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Review Article

From bacterial battles to CRISPR crops; progress towards agricultural applications of genome editing

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Genome editing is the precise alteration of DNA in living cells by the cutting or removal of specific sequences, sometimes followed by insertion of new sequences at the cut site. CRISPR–Cas9 has become firmly established as the genome-editing method of choice, replacing the systems that had been developed and in use since the early 1990s. The CRISPR–Cas9 system has been developed from a mechanism used in prokaryotes as a defence against bacteriophage but actually functions in cells of all types of organisms. It is widely used in research as a gene knockout and editing tool; applications in veterinary medicine (such as increased resistance to disease) and human medicine (such as correction of disease-causing mutations) are under development. In agriculture and horticulture, the potential for various aspects of crop improvement is very large. Selected aspects of this potential are presented here, with particular focus on crop quality and disease resistance. The article ends with a brief discussion of the regulatory 'environment' in the USA and the EU.

Introduction

Genome editing, the inactivation of specific DNA sequences by cutting the DNA or by complete removal (which may be followed by insertion of new DNA at the edited site), has been in use for nearly 30 years. In the very early 1990s, it was demonstrated that the Cre-Lox recombinase system could remove individual genes from plant genomes, including the antibiotic-resistance genes that were, and in some cases still are, used as selectable markers in plant genetic modification [1,2]. Systems were then developed that made use of deoxyribonucleases, especially the transcription-activator-like effector nucleases — TALENs — and the zinc finger nucleases — ZFNs [3]. The cells' endogenous non-homologous end-joining then repairs the cut site *in situ* thus effectively causing a functional 'knock-out' of the targeted sequence. Alternatively, a repair could be effected by directed insertion of another sequence at the cut site. These methods had wide applications in research, where gene knock-outs are very useful tools in determining gene function. However, these methods were relatively cumbersome in that they needed to be specifically designed for each target sequence and could only be directed at one target at a time. The arrival in 2012 of a simpler system that could be targeted to multiple sites simultaneously was therefore very welcome. That system was based on a DNA sequence known as CRISPR and an associated deoxyribonuclease, Cas9.

History and development of the CRISPR–Cas9 genome-editing system

The development of CRISPR–Cas9 is a very nice example of a natural system being re-purposed for human use. This fascinating and important story starts in the 1980s when repetitive DNA sequences were found in bacterial genomes (e.g. [4]). Up to that time they had been thought to be a feature only of eukaryotic genomes. Further analysis showed that the 30-base-pair repetitive DNA elements were clustered and that their sequences were palindromic (read the same in both directions). The term CRISPR (Clustered Regularly Interspersed Short Palindromic Repeats)[1] was introduced in 2002 [5]

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but their function remained unknown. Mojica et al. [6] discovered that the spacer sequences separating the repeats were similar to the DNA of bacteriophage; they went on to suggest that they may be part of a bacterial immune system against phage. It took another two years of research in laboratories in Europe, the USA and Canada to confirm this and another three years to elucidate the detailed mechanism. The essentials are as follows: the spacer sequences, which have been synthesised in response to a previous exposure to a foreign genome, are copied into RNA (crRNA) on re-exposure to that genome. The crRNA, aided by another RNA species, tracrRNA or guideRNA, guides Cas9 (a **C**RISPR-associated nuclease) to the invading genome and inactivates it by cutting it at precise sites [7,8].

Moving forward to 2011, Siksnys and his team at the University of Vilnius in Lithuania showed that CRISPR systems can work when put into other species [9]. As mentioned above they later published details of the mechanism by which CRISPR–Cas9 recognises and cuts DNA, [7]. Doudna's group in California made the same discovery at about the same time [8]. The way was thus paved for development of CRISPR–Cas9 as a general genome editing system, based essentially on Cas9 and guideRNA (sgRNA); sgRNA complementary to the targeted gene is used to position the nuclease which then cuts both strands of DNA. A single RNA molecule, sgRNA complementary to the target sequence, is sufficient for effective targeting. This adds further to the ease of use of the system. Use of two complementary sgRNA sequences results in two double-stranded cuts and therefore excision of the tract of DNA between them[2]. Thus we have gone from the unexpected discovery of DNA sequences, to elucidation of a bacterial immune system to the development of techniques for removing almost any sequence from almost any genome. The power of the system was well illustrated by Church's group at Harvard [10] who used it to remove 62 retroviral sequences from the pig genome, in the context of research on the use of pig organs for human transplants. However, that was over-shadowed by a spectacular experiment in 2018 when Kruglyak and colleagues [11] were able to make 10 000 mutations in a yeast cell culture by pairing each RNA guide sequence with its own DNA patch.

Furthermore, genome editing, by whatever method, also provides a way of targeting genes into specific loci in the genome. The variation of insertion site and the inability to control where a transgene is inserted were two frustrating features of earlier genetic modification techniques. This meant, for example, that both the expression of the transgene and its heritability had to be assessed across a range of genetically modified (GM) individuals in order to select the best expressers. With the ability to generate cuts or gaps at specific places comes the added ability to insert new sequences at those specific places [12].

Before discussing some of the applications it needs to be said that the range of discoveries related to CRISPR–Cas9 and the expanding range of possible applications has led to a complex situation regarding intellectual property. There have been claims and counter-claims from different 'inventors' and the patent jurisdictions in the USA. and the EU have sometimes reached different conclusions regarding precedence. Individuals and organisations that develop applications for the technology will need to navigate this complicated IP environment (see [51]).

A short excursion into medicine

No account of the development of CRISPR–Cas9 genome editing would be complete without a brief consideration of its possible use in human medicine. We can identify several different types of a pathological condition in which knock-out or removal of a gene or removal and replacement of a gene will bring a therapeutic benefit, in some instances amounting to a full cure [13,14]. Furthermore, a precedent was set in 2015 when a TALENs-based genome-editing procedure (see Introduction) was used successfully in an experimental treatment to cure a baby girl of acute lymphoblastic leukaemia when all standard treatments had failed [13,15].

The advent of CRISPR–Cas9 genome editing makes it likely that there will be more applications of this type but there are in addition, several other therapeutic uses of genome editing. I have discussed these briefly elsewhere [13,14] and have also noted the ethical issues that may arise. In the main, these issues are similar to those that arise in 'conventional' genetic modification of humans, namely

- Risk and safety
- Use for therapy but not for enhancement
- Use in somatic cells but not in the germ-line (the latter would make the changes heritable)

[1]Archaeal DNA also contains these sequences

[2]Alternatively, a single cut can be made; the DNA is repaired by the endogenous end-joining mechanism as mentioned earlier in relation to other genome-editing methods. This is adequate for functional gene knock-out but not for complete removal of a sequence.

In respect of the latter point, there is currently a near-universal moratorium on germ-line editing of humans (primarily on grounds of risk), albeit that it was broken by a Chinese scientist, He Jiankui in 2018 [16,17]. He has subsequently been removed from his job by the Chinese authorities. However, there is extensive research on the development of somatic (non-heritable) genome editing using the CRISPR–Cas9 system. Indeed, a previous issue of this journal was devoted to the topic, with a very useful overview by Qasim [18].

Into the fields: use of CRISPR–Cas9 genome editing in crop plants

Setting the scene

It may seem a big jump from medicine to agriculture but the overall pattern of possible applications is very similar, as is emphasised by Zhang et al. in a recent review [19]: we can identify targets for genome editing that would improve overall genetic fitness, prevent unwanted physiological or biochemical reactions and impart resistance to specific pathogens. In animal agriculture, the latter principle was illustrated by the removal from pigs of the gene encoding the receptor protein for the virus that causes Porcine Reproductive and Respiratory Syndrome (PRRS), thereby making the animals resistant to the disease [20]. If this approach is effective in animals, there is no a priori reason why it should not work in plants.

Effective use of the CRISPR–Cas9 system in plants was first demonstrated in 2013. Feng et al. [21] used transgenic *Arabidopsis* and rice plants in which the CaMV 35S promoter 'drove' the *Cas9* gene. Synthesis of the guide RNA (sgRNA) was under the control of the AtU6-26 promoter in *Arabidopsis* and the OsU6-2 promoter in rice. In both plants, the system was successfully directed at a reporter transgene which was inactivated by the editing process. Similarly, Nekrasov et al. [22] were able to demonstrate inactivation of a reporter gene in leaf tissue of *Nicotiania benthamiana* while Jiang et al. [23] demonstrated CRISPR–Cas9-based genome editing in two dicots, *Arabidopsis* and tobacco and in two monocots, rice and *Sorghum*.

Feng et al. [21] went on to target endogenous genes in both *Arabidopsis* and rice; the genes selected were involved in hormonal signalling; gene disruption gave readily identifiable growth phenotypes, demonstrating the efficacy of the genome editing. A very similar approach was taken by Xie and Yang [24] who targeted a stress-responsive mitogen-activated kinase. Their reason for doing this was that the particular MAP kinase is a negative regulator of a resistance response and thus inactivating it may increase disease resistance.

As discussed later in more detail, there has been ongoing concern, especially in Europe, about plants carrying 'foreign' DNA such as the *Cas9* gene or the CaMV 35S promoter. One way to deal with this problem is to back-cross the edited line in order to eliminate the transgene. Another way of circumventing this concern was taken by Woo et al. [25] who pre-assembled complexes containing the Cas9 nuclease and appropriate guide RNAs. The complexes were transfected into protoplasts of *Arabidopsis,* tobacco, lettuce and rice. Plants regenerated from the protoplasts showed mutation rates in the targeted genes of up to 46%. The authors note that the mutations were heritable and were 'indistinguishable from naturally occurring genetic variation' and this was achieved without any integration of 'foreign' DNA.

Applications

As has been hinted at already, the range of possible applications of the CRISPR–Cas9 system to crop plants is very large. The applications actually extend beyond straightforward genome editing, as discussed by Belhaj et al. [26] and by Xhang et al. [19] but here I focus mainly on genome editing itself. The main aims in genome editing of crop plants are

- Improvement of crop quality
- Improved resistance to diseases and other biotic stresses
- Improved tolerance of or resistance to abiotic/environmental stresses. This includes creating crops that are resilient to climate change
- Improvement of crop performance under normal field or greenhouse conditions

All of these are 'umbrella' aims and embody a range of more specific targets. The last group of aims is especially wide and already includes 're-domestication' of wild tomatoes to make the cultivated crop more suited to current needs [27]. A few genome edits can replace many years of plant breeding!

Because of the very wide range of possible applications, I will confine my discussion to the first two of these topics, looking at a selection of examples that illustrate the current and ongoing situation. Thinking first about

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crop quality, a recent development in crop GM technology was the commercial production of the 'Arctic Apple' which does not go brown when sliced [28]. The reason for this is that the gene encoding polyphenol oxidase (PPO) has been silenced by RNA interference. A similar approach was used to silence PPO in the production of non-browning potatoes [29]. RNA interference is similar to and certainly has the same result as the antisense technology that was used in production of the FlavrSavr™ tomato in the 1990s [30]. However, inactivation of the PPO could be carried out just as easily (and possibly *more* easily) with genome editing techniques. It will be interesting to see whether, if further varieties of non-browning apples or potatoes are produced, as seems very likely, genome editing will be employed.

Non-browning lettuce with a shelf-life nearly two weeks longer than its conventional counterparts has been produced by a US company, Intrexon, under the name Green Venus™ [31]. The company have not revealed specifically how this variety was produced, except to say that it is not transgenic; the implications of this statement and of information on the company website are that genome editing was used to produce the new lettuce. Furthermore, the reactions of both the USDA and the EU's regulatory bodies imply that it is known that the production involved genome editing. And we certainly do know that non-browning mushrooms, developed in Yinong Yang's Laboratory at Pennsylvania State University were produced by genome-editing to knock out polyphenol oxidase [32]. The USDA announced in 2016 that these mushrooms did not need their approval [32] (see below) but they have not yet come to market (as at mid-2019).

Another example of quality improvement is a change in the fatty acid composition of seed lipids in soybean [33]. Soybeans in which 80% of the stored tri-glycerides contain the mono-unsaturated fatty acid, oleic acid (18.1; cis9) were launched commercially early in 2019 by the agri-food company Calyxt Inc. Furthermore, the content of saturated fatty acids is reduced by up to 20%; processing of the stored lipids to make cooking oil produces no *trans*-fatty acids. The oil is marketed as being a healthier option than normal soybean oil (trans-fats have been implicated in heart disease) with a much longer 'fry life' than the normal oil. Interestingly, company uses the term 'non-GMO' as a selling point for this high-oleate variety [33].

Moving on to consider the generation of resistance to biotic stresses, especially diseases, in many instances we know how a pathogen interacts with its host. Knock-out of the 'host susceptibility genes', many of which encode proteins recognised or activated by the pathogen, will confer resistance to the organisms that interact with or activate those proteins [34]. A current urgent projected application of this approach relates to the Cavendish banana [35]. This banana variety is grown all over the world and accounts for ~85% of the exports of the crop to countries where bananas are not grown. A new strain of *Fusarium oxysporum* ('Fusarium wilt') has evolved ('Tropical Race/TR 4) to which Cavendish is susceptible; the disease is spreading rapidly in banana-growing areas. Scientists in Australia have now used GM techniques to construct a TR4-resistant line, using a gene from a naturally occurring resistant banana subspecies from south-east Asia [36]. However, with disease resistance of this type there is always the danger that the pathogen will evolve to overcome the resistance. Because of that, a genome editing approach has also been taken in which the ability of the plant to interact with the fungus is knocked out. Field trials of these genome-edited bananas started in three continents in 2019 [37].

The *Fusarium*-resistant banana is one example of many disease-resistant crop lines that have been generated by genome editing. In a review published in 2019, Wang et al. [38] list nine diseases to which tomatoes have been made resistant. In addition, they list two diseases in grape, three in cucumber, and one each in papaya, cocoa (*Theobroma cacao*) and banana, for which resistant host strains have now been generated. The banana example is especially interesting. It concerns resistance to banana streak virus (BSV); it is an endogenous virus in the genus *Musa* meaning that its genome is integrated into the genome of its host. Resistance was generated by editing the integrated genome copy [39].

Genome editing is also applicable to staple crops. Rice is the most widely consumed cereal crop in the world. About 490 million tonnes are consumed annually, providing essential nutrition for more than three billion people [40]. It has thus been a major subject of research on the use of genome editing [41]. In particular, it has been a very useful subject for an alternative CRISPR-based system using an alternative nuclease, Cpf. Both CRISPR–Cas9 and CRISPR-Cpf systems have been employed to improve a very wide range of aspects of crop quality. In relation to disease resistance, one of the most important targets has been rice blast disease, caused by *Magnaporthe oryzae*. In some rice-growing areas, very large crop losses occur which can amount to over 60% of the total yield in some years. Some success has already been achieved in improving resistance in rice lines in which the host transcription factor ERF922 (ERF: ethylene response factor) has been disabled by editing with the CRISPR–Cas9 system [42].

The examples discussed above are illustrative of the very wide range of current and projected applications of genome editing using CRISPR-based systems. However, the ability of plant scientists to use these systems in crop improvement may be affected by factors external to the science itself, as is discussed in the next section.

Social and ethical aspects

One of the factors that has affected the uptake into agriculture and horticulture of GM crops has been the regulatory 'climate' in particular countries. This has been discussed very fully elsewhere [13]; here, discussion is confined to the USA and the European Union. In the USA, the US Department of Agriculture and the Food and Drugs Administration applied more stringent criteria for approval of GM-bred crops than to 'conventionally' bred crops but the criteria were not so stringent as to inhibit the commercialisation of these crops. When the first genome-edited crops were in the pipeline, the USDA announced that they would not be subject to the criteria imposed on GM-bred crops but would be treated in the same way as conventionally bred crops. The position was further clarified in 2018 [43]. Plant scientists have welcomed this announcement which paves the way for commercialisation of many of the applications of genome editing that were mentioned earlier. They also welcomed the more recent announcement from the USDA that after 25 years of experience with GM-bred crops, criteria for their evaluation are now to become less stringent [44].

In Europe, the situation is more complex. When the first GM crops, and/or products of GM crops, nearly all grown in the USA, came to market in the mid to late 1990s, environmental groups in the U.K. and in much of Western Europe waged a very active campaign of opposition. The original reasons for their opposition have never been really clear, despite the policy statements of the organisations themselves but nevertheless, the campaigns were very effective in turning public opinion against crops bred by GM methods [45]. This led in turn to the adoption by the EU, based again on active campaigning by 'Green' MEPs, of the strictest interpretation of the Precautionary Principle in relation to the commercial growth of GM-bred crops. The safety evaluation imposed on such crops is very lengthy and cumbersome [46,47]. Since the late 1990s, only two crop varieties bred by GM techniques have been approved for commercial growth and only one of those, insect-resistant maize (carrying the Bt-insecticidal protein gene) is grown anywhere in Europe (mostly in Spain) [13,47].

There is undoubtedly growing opposition in the plant science community to the EU position [47], believing that it disadvantages European farmers (because they are competing with farmers from countries in which growth of GM-bred crops is permitted) and that it inhibits some aspects of plant science research. It was hoped that a different approach would be adopted for genome-edited crops. In general, these are not carrying an added trait neither, as mentioned earlier, do they need to contain any 'foreign' DNA [25]. In many cases, they are indistinguishable from naturally occurring (albeit rare) mutants of the same crop (also as mentioned earlier). In general, plants bred by mutagenesis techniques are exempt from the EU's strict regulation of GM crops. However, in a test-case ruling, the European Court of Justice (EJC) has decided that genome-edited crops do not meet the criteria for the 'mutagenesis exception.' They therefore recommend that genome-edited varieties should be regulated in the same way as GM-bred varieties [48], reflecting the negative view amongst those who oppose 'biotech' crops (e.g. [49]). This has caused great consternation in the European plant science community and has elicited strong reactions [47,50]. Nevertheless, for the present, the commercial growth of genome-edited crops remains on hold in the European Union. Whether, eventually, the European Commission accepts the verdict of the EJC remains to be seen, although based on previous experience they are likely to do so.

Summary

- CRISPR–Cas9 is an easy-to-use genome editing system based on a bacterial defence mechanism.
- It is widely used in research on gene function; applications in veterinary and human medicine are in the pipeline.
- Its potential for use in agriculture and horticulture is huge, especially in relation to disease and stress resistance and improvements of crop quality and performance.
- The first genome-edited crops are now on the market in the USA and Canada.

Abbreviations

BSV, banana streak virus; EJC, European Court of Justice; ERF, ethylene response factor; GM, genetically modified; PPO, polyphenol oxidase; PRRS, Porcine Reproductive and Respiratory Syndrome.

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Competing Interests

The Author declares that there are no competing interests associated with this manuscript.

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