Application of CRISPR/Cas9 technology to improve the important traits in coffee

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Abstract. Coffee is an essential estate crop that has a tremendous economic impact globally and is cultivated in many countries worldwide. However, due to the long process, the genetic improvement of coffee to develop new varieties through conventional breeding is slow and difficult. In addition, its production is constrained mainly by biotic and abiotic stresses. Therefore, developing superior coffee varieties with tolerance to abiotic and biotic stresses and improved quality is vital. The invention of genome editing technology has evolved the technologies in agriculture. CRISPR/Cas9 technology is a genome editing technique that more commonly applied for its simplicity, robustness, and high efficiency, compared to other techniques such as ZFN and TALEN. Furthermore, to develop new superior varieties of coffee, the CRISPR/Cas9 system provides an opportunity to improve by adding desirable traits or removing unwanted characteristics. Therefore, genome editing techniques in coffee breeding can help to increase yield, resistant/tolerant of biotic and abiotic, reduce input costs, and improve product quality. This article discusses the latest innovations of the CRISPR/Cas9 technique applicable in agriculture and prospectively on genome editing to improve important characters in coffee.

Keywords: coffee, genetic improvement, genome editing, CRISPR/Cas9

1. Introduction

Coffee is one of the important estate crops worldwide and the most traded internationally after crude oil. Coffee is grown in more than 80 tropical and subtropical countries worldwide, mainly in Africa, Asia and Latin America covering more than 10.2 million hectares. The income from the coffee harvest supports the economy of these coffee-producing countries. In fact, more than 100 million people rely on their livelihood on coffee cultivation, directly or indirectly for. In addition to having an essential role in Coffee is also an important export commodity and bring significant amount of foreign exchange for Indonesia’s economy. Indonesia ranks fourth in coffee bean-producing countries after Brazil, Vietnam, and Colombia [1]. In 2019, the national coffee plantation area was 1,239,22 million ha, of which 98% were smallholder plantation of 1,215 ha, and the rest belonged to Government Estate (14.5 thousand ha) and Private Estate (9.7 thousand ha) [1]. In Indonesia, Robusta coffee is the most cultivated coffee and the highest contributor of national coffee production. In 2018, national coffee production reached 722.46
thousand tons, and Robusta coffee contributed 73.06% (527.80 thousand tons) and the rest was of Arabica coffee at 26.94% (194.66 thousand tons) [1].

The coffee belongs to the genus Coffea of the Rubiaceae family. The genus Coffea L. has more than 100 species [2], but only two species are commercially cultivated, namely *C. arabica* (Arabica coffee) and *C. canephora* (Robusta coffee). Another type of coffee, Coffea liberica, is also cultivated but at lesser scale and consumed in small quantities. Almost all types of coffee, especially Robusta coffee, are genetically diploid (2n = 2x = 22). In addition, coffees are generally self-incompatible except for naturally occurring allotetraploid *C. arabica* (2n = 4x = 44) self-fertile species [3]. *C. arabica* is preferred by consumers because it has a quality taste, distinctive aroma, and low caffeine. Meanwhile, Robusta coffee has a stronger bitter taste and higher caffeine content [4]. The brew quality and taste of *C. arabica* is more known than *C. canephora*, but developing both species for better quality is the aim of most coffee breeding programs. The latest technology in genome editing and sequencing technology enable the breeders to gain more knowledge on the coffee genome and molecular inheritance and create more efficient breeding programs [5].

Improving the plant traits of coffee through conventional breeding approaches requires a long and time-consuming process that involves selection, crossing, and identification and evaluation of individual crosses. At least, the improvement of new coffee varieties takes a minimum of 30 years. Other obstacles faced in conventional breeding of coffee include the lack of precision in the breeding process, long-lived plants, high field-testing costs, and differences and discrepancies in the ploidy level of coffee species [6]. So far, the improvement of coffee traits is directed at increasing productivity, resistance to biotic and abiotic stresses, and breeding quality and nutritional value. Improvements to coffee plants have been successful in increasing crop yields. However, breeding that is directed at improving product quality, especially related to human health, is currently a concern to provide various nutrients such as protein, fiber, vitamins, minerals, and bioactive compounds [7, 8, 9, 10].

The discovery of endonuclease enzymes offers a key element for use as tools to genome editing in plant. Meganuclease I-SceI specifically recognizes 18-bp DNA sequences and cleaves DNA to lead to double-strand breaks (DSB) [11]. Through gene silencing and over-expression, previous crop improvement techniques often lead to unwanted gene expression or integration of the transgene into other genes in plants, thus impairing their function. The most recent gene modification techniques, particularly the CRISPR/Cas9 system, is an efficient platform in modifying the target traits. This technique also expands the scope and prospects of genome editing. Recently, genome editing technology has shown a distinct and spectacular advantage in plant breeding as it can modify plant genes in precise and predictable ways [12, 13].

This study will review advancement of genome editing systems especially CRISPR/Cas9 in agriculture and future prospective of genome editing application in improving important characters in coffee.

### 2. Genome editing techniques

Genome editing technology using site-specific nucleases helps a more efficient and precise research on reverse genetic, genome engineering, and targeted transgene integration. The technology involves recognizing directed double-stranded DNA breaks (DSB) using a modified nuclease enzyme, inducing cellular DNA repair mechanisms [14]. Genome editing techniques can be used to alter selected nucleotides from billions in the genome of living cells, change alleles, or insert new genes in targeted regions of the genome to and improve the plant quality. Aside from increasing the nutritional value of plants, this technology is also the most effective in generating pest-resistant and adaptive crops under challenging climates [15]. Thus, the primary mechanism of the genome-editing technique is based on the precise cutting of double-stranded DNA (double strand breaks, DSB) by artificial nucleases at the target sites of specific genes. The DSB will induce the DNA repair process in cells [16].

Currently, the clustered regular interspaced short palindromic repeats (CRISPR)/CRISPR related protein (CRISPR/Cas) technique is the most popular genome-editing system technique. It is widely applied for agriculture or medicine compared to the two previous genome editing techniques, namely
zinc finger nucleases (ZFN) and transcription activator-like effector nuclease (TALEN). The main components of the all three genome editing systems are similar and contain two main domains, the recognition domain and the cleavage domain. First, the recognition domain directs the cleavage of the domain to a genome-specific site. Furthermore, the DNA from a particular site is cut by the cleavage domain. Then, these DNA breaks can be repaired by nonhomologous ends joined (NHEJ) or homologous recombination (Figure 1). The repair will be accompanied by the insertion of gene fragments, gene mutations, and changes in chromosomal structure, which ultimately lead to changes in the function of the original gene [17]. DSB induction by genome editing can be repaired by two pathways: nonhomologous end join (NHEJ) and homologous recombination (HR). The NHEJ pathway can result in frameshift mutations when repair occurs in the gene's open reading frame (ORF) region and induces random insertions or deletions (indels). The presence of indel can cause premature stop codon and so that the gene is knocked out. The NHEJ pathway can also generate end overhangs so that template DNA with compatible overhang ends can be inserted [18, 19].

ZFN and TALEN technique are the first and second-generation systems of SSN. The genome-editing mechanism of the systems relies on the ability of the protein to recognize specific DNA sites and the nuclease activity of the FokI domain to cleave the target sequence. [20]. Therefore, ZFNs and TALENs bind to DNA via protein and DNA interactions. Meanwhile, CRISPR/Cas identifies target sites through RNA and DNA interactions. These robust genome editing systems can support various research areas, including plant breeding. For example, they enable scientists to cultivate and produce new plant varieties by enhancing their agronomic traits or resistance to biotic and abiotic stresses.

Figure 1. Targeted genome engineering using sequence-specific nucleases can induce DSBs and repair by NHEJ or homologous recombination.

3. ZFNs-based genome editing
ZF motifs occur in numerous transcription factors which are a class of specific proteins with the function to recognize the DNA sequence and promote gene transcription. A zinc finger is a common transcription factor containing conservative protein structures and found in eukaryotes [15, 16]. In the ZFNs system, zinc finger protein and the restriction enzyme FokI components form complexes that play an important role in genomic DNA cleavage. Each zinc finger protein contains about 30 amino acids, which can recognize triplet nucleotides. To recognize and bind to a specific DNA sequence of 9-18 bp, three to six zinc finger proteins are required to be linked together to form a protein domain. Therefore, FokI must form a dimer, so a pair of ZFNs must be designed on both sides of the cut site. And a 5–7 bp spacers of the two binding sequences were sufficient for FokI dimerization. This characteristic can also reduce the occurrence of non-specific genome editing or off target effect [17]. Sequence specificity of ZFN technique is given by the DNA-binding domain of each polypeptide, and DNA cleavage is performed by the nuclease domain FokI [20].
The zinc finger nucleases system was the first most applicable site-specific genome editing tool and available on market. ZFN has been used in various plant breeding such as Arabidopsis, tobacco, soybean, and rice [21, 22, 23]. The application of ZFNs has succeeded in modifying specific endogenous genes in tobacco using ZFN technology and giving tobacco herbicide-resistant abilities [24]. Scientist used ZFN technology to modify the inositol 1,3,4,5,6-pentachyphosphate2-kinase (IPKI) gene of maize to obtain herbicide-tolerant maize [25].

However, application of ZFN technology has several weak points, such as the complexity and costly of constructing protein domains for each specific genomic locus and the possibility of inaccurate target DNA cleavage or off-target effect due to single nucleotide substitution or inappropriate interactions between domains [26]. Other limitations include the difficulty of designing and constructing zinc finger proteins, challenging specific recognition activity, and long experimental period.

4. TALENs-based genome editing
TALEN system was created from the transcriptional activator-like effectors generated by phytopathogenic bacteria of the genus Xanthomonas [27]. Effector proteins belong to the family of DNA-binding proteins and can be used to trigger the expression of their target genes such as eukaryotic genome transcription factors. Previous studies have shown that TALE has the ability to bind to DNA sequences from certain host plants and to induce host expression of plant-specific genes that may help protect against an invasive pathogen [28]. Similar with ZFNs, TALENs are chimeric proteins assembled by combining a manipulated DNA-binding domain with the catalytic domain of the FokI endonuclease, which cut as dimers [29]. Both TALENs and ZFNs, have a similar mechanism in that two monomers bind opposing strands of DNA separated by a spacer of a suitable length, allowing FokI to dimerize and cut DNA. In addition, ZFNs and TALENs both bring the restriction endonuclease catalytic domain FokI, which generates DSBs with cohesive ends. Therefore, engineered nucleases bind to target sequence sites in the nucleus and the FokI domains will dimerize to form a double-strand break in a spacer sequence [15].

Study has demonstrated that TALENs could work in tobacco cells [30]. This suggests that TALENs can be applied for genome manipulating in plant cells. TALENs have been used to edit gene of various diverse plant species, namely Arabidopsis, potato, rice, barley, wheat, and maize [31, 32, 33, 34, 35, 36]. It is proven that TALEN technology has been widely used for crop breeding such as gene knockout, gene replacement, functional superposition, and controlling gene expression, achieving extraordinary results [37, 38].

Compare to ZFN system, TALEN is widely applied in various fields. First, the TALEN target site has fewer limiting factors; only the first base of the target site is T. Second, the binding of the TALEN and the target site is not affected by the upstream and downstream sequences of the target site. In addition, TALENs are easy to assemble, and there is no need to screen the library, so they also save time. Finally, the build costs of TALENs are lower than ZFNs; therefore, they are more suitable for most laboratories. TALENs have disadvantages including a new chimeric protein should be constructed for each new target sequence for a certain trait. This has been a main obstacle to the routine use of this system because constructing new protein is a very complicate work, costly, not time-effective and is inapplicable in most laboratories [16].

5. CRISPR/Cas9-based genome editing
Due to its specificity, simplicity, and versatility, the CRISPR/Cas9 technique is playing an essential role in advancing genome editing [39, 40, 16]. Initially, CRISPR was known to provide bacterial protection against viral invasion. However, currently, the CRISPR system that has been developed and widely applied for multipurpose purposes is CRISPR/Cas9 type II from Streptococcus pyogenes. The CRISPR/Cas9 technology, at present, has become a promising technology to be applied in various research programs, including plant breeding.

The CRISPR/Cas9 arrangement includes two elements, the Cas9 protein, which contains two domains, namely the RuvC-like domain and the HNH domain, and the guide RNA to be combined with
CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). In contrast to the ZFN and TALEN systems, attachment to CRISPR/Cas9 occurs complementarily between gRNA and target site DNA. gRNAs are transcribed and processed into crRNAs about 20 bases long, followed by a protospacer contiguous motif (PAM) in the target gene. The coupling of gRNA and Cas9 nuclease will result in DSB upstream of the PAM motif [41]. In its application, genome editing in plants with CRISPR/Cas9 requires several stages, including identification of gRNAs, design and construction of transformation modules, delivery of construction modules in plant cells, plant regeneration, and screening of gene-edited plants [42].

Compared with ZFN and TALEN, CRISPR/Cas9 technology has a simpler construct, lower cost, and higher editing efficiency, and it is easy to use for editing of multiple sites. The CRISPR/Cas9 technology has greatly simplified the process, making it much easier for use in plants. Unlike ZFNs and TALENs, CRISPR/Cas9 is more direct and involves designing a 20-nt gRNA complementary to the DNA stretch within the target gene. However, this system still has some limitations including the target site that should contain sequences of PAM is the major limitation. Although the NGG sequence is common in various biological genomes, some genes still do not contain PAM, so the CRISPR/Cas9 system cannot edit such genes.

### 6. Opportunities the use of CRISPR/Cas9 for coffee improvement

To date, the CRISPR/Cas9 technique has been applied to improve various important traits including increased yield, resistance to pest and diseases, and improved nutrition in more than 20 plant species [43]. The use of the CRISPR/Cas9 technology by silencing unique genes is crucial in plant improvement. The CRISPR/Cas9 technology has been applied to develop plant resistance to certain pathogen and also to increase tolerance to major abiotic stresses such as drought and salinity. The application of the CRISPR/Cas9 for gene manipulation in numerous plants can be seen in Table 1.

#### Table 1. Application of CRISPR/Cas9 for improving interest trait based on genome editing in various plant.

| Crop              | Target gene | Trait                                      | Reference |
|-------------------|-------------|--------------------------------------------|-----------|
| A. thaliana       | elF(iso)4E  | Turnip mosaic virus (TuMV) resistance      | [44]      |
| N. benthamiana    | ORFs & IR sequence sDNA | Tomato yellow leaf curl virus (TYLCV) | [45]      |
| Rice              | OsEF922     | Blast resistance                           | [46]      |
| Wheat             | MLO         | Powdery mildew resistance                   | [33]      |
| Cucumber          | elF4E       | cucumber vein yellowing virus (CVYV)       | [47]      |
| Maize             | ARGOS8      | Increased grain yield under drought stress | [48]      |
| Tomato            | SIMAPK3     | Drought tolerance                          | [49]      |
| A. thaliana       | MIR169a     | Drought tolerance                          | [50]      |
| Rice              | OsMPK5      | Various abiotic and biotic stress resistance| [51]      |
| Rice              | OsPRX2      | Potassium deficiency tolerance              | [52]      |
| Maize             | ZmIPK       | Phytic acid synthesis                       | [53]      |
| Wheat             | TaVIT2      | Fe content                                 | [54]      |
| Potato            | ALS1        | Herbicide resistance                       | [55]      |
| Cassava           | MePDS       | Carotenoid biosynthesis                     | [56]      |

Several obstacles in improving coffee plants include the very narrow genetic background of the C. arabica species. This condition results in homogeneous agronomic characteristics, high susceptibility to pests and diseases, low productivity, low adaptation to abiotic stresses and extreme climates, and low quality. Thus, increasing the genetic diversity of Arabica varieties and improving resistance to pathogens
and environmental limitations are the focus on developing superior coffee varieties. Therefore, developing reliable, rapid tools for accelerating a variety release of coffee is urgent. CRISPR/Cas9 technology is one method that is able to develop a new coffee variety with specific trait including high yield and good quality.

The potential use of CRISPR/Cas9 technology to improve several essential aspects in coffee will be explained below.

6.1 Improvement coffee with low caffeine content
Caffeine in coffee is clinically proven to increase endurance, energy and even improve the cognitive level of the brain when consumed in reasonable doses. However, uncontrolled and excessive caffeine consumption can cause adverse effects that can cause harm to the health of the consumer itself such as headaches, tremors, nausea and vomiting even increased risk liver damage. Therefore, the production of coffee with low caffeine is urgent for safe and healthy consumers. Studies to produce coffee plants with suppressed caffeine synthesis using RNA interference (RNAi) system by silencing the XANTHOSINE METHYLTRANSFERASE gene (CaMXMT1) have been carried out [57]. The results showed a reduction of caffeine and theobromine in modified plants up to 70%. However, application of the RNAi technique has limitations, including that it suffers from high off-target effects and results in a modified phenotype.

There has been no report on the use of CRISPR/Cas9 to produce low caffeine coffee. Application of CRISPR/Cas9 technology to knockout the down-regulatory element such as CaMXMT1 gene will produce a variety of coffee with a reduction of theobromine and caffeine content.

6.2 Enhancement in cup quality
For this purpose, detailed knowledge of the chemical elements and metabolic pathways involved in quality determination is required. There have been researches on the roles of sucrose, chlorogenic acid (CGA), and trigonelline as the three main constituents on coffee quality. The sucrose content in coffee beans can be related to the taste of coffee. The higher sucrose content in coffee beans, the stronger will be the cup flavour [58].

Therefore, it is necessary to improve the sucrose content in coffee beans to improve the quality of the cup. Knockout of the sucrose synthase (CcSUS2) gene from C. canephora using CRISPR/Cas9 can increase the sucrose content of coffee beans. In addition, the CGA in coffee is associated with disease resistance and is known to have antioxidant properties [59]. Mutagenesis of genes involved in CGA synthesis using CRISPR/Cas9 can improve this trait by up- or down-regulating the pathway.

6.3 Herbicide-tolerance coffee
ACETOLACTATE SYNTHASE (ALS) is a target of sulfonylurea (SU) and imidazolinone (IMI) herbicides. Plants that have site-specific mutations, especially in the ALS gene, become resistant to herbicides. Genome editing techniques can produce selected point mutations in plants. CRISPR/Cas9-mediated genome editing can direct specific DNA sequences into target loci and introduce point mutations in the genome. With CRISPR/Cas9 technology, point mutations can be introduced in the ALS gene to engineer plants resistant to herbicides.

6.4 Drought stress tolerance
So far, functional or regulatory genes and transcription factors have been directed to assemble drought-tolerant plant varieties through genetic engineering techniques. Genes targeted for drought tolerance include proteins for transporting metabolites, coding enzymes involved in detoxification or osmotic response metabolism, enzymes active in signaling, and regulating plant energy levels [61]. Thus, manipulating genes related to transcriptional regulation and signal transduction can help increase drought tolerance in coffee.
CRISPR/Cas9 system can be a useful technique in producing drought stress tolerance in coffee. Manipulating transcription factors or signaling pathways such as DREB, ERF, and MAPK related to environmental sensitivity can generate drought-tolerant varieties of coffee.

6.5 Improving of fruit ripening
Coffee cherries that ripen uniformly are definitively correlated with cup quality. Therefore, it is crucial to control the action of genes involved in the final step of the ripening process to promote the uniform ripening of coffee cherries. Two genes control coffee fruit ripening, mainly in ethylene biosynthesis, ACC synthase and ACC oxidase. Therefore, knocking out the gene that produces an early ethylene burst using CRISPR/Cas9 is one way to control coffee fruit ripening [62].

7. Conclusion and Future Perspective
The ZFNs, TALENs, and CRISPR/Cas9-based genome editing technologies offer researchers the capability to accurately and rapidly introduce the interest traits than classical breeding. The CRISPR/Cas9 is an essential revolution technology that has allowed scientists to create new approaches for coffee breeding program. The application of CRISPR/Cas9 technique in coffee improvement can increase yield, nutritional quality, pest and disease resistance, and other characters that will be protruding fields of research in the future. Recently, CRISPR/Cas9 is being used dynamically in various plant systems for purposeful studies and overcoming biotic and abiotic stresses, and improving other important agronomic traits. Therefore, CRISPR/Cas9 based genome editing will increase acceptance and be an important technique to develop a new variety of coffee that will help achieve high productivity and good quality.

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