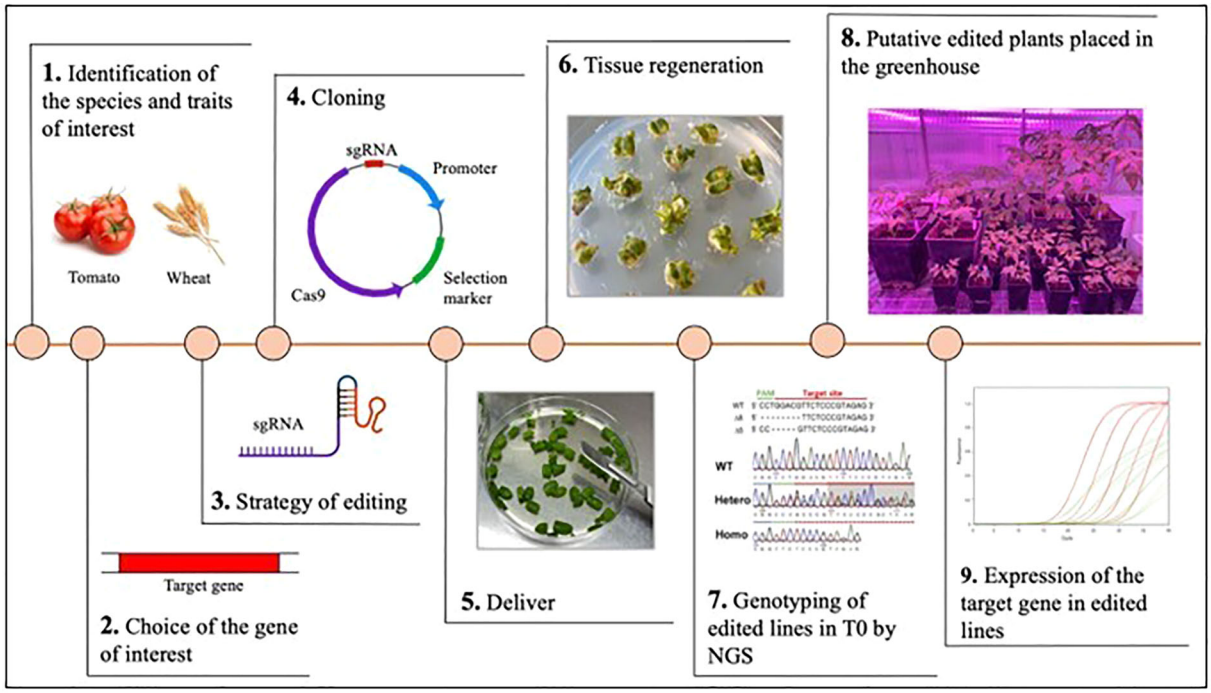

FIGURE 4 | CRISPR/Cas9-mediated genome editing workflow in plants. CRISPR/Cas9-mediated genome editing in plants involves several steps, including selection of the gene of interest, design and synthesis of sgRNAs, cloning, delivery of the vector into plant cells, transformation, plant regeneration, and screening of the edited plants. sgRNA, single-guide RNA.

The efficiency of the CRISPR/Cas9 system has been demonstrated in soy, with the development of hypoallergenic soybean plants of the Japanese varieties Enrei and Kariyutaka (Sugano et al. 2020). Two gRNAs were used to mutagenize the major soy allergens vicilin-like glycoprotein Gly m Bd 28 K and the oil body-associated protein Gly m Bd 30 K. The simultaneous site-directed mutagenesis of the two genes, mediated by *Agrobacterium* transformation, resulted in frameshift mutations at the target loci that caused reduced expression of the genes (Sugano et al. 2020). In 2022, Biswas et al. successfully targeted the 2S albumin protein Ara h 2, a major allergenic gene in peanut, whereas Assou et al. (2022) used CRISPR/Cas9 to silence the allergen Bra j I, a seed storage protein gene of the same 2S albumin class in the allotetraploid brown mustard (*Brassica juncea*), obtaining lines with reduced/suppressed allergen.

Another efficient application of CRISPR was achieved in wheat (Sánchez-León et al. 2018) to reduce gluten proteins that trigger coeliac disease, an autoimmune disorder that is affecting more and more people. The authors modified the α-gliadin gene array obtaining non-transgenic wheat lines with low gluten content as confirmed by an 85% reduction in immunoreactivity detected by ELISA.

In durum wheat, a CRISPR-Cas9 multiplexing strategy was successfully adopted to reduce the level of structural and metabolic proteins α-amylase/trypsin inhibitors (ATIs) responsible for wheat allergy (baker's asthma) and non-celiac wheat sensitivity (NCWS) by editing the ATI subunits WTAI-CM3 and WTAI-CM16 genes in the Italian cultivar Svevo (Camerlengo et al. 2020).

2.2 | Anti-Nutritional Factors

Plant foods are rich in nutrients and molecules that are beneficial to human health but may also contain compounds that are considered antinutritive. These are substances that can impair human and animal growth by inhibiting or blocking important cellular metabolic pathways and reducing the absorption and nutrients utilization (vitamins, minerals, and proteins) at the intestinal level (Ali et al. 2022). Antinutrients are mainly found in the seeds of cereals and pulses. The most important plant anti-nutrients are cyanogenic glycosides (CGs), nonprotein neuroexcitatory amino acids (β-ODAP), raffinose family oligosaccharides (RFOs), phytic acid (PA), alkaloids, and steroidal glycoalkaloids (SGAs). Applications of the CRISPR/Cas9 system to reduce anti-nutritional content in major crops and to preserve their nutritional value without altering important agronomic traits are examined below (Table 1).

2.2.1 | Cyanogenic Glycosides

CGs are found in more than 2500 plant species belonging to the Fabaceae, Rosaceae, Linaceae, and Compositae families (Vetter 2000). A renowned cyanogenic diglucoside is amygdalin, which accumulates in large quantities in bitter almond kernels (Dicenta et al. 2002; Sánchez-Pérez et al. 2008). CGs are organic compounds formed in plants secondary metabolisms and consist of an α-hydroxy nitrile-type aglycone and a sugar part (mainly D-glucose) (Vetter 2000). The enzymes β-glucosidase and α-hydroxynitrileses cause the hydrolysis of glycosides to sugars, aldehydes, or ketones and hydrogen cyanide. This compound is

TABLE 1 | List of anti-nutritional molecules and allergenic proteins successfully edited by CRISPR/Cas9 in cultivated plants.

Traits	Plant species	Goal	Target sequence	Molecular function	CRISPR/Cas9 strategy	References
Elimination of anti-nutritional compounds	Cassava	Reduction of cyanide levels	CYP79D1 and CYP79D2 genes	Biosynthesis of cyanogen	Yes	Gomez et al. (2023)
	Grass pea (*Lathyrus sativus* L.)	Reduction of β-ODAP content in seeds	BAHD-AT3 gene	β-ODAP synthesis	Yes	Saha et al. (2023)
	Soybeans (*Glycine max*)	Reduction of RFOs content in soybeans	GmGOLS1A and GmGOLS1B genes	Galactinol synthesis	Yes	Le et al. (2020)
			RS2 and RS3 genes	Raffinose synthesis	Yes	Cao et al. (2022)
	Rice (*Oryza sativa*)	Reduction of phytic acid	*OsITPK1*-6 genes	Phytic acid biosynthesis	Yes	Jiang et al. (2019)
	Rapeseed (*Brassica napus*)		*BnITPK1* and *BnITPK4* gene families	Phytic acid biosynthesis	Yes	Sashidhar et al. (2020)
	Wheat (*Triticum aestivum*)		*TaIPK1* gene	Phytic acid biosynthesis	Yes	Ibrahim et al. (2022)
	Soybeans (*G. max*)		*GmIPK1* gene	Phytic acid biosynthesis	Yes	Song et al. (2022)
	Potato (*Solanum tuberosum*)	Reduction of SGAs	*St16DOX* gene	SGAs biosynthesis	Yes	Nakayasu et al. (2018)
Elimination of allergenic proteins	Soybeans (*G. max*)	reduction of the major soy allergens	Gly m Bd 28 K and Gly m Bd 30 K genes	Vicilin-like glycoprotein and oil-body-associated protein	Yes	Sugano et al. (2020)
	Peanut (*Arachis hypogaea* L.)	Reduction of the major peanut allergen	*Ara h 2 gene*	2S albumin protein	Yes	Biswas et al. (2022)
	Mustard (*Brassica juncea*)	Reduction of the major mustard allergen	*Bra j I* gene	2S albumin protein	Yes	Assou et al. (2022)
	Wheat (*Triticum durum*)	Reduction of α-gliadin	α-Gliadin gene array	Gluten proteins	Yes	Sánchez-León et al. (2018)
	Wheat cv Svevo (*Triticum durum*)	Reduction of α-amylase/trypsin inhibitors (ATIs)	ATIs genes	ATI subunits WTAI-CM3 and WTAI-CM16	Yes	Camerlengo et al. (2020)

Abbreviations: BAHD-AT, BAHD acyltransferase; RFOs, raffinose family oligosaccharides; SGAs, steroidal glycoalkaloids; β-ODAP, nonprotein neuroexcitatory amino acids.

toxic to human health and causes chemical asphyxia (oxygen deprivation) in cells (Kerns and Kirk 1998). Cyanide poisoning affects the respiratory, neurological, and cardiovascular systems and causes symptoms such as stupor, coma, convulsions, hemodynamic shock, and cardiorespiratory arrest (Nelson 2006).

GE to prevent cyanogenesis was used for the first time on South American *Euphorbiaceae cassava* by Gomez et al. (2023), targeting two paralogous genes encoding the enzymes that catalyze the first step of cyanogen biosynthesis, the CYP*79D1* and *CYP79D2* genes (Andersen et al. 2000). The authors used CRISPR-Cas9

constructs with RNAs guide targeting both genes, individually and in combination, to edit the popular West African landrace TME 419 and the variety resistant to cassava mosaic disease TMS 91/02324 (Gomez et al. 2023). Double knockouts eliminated cyanogenic potential in all cassava accessions. In contrast, single-gene knockout lines revealed the differential contribution of the two CYP79D genes to the cyanogenicity, showing that the loss of the CYP79D2 gene alone is sufficient for a drastic and stable reduction in cyanide levels.

2.2.2 | Nonprotein Neuroexcitatory Amino Acid

Neurolathyrism is a disorder producing irreversible limb paralysis in humans and animals (Spencer et al. 1986). One of the major causes of neurolathyrism is the β-ODAP, β-*N*-oxalyl-L-α,β-diaminopropionic acid, which is found in the seeds of grass pea (*Lathyrus sativus* L.) (Jiao et al. 2011). This species is an important source of starch and protein for populations in marginal areas of Asia and Africa and is considered superior to other legumes in terms of several agronomic traits such as yield, nitrogen fixation index, tolerance to water stress, and salinity (Vaz Patto et al. 2006; Korus et al. 2008). The disease caused by β-ODAP has been affecting famine-stricken populations worldwide for centuries. It is still present in Eritrea, Ethiopia, and Afghanistan, where *Lathyrus* seeds have long been the only source of food. Conventional breeding and transgenic approaches have produced varieties with low β-ODAP content (<0.1% seed dry weight) (Kumar et al. 2016; Dixit et al. 2016), but only the application of CRISPR/Cas9 editing technology has recently enabled a breakthrough.

The final step of β-ODAP synthesis is catalyzed by the enzyme BAHD acyltransferase (BAHD-AT), which is encoded by several BAHD-AT genes (Goldsmith et al. 2022). In particular, the BAHD-AT3 gene is the most highly expressed compared to the other isoforms (Edwards et al. 2023). Saha et al. (2023) designed and selected three gRNAs with high mutation specificity for the BAHD-AT3 gene and performed *Agrobacterium*-mediated genetic transformation of the Indian commercial cultivar Nirmal. Analysis of ODAP content in leaves and mature seeds of the wild type (WT) and mutants revealed 99% and 97% reduction in β-ODAP content in leaves and seeds, respectively, while retaining the original agronomic traits.

2.2.3 | Oligosaccharides of the Raffinose Family

RFOs are soluble carbohydrates found in all plants (Elango et al. 2022), but especially in the seeds of pulses such as soybeans, lentils, and chickpeas. In humans, RFOs have a positive effect on the intestinal microflora by promoting the growth of beneficial bacteria such as *Bifidobacterium* in the large intestine (Martínez-Villaluenga et al. 2008). They also have anti-allergic, anti-obesity, and antidiabetic effects and prevent nonalcoholic fatty liver disease (Elango et al. 2022). In plants, RFOs are involved in numerous metabolic processes, such as phloem transport and defense response to stress factors (Hannah et al. 2006; Nishizawa et al. 2008; Obendorf and Górecki 2012; Gil et al. 2012; Sengupta et al. 2015). However, they are also considered anti-nutritional molecules as they can cause flatulence (Elango et al. 2022), which is why legumes are only consumed in small amounts in human and animal diets. So far, reducing their content in legumes is an important target for breeding programs.

In soybeans, the main RFOs are raffinose, stachyose, and verbascose, which are mono-, di-, and tri-galactosides of sucrose, respectively (Kennedy et al. 1985; Hou et al. 2009). Previous studies have shown that downregulation of some genes involved in RFO biosynthetic pathways leads to a reduction in raffinose and stachyose without other changes to plant developmental phenotypes (Valentine et al. 2017). Le et al. (2020) used the CRISPR/Cas9 system to induce knockout mutations of two galactinol synthase (GOLS), an enzyme involved in the biosynthesis of RFOs, the GOLS-encoding homeologs *GmGOLS1A* and *GmGOLS1B*, to study their potential role in soybean RFO metabolism. After generating stable null segregants in the T2 generation and investigating the disruption of the genes encoding GOLS, a significant reduction in stachyose, raffinose, and sucrose content was observed. No effects on plant phenotype and seed germination were observed, thus confirming the efficiency of CRISPR/Cas9 as a tool for improving the nutritional quality of soybeans.

Cao et al. (2022) utilized the CRISPR/Cas9 multiplex gene editing system to delete the *RS2* and *RS3* soybean *Raffinose synthase* (RS) gene, reducing RFOs in mature soybean seeds. The authors designed four single-gRNA (sgRNA) targets to induce mutations on RS genes using the TCTU-tRNA system and carried out the genetic transformation mediated by *Agrobacterium rhizogenes* in the hairy roots. After transformation and segregation of the progeny, stable null alleles with loss of function of *RS2* and *RS3* genes and low RFO content in seeds were obtained at the T2 generation. Using CRISPR/Cas9-mediated multiplex strategy, low-RFO and high-sucrose soybean plants with inherited mutations in multiple genomic loci were successfully generated, improving soybean crop quality.

2.2.4 | Phytic Acid

PA (myo-inositol 1,2,3,4,5,6 hexakisphosphate [IP6] or hexaphosphorylated myo-inositol) is the main phosphorus storage compound in cereal seeds accounting for 60%–90% of the total amount (Karmakar et al. 2020). However, it is also an anti-nutritional factor as it acts as a chelator of nutrient cations such as magnesium, zinc, calcium, copper, iron, and potassium, reducing their absorption in the digestive tract of humans and monogastric animals. The enzyme phytase, which promotes the catalysis of PA to inositol phosphatase (IP) and inorganic phosphorus (Pi), is absent in humans and animals, resulting in the release of phosphorus into the environment, thus contributing to water pollution (Handa et al. 2020).

To reduce PA concentrations in plants without affecting plant germination and vigor, various molecular strategies have been applied, such as the disruption of the enzymes involved in its biosynthesis (Silva et al. 2019). However, disruption of these enzymes has a pleiotropic effect (Sahu et al. 2023). The challenge of reducing PA content and minimizing these effects was achieved through the use of CRISPR/Cas9 technology in *O. sativa* (Jiang et al. 2019), *Brassica napus* (Sashidhar et al. 2020), *Triticum aestivum* (Ibrahim et al. 2022), and *Glycine max* (Song et al. 2022). In these species, mutant lines with reduced PA levels and an

increase in P and nutrient ions were obtained by knockout of ITPK (inositol 1,3,4-triphosphate 5/6-kinase) genes, one of the key enzymes involved in PA biosynthesis.

2.2.5 | Alkaloids and SGAs

Alkaloids are the largest group of secondary metabolites produced by plants. Currently, more than 20,000 different alkaloid molecules are known (Yang and Stöckigt 2010). Alkaloids are low molecular weight nitrogen-containing compounds (Matsuura and Fett-Neto 2015) playing numerous roles in plant response mechanisms to biotic and abiotic stresses and in reproduction. They have antioxidant, antitumor, analgesic, and anti-inflammatory effects on human health, which is why they are still of great interest in pharmacology and medicine today. Caffeine, nicotine, morphine, and codeine are among the potentially toxic alkaloids. Caffeine, which is found in beverages such as coffee (*Coffea arabica*) and tea (*Camellia sinensis*) or foods such as cocoa (*Theobroma cacao*), is used to increase and improve physical and mental alertness. Nicotine (*Nicotiana tabacum*) is a central nervous system stimulant contained in cigarettes, whereas morphine (*Papaver somniferum*) and codeine (*P. somniferum)* are powerful analgesic and sedative, respectively (Matsuura and Fett-Neto 2015). SGAs, nitrogen-containing compounds, are found in solanaceous species such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), and aubergine (*Solanum melongena*) (Heftmann 1983). The main SGAs expressed in potatoes are α-chaconine and α-solanine (Friedman 2006), whereas in tomatoes are α-tomatine, dehydrotomatine, and esculeosides (Fujiwara et al. 2004; Moco et al. 2007). Although SGAs are involved in defense mechanisms against various pathogens, they have toxic effects. SGAs can cause gastrointestinal and neurological disorders and can be lethal to humans at high concentrations, through mechanisms, including membrane disruption and inhibition of acetylcholinesterase activity (Roddick 1989). The major precursor of SGAs is cholesterol (Itkin et al. 2013), and their biosynthesis requires several enzymes involved in hydroxylation, oxidation, and transamination reactions.

The new GE technologies were applied to potato to obtain plants without SGAs by silencing the gene encoding the enzyme St16DOX (2-oxoglutarate-dependent dioxygenase, 2OGD), which is involved in the biosynthesis of SGAs. Silencing of this gene was achieved by RNA interference and led to a significant reduction in endogenous SGA levels (Nakayasu et al. 2017), although not to their elimination. Further research enabled Nakayasu et al. (2018) to knock out the *St16DOX* gene by using nine targeted gRNAs. Genetic transformation via *A. rhizogenes* in potato hairy roots allowed to obtain two *St16DOX*-damaged potato lines showing complete elimination of the SGAs.

3 | CRISPR/Cas9 System to Increase Beneficial Phytocompounds Amount

Taste, fruit size, color, and nutritional composition are the main factors determining the quality of a plant-based food. Moreover, they are associated with health-promoting properties, an aspect that is becoming increasingly important for the modern consumer. Phytochemicals are among the most important nutritional factors that play a central role in various functions of the animal body and have potential benefits for the promotion and maintenance of health (Abbas et al. 2015; Lee et al. 2017; Barnes 2001; Toniolo et al. 2001). Therefore, in recent years, several studies have focused on nutraceuticals, functional foods, and the development of healthier crop varieties. As outlined below, genes involved in the synthesis of macronutrients, vitamins, and flavonoids can be successfully edited by CRISPR/Cas9 GE.

3.1 | Fatty Acids

Vegetable oils are an important component of the human diet and cover up to 40% of the daily caloric requirement. Today, more than 20 oilseed crops are cultivated in the world, including palm oil, soybean, and rapeseed/canola, but also sunflower, peanut, camelina, cotton, coconut, and olive (Mnasri et al. 2023; Miazzi et al. 2020). In addition to their essential role in human nutrition, vegetable oils are also of great economic importance as they are actively used for animal feed, renewable energy resources, and as a raw material for several industrial products (i.e., cosmetics, pharmaceuticals, detergents, plastics, waxes, and paints) (Wittkop et al. 2009; Rahman and de Jiménez 2016).

The quality of the oil depends mainly on the FA profile and on specific concentrations of flavonoids, tocopherols, and sterols. Normally, edible oils have a high proportion of oleic acid or stearic acid compared to a high proportion of linoleic acid, which oxidizes and becomes rancid during cooking. To date, GE approaches have targeted the enzymes FAD2 and FAD3 involved in the biosynthesis of FAs in several crops such as rapeseed, camelina, soybean, peanut, and pennycress (Haun et al. 2014; Demorest et al. 2016; Jiang et al. 2017; Wen et al. 2018; Al Amin et al. 2019). In camelina, this application resulted in an increase of up to 50%–83% of oleic acid (18:1) in the seed oil compared to the 10%–25% in the wild-type, and a significant decrease in the less desirable PUFAs linoleic acid (18:2) (from ~16 to <4%) and linolenic acid (18:3) (from ~35 to <10%) (Jiang et al. 2017). Despite these good results, the CRISPR/Cas technique applied to the *FAD2* gene to increase 18:1 FAs was not successful in *B. napus* (Okuzaki et al. 2018). In contrast, the seed oil content of rapeseed was successfully increased by CRISPR/Cas9 technology targeting the *BnSFAR4* (seed FA reducer 4) and *BnSFAR5* (seed FA reducer 5) genes (Karunarathna et al. 2020). GE has also been used in *B. napus*, *Crambe*, and *Camelina* to reduce the 22:1 monounsaturated FA which is an anti-nutritional factor, by downregulating the *FAE1* gene without deleterious effects on seed physiology or plant growth (Ozseyhan et al. 2018). Similarly, the homozygous disruption of the *FAE1* homologue gene in the diploid pennycress (*Thlaspi arvense*) reduced the 22:1 content, suggesting that the modulation of *FAE1* gene is the main contributor to this trait. In addition, the oil obtained had a composition comparable to that of the canola oil, which is an interesting result with regard to the use of this species as an alternative oilseed crop (McGinn et al. 2019).

3.2 | Carotenoids and Vitamins

"Micronutrient deficiency (or hidden hunger) is described as a form of malnutrition caused by low intake and absorption

of vitamins and minerals that are essential for the health and development of children, and the well-being of adults" (von Grebmer et al. 2014). Although the effects are not immediately noticeable, this deficiency can lead to serious illness in the long term. It is estimated that around 2 billion people worldwide suffer from hidden hunger (Bailey et al. 2015; Rautiainen et al. 2016), mostly in the form of anemia (McLean et al. 2009). Furthermore, it is estimated that even in populations that are adequately supplied with food, people can suffer from hidden hunger because their diet is "rich" but does not provide all the nutrients they need (WHO 2006; Kennedy et al. 2003; Amoroso 2016). The situation is most serious among poor population groups and in emerging countries, where micronutrient deficiencies are a critical public health problem (Gödecke et al. 2018). In such contexts, malnutrition is estimated to be the cause of death for around 7 million children per year. In particular, the lack of essential micronutrients such as zinc, iron, iodine, and vitamin A causes serious health problems, especially in women and children (von Grebmer et al. 2014).

Vitamin A is essential for several biological functions such as vision and dark adaptation, embryo growth, innate and adaptive immunity, and general well-being in humans (Strobbe et al. 2018; Timoneda et al. 2018). To date, vitamin A deficiency (VAD) is considered one of the most serious health problems worldwide, as it can cause a variety of symptoms such as xerophthalmia, night blindness, childhood blindness, and an increased risk of morbidity and mortality, especially in young children (Sommer 2008; Reddy et al. 2022). Unlike photosynthetic organisms, which can synthesize isoprenoid pigments, humans are unable to produce carotenoid compounds *de novo*, so vitamin A precursors and carotene must be ingested through food (Maoka 2020).

Over the last two decades, extensive efforts have been made to improve the nutritional value of plant-based foods by increasing the carotenoid content in staple crops (Zheng et al. 2020). Although conventional selection is a tedious and slow process, this strategy has been used for the biofortification in carotenoids of crops such as corn, which are characterized by high genetic diversity of carotenoid content. However, in other crops such as rice, which do not have such variability, other approaches were required to increase the biosynthesis of carotenoid, including the overexpression of one or more biosynthetic enzymes that determine the structure and content of carotenoids. The best known examples concern the crop canola (Shewmaker et al. 1999), rice (Ye et al. 2000; Paine et al. 2005), potatoes (Diretto et al. 2007), maize (Zhu et al. 2008), cassava (Welsch et al. 2010; Sayre et al. 2011), wheat (Wang et al. 2018), sorghum (Che et al. 2016), and bananas (Paul et al. 2017).

Another strategy is to suppress the conversion of their precursors by competing enzymes or the further metabolization of the desired carotenoids, especially β-carotene, by silencing or disrupting the corresponding genes (e.g., *LCYe*, *BCH*, *ZEP*, and *CCD4*) (Romer et al. 2005; Diretto et al. 2006; Pons et al. 2014), or stabilizing the storage of carotenoids in plastids by genetically modifying genes such as *ORANGE* (*OR*), which influence the absorption capacity of plastids (Li et al. 2012; Carrera et al. 2007).

Due to its unique characteristics, CRISPR-based genome-editing technology is now the most efficient and widely used system for introducing targeted nucleotide changes into genomes, which not only enables rapid and transgene/DNA-free modified organisms but also provides the ability to multiplex multiple targets simultaneously, meeting the requirements for the development of a "golden" staple crop (Zhu et al. 2020; Zheng et al. 2021). Several genome-edited, carotenoid biofortified crops have been developed using the CRISPR/Cas systems to cope with VAD. In 2015, Tzuri et al. (2015) succeeded in obtaining the Golden flesh melon through a CRISPR base editor that induces an aminoacidic change Arg-His in the melon orange protein gene (*CmOr*). A few years later, the knockout of the *STAYGREEN* (*SGR*) gene in tomato, a negative regulator of carotenoid biosynthesis, led to a significant increase in the carotenoid content in the fruit (lycopene and β-carotene), as well as an increase in other valuable health-promoting secondary compounds (Li et al. 2018; Gianoglio et al. 2022). In 2020, the results of two other golden crops were published: a Golden Banana, in which the lycopene epsilon-cyclase (LCYε) was knocked down by creating indels using CRISPR-Cas9, thus resulting in a higher amount of β-carotene, and a new Golden rice cultivar Kitaake. In the latter case, CRISPR-Cas9 was used to knock in a 5.2-kb carotenogenesis cassette consisting of *phytoene desaturase (PDS)* and maize *phytoene synthase* (*PSY*) genes, resulting in almost 8 µg/g dry weight ß-(DW) carotene in the endosperm (Dong et al. 2020). The application of CRISPR/Cas9 led to the loss of functionality of the carotenoid isomerase gene in Chinese kale, resulting in the accumulation of lycopene and a color shift from green to yellow (Sun et al. 2020). In chicory, watermelon, and cabbage, CRISPR/Cas9-based gene knockout or knockdown also resulted in PDS mutants with an albino phenotype (Tian et al. 2017; Bernard et al. 2019; Ma et al. 2019; Lee et al. 2020).

As reviewed by Kumar et al. (2022), vitamin E (tocopherol), a powerful fat-soluble antioxidant, is another important vitamin that is essential for human nutrition and general human well-being. Inadequate vitamin E intake has been shown to be associated with cardiovascular disease and some types of cancers, among others (Rizvi et al. 2014). A daily vitamin E intake between 15 and 30 mg is recommended for humans (Ungurianu et al. 2021). CRISPR-Cas9 technology has been successfully applied in barley to overexpress the genes *homogentisate phytyltransferase* (*HvHPT*) and *homogentisate geranylgeranyltransferase* (*HvHGGT*), resulting in a significant increase in the content of tocopherols and tocotrienol (Zeng et al. 2020), thus suggesting that targeted mutagenesis of these genes can also be exploited in other crops to increase vitamin E content.

3.3 | Antioxidants

Numerous natural compounds contained in foods have antioxidant properties (Abbas et al. 2015; Lee et al. 2017). In addition to antioxidants such as vitamins C and E, other compounds such as phenolic acids and flavonoids have a stronger antioxidant function than essential vitamins. Polyphenols are broadly classified into four classes, including flavonoids, flavones, isoflavones, and anthocyanidins, stilbenes, lignans, and phenolic acids (Panche et al. 2016). In plants, polyphenols act as antioxidants, antimicrobial agents, photoreceptors, visual attractors, feeding repellants, and light screening agents, and they have many important biological effects, such as attenuating oxidative stress, protecting against

degenerative diseases, and reducing the risk of cardiovascular diseases (Rahman et al. 2006). A large body of evidence suggests that consistent intake of flavonoid-rich foods can be beneficial to the health of both humans and animals, helping to reduce overweight and obesity and various associated chronic diseases (Scarano et al. 2020; Spika et al. 2022; Sgaramella et al. 2023).

With the aim of increasing polyphenol content, the CRISPR/Cas9 system has been used to reduce or eliminate negative regulators of phenylpropanoid/flavonoid pathway or, conversely, to enhance the function of activators gene expression involved in polyphenol biosynthesis. As an illustration, deletion of the regulatory gene *SlMYB12* using CRISPR/Cas9 has resulted in the accumulation of flavonoids in pink-colored tomato fruits (Deng et al. 2017), whereas the *SlAN2* tomato mutants obtained by targeting the *AN2 MYB* transcription factor gene showed anthocyanin accumulation in the fruits (Zhi et al. 2020).

CRISPR/Cas9 was used to induce mutations also at the regulatory *BnTT8* gene in *B. napus*, resulting in the suppression of phenylpropanoid/flavonoid pathway and the deposition of proanthocyanidin in the seed coat, which took a desirable yellow color (Zhai et al. 2020).

Čermák et al. (2015) targeted the *SlANT1* gene by delivering CRISPR/Cas9 with a geminivirus-based replicon system fuse with a constitutive promoter. This approach exploited the HR repair mechanism and led to the development of novel edited tomato lines that accumulate anthocyanins. The heritability of the newly mutated alleles was confirmed in a segregating population exhibiting purple phenotypes. Similarly, the CRISPR/Cpf1 approach resulted in overexpression of the *SlANT1* gene and plants with an anthocyanin-pigmented phenotype that was retained in the progeny (van Vu et al. 2020). The HR repair mechanism was also used to reactivate anthocyanin pigmentation in tomato seedlings by CRISPR/Cas9-induced deletion of the *SlDFR* target gene (Danilo et al. 2018). Moreover, CRISPR/Cas9 was successfully established in carrot cells, where a mutation in the anthocyanin biosynthesis gene *F3H* led to reduced anthocyanin accumulation (Klimek-Chodacka et al. 2018). Finally, in mushrooms and potatoes, CRISP/Cas targeting the *Polyphenol oxidase* (*PPO*) genes encoding for polyphenol-degrading enzymes in fruits and vegetables resulted in non-browning products (González et al. 2020; Waltz 2016).

3.4 | Gamma-Aminobutyric Acid (GABA)

Gamma-Aminobutyric acid (GABA) is a non-proteogenic amino acid with four carbon atoms that acts as a signaling molecule with various functions in plants and animals (Khan et al. 2021). GABA is involved in numerous physiological and molecular processes in plants, including regulation of cytosolic pH, modulation of nitrate uptake, regulation of pollen tube growth during plant reproduction, and tolerance to abiotic stress (Zhen et al. 2018; Su et al. 2019; Priya et al. 2019; Sita and Kumar 2020). In humans, GABA acts as an inhibitory neurotransmitter and when GABA levels drop, nervousness, depression, and insomnia can occur (Bachtiar et al. 2015). Recently, more attention has been paid to this molecule as it is functional and beneficial to human health. In Japan, for example, there are numerous dietary supplements and foods that are enriched with GABA, which is considered an analogue of vitamin C (Waltz 2022). In the GABA shunt, the precursor glutamate is converted into succinic semialdehyde (SSA) via GABA transaminase (GABA-T) and is oxidized to succinate by the enzyme succinate-semialdehyde dehydrogenase (SSADH) (Daş et al. 2016). Li et al. (2018) used a multiplex CRISPR/Cas9 strategy targeting the shunt GABA metabolic pathway in tomato plants. By using six gRNAs for five target genes involved in the shunt and the genetic *Agrobacterium*-mediated transformation on cv. Ailsa Craig and cv. Micro-Tom, they obtained leaves and fruits with higher GABA content than the WT plants. In addition, an increase in vegetative and reproductive growth of the edited plants was observed.

Since 2020, the first "Sicilian Rouge tomato" with high GABA content produced with CRISPR/Cas9 GE has been commercialized in Japan by the company Sanatech Seed. The company's researchers used CRISPR to introduce mutations into the gene coding for a calmodulin-binding domain (CaMBD) of the GABA shunt. Knocking out this gene led to an increase in GABA through increased activity of the decarboxylase enzyme, which catalyzes the decarboxylation of glutamate to GABA (Waltz 2022).

4 | Challenges of CRISPR/Cas9 Technology in Vegetable Crops

GE techniques can be used to make precise, efficient, and targeted modifications in the genomic loci of plants. Despite these excellent results, further efforts are needed to improve their efficiency and overcome the associated problems, such as off-target sites, that is to say, the cleavage of nontarget genomic sites by the endonuclease. The occurrence of such effects is influenced by two factors. First, the chosen sgRNA may bind to nontarget sequences. The specificity of the binding of sgRNA to its target sequence is critical for the success of CRISPR and Cas9 methods. Nonetheless, due to the intricate nature of genomes (i.e., polyploid ones), sgRNA may also associate with similar sequences in addition to the target site, resulting in the endonuclease cleavage activation by the endonuclease of nontarget sites. Second, endonucleases might detect nonstandard PAM. For example, the CRISPR/Cas9 system should cut three bases upstream of the PAM site. It has been shown that Cas9 can sometimes also recognize a nonstandard PAM resulting in off-target effects. Although a low frequency of off-target mutations has been reported in plants due to their presence in noncoding regions, the prevention of DSBs in nontarget regions remains a challenge. Researchers can indeed operate both by increasing the specificity and fidelity of Cas9, as well as carefully checking the genome sequences nearby to the target PAMs when designing the sRNA targeting sequence to avoid nonstandard PAMs. Several bioinformatics tools have been used to design highly specific sgRNAs with minimal off-target activity such as CRISPR-P V2, CRISPOR, and Cas-OFFinder.

Other issues related to the application of GE are the barriers to gene activation/overexpression, the efficacy of delivery systems, the need to develop protocols for transformation, and regeneration for all species as well as a high-throughput screening system for the new mutant lines. In particular, the efficiency of editing depends on the plant species, which in turn influences the type

of tissue to be used for transformation and the transformation method itself. Furthermore, the regeneration capacity of in vitro cultures is hampered by recalcitrant cultures that require more laborious protocols to maximize the result.

High-throughput screening systems are also important for the identification of new mutant lines. Currently, methods based on classical PCR are the most used, but they may have some limitations in the detection of single nucleotide variants (SNVs). Although they are cheap and easy to use, PCR-based techniques are not sufficient to identify SNVs. Therefore, they are often combined with sequencing techniques that allow the nucleotides involved in the mutation to be precisely identified. Sequencing is still the most suitable method for identifying mutations in polyploid plants and for identifying off-targets. These aspects increase the need for ever more modern and highly efficient bioinformatics tools based on sequencing. A huge help has been provided by next-generation sequencing (NGS) using second- and third-generation sequencing techniques capable of analyzing heterogeneous samples. Furthermore, sequencing techniques also enable whole-exome and/or whole-genome (WGS) sequencing to identify structural rearrangements such as inversions, translocations, insertions, and deletions and to detect rare off-targets. NGS techniques not only enable precise characterization of an edited line but are also advantageous in terms of cost.

In addition, the legal aspects and regulations for the production and commercialization of crops derived from NBTs need to be considered. Awareness of GE among European Union citizens has increased significantly in recent years, as has concern about the use of these breeding strategies. Despite Europe's formerly hostile position about genetically engineered organisms, last February the European Parliament voted to mitigate the normative concerning crops obtained through GE methodologies. The wide-ranging opportunities offered by these technologies can undoubtedly contribute to a more sustainable and resilient food system by developing new and improved plant varieties that are climate resilient, pest resistant, and higher yielding, with lower input use and, as reviewed in this article, of better nutritional quality. The negotiations with Member States on the Commission's proposal on new genomic techniques (NGTs) were favorably received by the majority of Members of the European Parliament, who agreed with the proposal to create two different categories of NGT plants subject to different regulations. It is indeed possible to distinguish between the so-called NGT-1 plants, which are considered equivalent to the conventional ones "when it differs from the parent plant by no more than 20 genetic modifications" and are therefore not subject to GMO legislation, whereas NGT-2 plants are subject to stricter requirements. Although this is only a small step forward for NBTs, it is a huge advancement considering the precautionary principles which had characterized Europe so far. Monitoring public opinion on GE of crops and food will become increasingly mandatory, as will have a decisive influence on the new regulatory framework proposed by the European Commission and ultimately on the extent to which European consumers will benefit from new biotechnologies.

Another important aspect of the application of this biotechnique on plants is its impact on biodiversity conservation to counteract the effects of climate change. CRISPR/Cas9 is a powerful tool to create genotypes with higher efficiency in nutrients uptake, reducing the need for harmful agrochemicals and greenhouse gas emissions, or with higher nutritional value and longer shelf life, thus reducing food waste. Although this biotechnological approach could lead to positive socioeconomic impacts in terms of costs and yield, the negative effects must also be considered. The use of editing technologies can lead to an uncontrolled increase of new allelic variants in natural populations of crops, both spatially and biologically through individual/species crosses. Therefore, a thorough risk assessment of potential negative effects not only on a biological and ecological level, but also on an economic and cultural level, is imperative to increase public involvement.

5 | Conclusion

The CRISPR/Cas9 represents an effective strategy for improving crops by passing the expensive and time-consuming conventional breeding programs and the ethical problems of transgenic approaches. Transgene-free GE is a promising alternative for rapid germplasm innovation and the introduction of new traits for agrobiodiversity. The results obtained with the GE technologies reviewed above are examples of how the NBTs can be easily applied to crops to improve some beneficial phytonutrients while reducing and/or mitigating anti-nutritional factors harmful to human health.

Author Contributions

Maria Dellino: conceptualization, writing–original draft, writing–review and editing. **Claudio de Giovanni**: conceptualization, writing–original draft, writing–review and editing. **Monica Marilena Miazzi**: writing–original draft, writing–review and editing, supervision. **Cinzia Montemurro**: funding acquisition, writing–review and editing, supervision. **Domenica Nigro**: writing–review and editing, funding acquisition, supervision.

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Conflicts of Interest

The authors declare no conflicts of interest.

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