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CRISPR in Plant Breeding

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An Overview of the Clustered Regularly Interspaced Short Palindromic Repeats System and its potential impacts on Plant Breeding

by

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Table of Contents:

Introduction:

Plant breeding has come a long way since humans first began harvesting seeds with desirable traits as long as 10,000 years ago. Since then there have been multiple advancements such as Gregor Mendel's experiments in the 19th century which were a major milestone in the study of genetics and its application in developing new commercial varieties of agriculturally important species. From conventional cross breeding to genetic engineering, plant breeders have continually pushed the limits of science and technology when pursuing improved varieties. There has been a relatively new development that uses a prokaryotic defense mechanism to edit genes in the most accurate, efficient, and cost effective way yet, it is called CRISPR. CRISPR stands for clustered regularly interspersed short palindromic repeats, and it has emerged as a groundbreaking tool for plant breeders. The aim of this review is to show how CRISPR was discovered and developed, as well as to give an all-encompassing overview of how CRISPR can be used in plant breeding. This includes summarizing important past and current research, as well as hurdles that will need to be overcome in being able to fully realize the potential that CRISPR has.

Background:

The story of CRISPR began when a research team in Japan led by Yoshizumi Ishino first discovered clusters of 29 nucleotide long repeating DNA in *Escherichia coli* in 1987, however at the time they did not know the function of the repeats (Ishino et al., 1987). Francisco Mojica in Spain worked on CRISPR throughout the 1990's and recognized that these repeated sequences were present in 90% of sequenced Archaea and over 40% of Bacteria (Mojica et al., 2000). He was even first to coin the term

CRISPR with Ruud Jansen in 2002 (Jansen et al., 2002). Mojica continued to study the CRISPR regions and hypothesized correctly that the spacers between the repeats matched regions of the genomes of bacteriophages and that they acted as a sort of immune system (Mojica et al., 2005). Similar findings by another group in France were published around the same time (Pourcel et al., 2005). Jansen identified CRISPR associated genes (*Cas*) adjacent to the repeat elements which served as a basis for later classification of three CRISPR systems (Jansen et al., 2002). CRISPR loci in system types I and III contain multiple Cas proteins while the type II system has many fewer Cas proteins to aid in recognition and destruction of the target nucleic acids (Makarova et al., 2012).

Another key advancement came in 2005 when Alexander Bolotin was studying *Streptococcus thermophilus* and discovered new Cas genes that his team predicted had nuclease activity and is now known as Cas9 (Bolotin et al., 2005). Soon after, Philippe Horvath and colleagues at Danisco were able to demonstrate how CRISPR worked in *S. thermophilus* by seeing how the bacteria responded to a phage attack, which was by the CRISPR array being transcribed into CRISPR RNA and transactivating RNA to form a complex with Cas9, then finding the matching sequence to the crRNA in the phage DNA and inactivating the invading phage (Barrangou et al., 2007).

The pace quickened on CRISPR research over the next few years and soon the basic functions and mechanisms of CRISPR systems were becoming much more well understood. The next big steps towards gene editing came when Sylvain Moineau and his team showed that only Cas9 in a type II system can mediate target DNA cleavage (Garneau et al., 2010), and then Elitza Deltcheva revealed that a key part of the process

is the transactivating RNA (tracrRNA) hybridizing with crRNA to allow RNA guided targeting of Cas9 (Deltcheva et al., 2011). Finally, Rimantus Sapranauskas of Lithuania demonstrated that the type II CRISPR system could be transferred to other organisms by transferring it from *Streptococcus thermophilus* into *Escherichia coli* (Sapranauskas et al., 2011). These new studies led to the idea that it could be possible to use CRISPR as a genome editing system.

In 2012, Jennifer Doudna and Emmanuelle Charpentier were able to use CRISPR/Cas9 to cleave target DNA in vitro and perhaps more importantly, showed that a single guide RNA could be constructed by joining crRNA with the guide sequence to a tracrRNA, further simplifying the system (Jinek et al., 2012). At this point it was only being used in prokaryotic organisms. In 2013 Feng Zhang of the Broad Institute of MIT and Harvard was able to successfully use CRISPR/Cas9 for genome editing in eukaryotic cells, proving that this naturally occurring prokaryotic system could have very far reaching applications (Cong et al., 2013).

These were just some of the key breakthroughs in understanding how this amazing process actually works. Over the past few years research has progressed at blistering speeds and has been applied to both prokaryotes and eukaryotes, including human cells. This is what would unleash CRISPR to become a vital tool in editing genomes for plant breeding purposes.

Mechanisms of the CRISPR/Cas9 System:

To understand the CRISPR defense mechanism it is important to first understand the natural systems that CRISPR evolved in. Bacteriophages are viruses that

exclusively attack bacteria. They consist of DNA encased in a protein envelope with a "head" and "tail" which is what attaches to the surface of bacteria. The phage injects its nucleic acid into the bacterium where it is then reproduced to the point where the bacterium essentially bursts and further spreads the phages.

The CRISPR sequence is comprised of palindromic repeats of 24-37bp (Grissa et al., 2007), which are separated by spacers, or short sequences that match parts of DNA from previous invading viruses. There is also a portion that has *Cas* genes, or CRISPR associated genes that encode for Cas proteins. The Cas proteins generally make helicases and nucleases that unwind and cut DNA respectively.

The CRISPR system is able to defend against viral invasion by executing three key processes; adaptation, expression, and interference. In adaptation, the bacteria needs to acquire and integrate a short segment of the viral DNA into the CRISPR array in order for it to begin its work. This is carried out by Cas1 and Cas2 proteins (Yosef et al., 2012). Once this new piece of DNA has been integrated into the array, it (or a segment from a previous invasion) and the repeats will be transcribed into small interfering CRISPR RNAs (crRNA) that are around 40 nucleotides in length. Finally, interference occurs when these combine with transactivating CRISPR RNA (tracrRNA) that guide the Cas9 nuclease to the corresponding region of the invading DNA and it can be cleaved. A protospacer adjacent motif (PAM) downstream of the target sequence on the tracrRNA helps the Cas9 bind and find the correct area to cut (Jinek et al., 2012). Once cut, a repair is done by the non-homologous end joining pathway (NHEJ) or homology directed repair (HDR). The NHEJ pathway is error prone and often

results in insertions, deletions, and substitutions, which causes the gene to be inactive, ending the threat of the virus (Symington and Gautier, 2011).

As research has progressed, there have been many different types of CRISPR systems identified besides the Type II system that use the effector protein Cas9. CRISPR is currently classified into two classes, six types, and 19 subtypes (Shmakov et al., 2017). Class 2 has been the best option for gene editing due to its relative simplicity in that it has single effectors to mediate interference as opposed to class 1, which uses multi-effector complexes. In addition to the Cas9 Type II system there is also Cas12 (A, B, C) Type V and Cas13 (A, B, C) Type VI. Although recent studies have shown there are a few Cas9 subtypes that can target RNA, Cas 13 is being studied with high interest due to some limitations of the main Cas9 system in regard to off target editing and ability to target RNA (Shmakov et al., 2017; Strutt et al., 2018).

Plant RNA viruses infect a wide range of plant species and are responsible for a large proportion of commercially important plant diseases (Nicaise, 2014). These do not have DNA intermediates in their life cycle and the classical CRISPR/Cas9 system would not be useful in targeting them, although some variants such as Cas9 from *Francisella novicida* can be reprogrammed to target specific RNAs (Price et al., 2015). The CRISPR/Cas13a system however, could provide a solution. In the Cas9 system, pre-crRNA is made before mature crRNA and requires accessory factors. Cas13 can process its own pre-crRNA transcripts and create mature crRNA with the transcribed spacer and part of the repeat sequence that associates with Cas13 to form the Cas13-crRNA complex (Mahas et al., 2018). After binding to the complementary sequence of the virus, cleavage occurs. Cas13 also has promiscuous cleavage activity which leads

to the destruction of non-target RNAs. Cas13 has recently been shown to be effective in conferring resistance against RNA phages such as *Turnip mosaic virus*, however further work is needed to increase its effectiveness specificity (Aman et al., 2018). Being able to target RNA genomes would be a superior method of fighting viral infections as it would not lead to permanent changes in the host genome (Mahas et al., 2018).

CRISPR has been shown to be relatively more efficient and easier to use than gene editing methods used previously such as zinc finger nucleases (ZFNs) and transcription activator like effector nucleases (TALENs). These are similar to CRISPR in that they can generate double stranded breaks in a targeted region, but they require extensive protein engineering for each targeted region as opposed to the single guide RNA of 20 bp used in CRISPR. These are much more labor and resource intensive when compared to CRISPR, and they also cannot target multiple genes simultaneously. The ability of CRISPR to be used for multiplexing where it can make multiple edits at different loci simultaneously is a major advantage it holds over ZFNs and TALENs (Li et al., 2013). Doing this with these other methods would require separate dimeric proteins specific for each target site.

Applications of CRISPR in Plant Breeding:

CRISPR has an extraordinarily diverse number of areas where it can be applied. It works in nearly every organism and has endless possibilities in plant breeding. A review of 52 peer reviewed articles on the agricultural application of CRISPR show that improved yield is the most studied trait by far, followed by biofortification and herbicide tolerance (Ricroch et al., 2017).

As previously discussed, CRISPR can perform simple insertions and deletions as well as more complex multiplex edits, and is effective in both RNA and DNA. And it does this in a relatively simple and cost effective way. For plant breeders, this is a great new tool in the toolbox for developing desired characteristics in their target plant species and it is able to do this quickly, especially in comparison to the traditional breeding process of crossing germplasm which can take years to produce an improved variety.

The most common early use for CRISPR/Cas9 was for insertions and deletions(indels) in the target genome. After the target DNA is cleaved, the break is often repaired by NHEJ which can lead to single or multiple base pairs lost or added, resulting in a non-functional gene. This is very useful when trying to determine the function of a gene. Multiplex transformations can also be performed where multiple gRNAs are used to do more than one edit at a time. Alternatively, gene insertion can be achieved through HDR by providing donor DNA that can be inserted where the break is made. This form of site specific nuclease use can be applied to stack multiple traits close to each other, which would be very helpful in lowering the segregation rate in use for further breeding activity (Ainley et al., 2013).

Another great advantage of CRISPR is that it is highly heritable and does not leave any transgenes behind. Studies in tomato and rice have shown that the CRISPR/Cas9 system can introduce homozygous mutations in the first generation (Brooks et al., 2014; Zhang et al., 2014). Hui Zhang found that 10.5% (6 of 57 plants) rice plants were homozygous after CRISPR/Cas9 transformation and 15.8% were bi-allelic (different mutations occurred on the two copies of the target gene), and these continued to be homozygous in the second generation. This high transformation rate

shows how valuable the process can be for saving breeding time compared to traditional breeding or other gene editing techniques. In addition to this, it's important that these subsequent generations are “transgene clean” due to the regulations in many countries that differentiate between new plant varieties and if they contain any foreign DNA.

One interesting area where CRISPR may be of some use is in gene drives. A gene drive is a technique where a gene can be propagated throughout a population overriding normal inheritance patterns. It has been proposed that gene drives could be useful in weed management by introducing deleterious mutations that impact fitness and weediness, or editing the weed genomes so populations are more sensitive to management interventions (Neve 2018). Paul Neve (2018) has suggested that the introduction of edited plants would lead to mating with wildtype plants where the heterozygous plants would carry the CRISPR system and it would be used to create a homozygous plant that would spread over time into the rest of the population. Systems like this carry ethical concerns about the spread of the plants going unchecked and leading to species extinction. However, a daisy chain system where a complex of genetic elements are arranged such that they depend on one another and could limit the spread to small populations could be effective in control (Smidler et al., 2017).

There are many interesting projects employing CRISPR currently underway that would be able to solve a lot of different problems experienced by growers around the world. Researchers at Wageningen University in the Netherlands are using CRISPR/Cas9 to remove antigens in gluten (Siegler, 2019). This would allow the millions of people in the US with celiac disease to be able to safely consume products

with gluten. Bananas, which are a staple crop for many and a sizable commercial product, have a variety of pathogens threatening to wipe out entire cultivars. Banana streak virus is integrated into the bananas genome and when the plant is stressed it can activate the virus. Leena Tripathi at the International Institute of Tropical Agriculture in Kenya has been able to use CRISPR/Cas9 to destroy the viral DNA in one variety and is working on making the bananas resistant to the virus (Tripathi et al., 2019). The popular commercial variety Cavendish has already been genetically modified to be resistant to a devastating fungal strain Tropical Race 4, but efforts to do it through CRISPR are preferred due to regulations on transgenic plants in many countries (Le Page, 2019).

Researchers at Syngenta have recently developed a creative new editing method they have called haploid induction editing (HI-Edit). In order to increase the efficiency of delivering CRISPR into cells of recalcitrant varieties that aren't easily transformed, they used haploid induction with pollen from plants transformed to carry the desired gRNA and Cas9 machinery (Kelliher et al., 2019). By adding CRISPR to a corn line that is relatively receptive to transformation and is also able to trigger haploid induction, the research team was able to create a method of increasing the efficiency of delivering CRISPR into different varieties while simultaneously taking advantage of traditional doubled haploid breeding systems which greatly decrease the amount of time it takes to generate inbred lines. They also showed that this method could be used in dicots as well as other monocots like wheat and recovered edited wheat embryos delivered by maize pollen.

Another inventive use for CRISPR/Cas9 utilizes what is known as catalytically dead Cas9 or dCas9. This method can be used to create efficient and programmable genome-wide scale transcriptional regulators. In the traditional CRISPR/Cas9 system, the two nuclease domains of Cas9, RuvC and HNH, cleave DNA, but in dCas9 these are inactive and can still bind to the target region, but will not cut the DNA (Qi et al., 2013). This repurposed system is known as CRISPR interference (CRISPRi) and converts a site-specific genome editing tool to a specifically targeted genome regulation tool that represses the gene (Piatek et al., 2014). Furthermore, fusing transcriptional regulators to the dCas9 enables precise regulation of gene expression. Similar to the gene editing abilities of CRISPR, this kind of gene regulation can also be accomplished by ZFN's and TALEN's, but the simplicity of using dCas9 and its ability to target multiple transcriptional regulators on the same gene or to different genes has made it much more effective.

Potential Concerns of Using CRISPR/Cas9:

One of the main concerns that arises when discussing any gene editing tool such as CRISPR is how can it be ensured that only the targeted gene is affected and that the process does not have any off target effects. Unwanted phenotypic changes could be found downstream and severely limit the efficiency of the process. The specificity of the CRISPR system depends mainly on the sgRNA sequence within 10-12 bp 5' of the PAM. If there are partial mismatches outside of the seed sequence, off target edits might be produced. There have been significant improvements in the specificity of sgRNA in recent years. Although the CRISPR/Cas9 system performs differently among species and varieties among those species, off target effects have been found to be

negligible in many, such as arabidopsis (Peterson et al., 2016) and rice (Zhang et al., 2014). Tools and guidelines are available for creating very specific sgRNA to reduce off target edits (Kim et al., 2016).

It is also important to consider the natural mutations that occur normally for plants while discussing CRISPRs possible off target effects. DNA damage can occur naturally through environmental stresses like pollution, desiccation and rehydration, UV radiation, etc., and this can lead to double stranded breaks (Waterworth et al., 2011). Traditional chemical mutagenesis used by breeders for years also causes unpredictable DNA damage. Repair of these can be comparable to DSB produced in off target sites by CRISPR.

In addition to off target editing, it is possible that CRISPR can be used to inadvertently create a gene drive. As previously discussed, gene drives can be used to aid in controlling weeds, but if the technology is not controlled properly it could lead to the spread of unwanted phenotypes into the environment. A daisy chain style gene drive is one possible solution to keep CRISPR mediated changes under control in local populations. Instead of one genetic element driving changes through the population, there would be multiple elements with each depending on the next to work, and eventually they would run out and the population would return to wild type (Smidler et al., 2017).

Regulation of Crops Created with CRISPR/Cas9:

In a world where genetically modified organisms are viewed as safe by 88% of AAAS scientists and only 37% of the general public (Public and Scientists Views 2015), and there is fierce debate on both sides, the introduction of crops produced by CRISPR

brings a whole new group of questions into the discussion. Contrary to many genetically modified organisms that have introduced foreign DNA into the genome, many early CRISPR edited organisms are simply loss of function mutations that do not contain any foreign DNA, and could happen naturally. This has led to different results among governmental regulators around the world. Some governments create their rules based on the process used to make the new variety (ex. Australia, New Zealand, European Union) whereas others are more concerned about the final product and not the technique used to reach it (ex, Argentina, Canada, USA). NHEJ mediated edits would be considered the least intrusive and could be accepted by more stringent countries due to no new DNA being present, in contrast HDR edits where transgenes are added would be more highly regulated.

The first CRISPR edited organism in the US to test the regulatory system was a white button mushroom developed by Yinong Yang at Penn State University that resisted browning. In October 2015 he sent a letter to the United States Department of Agriculture (USDA) inquiring if his mushrooms would be regulated as other GMOs had been in the past. “Because white button mushroom is not a plant pest or federal noxious weed, and the CRISPR edited mushroom regenerated from transiently transformed protoplasts contains no foreign plasmid DNA sequences, there is no scientifically valid basis to conclude that the CRISPR edited mushroom is, or will become, a plant pest as defined by the Plant Protection Act.” Yang stated in his letter. The answer from the USDA in April 2016 declared “APHIS does not consider CRISPR/Cas9 edited white button mushrooms as described in your October 30, 2015 letter to be regulated.” This was a landmark decision and set the precedent for future organisms. In March 2018 the

US Secretary of Agriculture Sonny Perdue released a statement clarifying the USDAs position on new genome editing techniques. He stated that the USDA does not regulate or have any plans to regulate plants that could otherwise have been developed through traditional breeding techniques as long as they are not plant pests or developed using plant pests (USDA, 2018).

A major setback to the progress and implementation of CRISPR edited organisms came in July of 2018 when Europe's highest court, the Court of Justice of the European Union (ECJ) decided gene-edited crops should be subject to the same stringent regulations as conventional genetically modified organisms (Court of Justice of the European Union 2018). According to the statement, crops created using CRISPR and other new gene editing technologies would be subject to a 2001 directive developed for older techniques of genetic modification. That directive was aimed at species where entire foreign genes were inserted into the genome and did not include mutagenesis which alters the DNA but does not introduce any foreign genetic material. But in the 2018 decision, all new gene editing techniques developed after 2001 are not exempt from the directive. This is likely to lead to global trade and export problems, and could be an important factor to consider when researchers are deciding where to invest in future breeding projects. Since the decision in the summer of 2018, there have already been costly effects on researchers in the EU, for example a Belgian startup using CRISPR on bananas has lost its funding and a Brazilian company has put its research on soybeans on hold because of its market in Europe (Wight, 2018).

Licensing Issues of CRISPR/Cas9 Technology:

As universities and companies increase their use of CRISPR in research and development of new products, an important component that needs to be considered is the licensing agreements and decisions that are still being worked out in global courts. Two of the key players in the discovery and development of the CRISPR/Cas9 system, co-researchers Jennifer Doudna of UC Berkeley and Emmanuelle Charpentier of the University of Vienna, and Feng Zhang of the Broad Institute of MIT have both filed patents for the technology. UC Berkeley filed the patent first, however the Broad institute requested expedited examination on their patent and received the patents first. The major difference in these patents is that UC Berkeleys demonstrated the use of CRISPR Cas9 in editing bacteria, while the Broad Institute showed that it could also be used in the more complex cells of Eukaryotic organisms. UC Berkeley claimed the Broad patent infringed on their own and an extensive and complex legal battle involving even more parties making claims such as University of Vilnius, Toolgen, Sigma Aldrich, and Rockefeller University has ensued (Storz, 2017). In February of 2017, the US Patent and Trademark Office (USPTO) decided in favor of the Broad Institute, and UC Berkeley appealed the decision. Finally, in September of 2018 the court upheld the decision and gave the Broad Institute control of CRISPR patents. In October 2017, the chemical company Dupont Pioneer and the Broad institute have announced that they have agreed to provide non-exclusive licenses to CRISPR-Cas9 intellectual property. This will simplify the licensing process for those working with CRISPR-Cas9 and should encourage further research (Cameron, 2017).

Conclusion:

There is a lot of exciting research being published every day showing the significant progress happening in the field of CRISPR research. Just recently there was a report showing that CRISPR was used to disable the genes crucial for meiosis in rice, forcing the plants to reproduce asexually and create seeds that are clones of the mother (Khanday et al., 2019). And there is the HI Edit process which shows how CRISPR can be combined with other breeding tools to make the entire breeding process more efficient. Other types and classes of CRISPR and effectors being studied could lead to even more diverse applications for the technology. It is significant advancements like these that have given the industry confidence that CRISPR will continue to have an impact on plant breeding in the future. It is one of many tools available for breeders and researchers to use, and should continue to become more useful as more is understood about how it works. Although there will always be additional hurdles to overcome such as increasing efficiency and specificity, getting global acceptance of plants edited in this way, and resolving licensing issues, CRISPR is poised to be around for a long time.

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