Modifying High Sucrose Tomatoes by Genome Editing A-Review

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Abstract. Sucrose which is the main product of photosynthesis in plants is resulted from carbon assimilation. The way to produce more sucroses as the sink product has been done a lot recently. However, the way such as overexpression of SPS or harvest environmental engineering needed a lot of samples and a certain period of time. Genome Editing promises giant leaps forward in advancing biotechnology, agriculture, and basic research whereby targeted mutations can be introduced into a plant genome in a highly specific manner through great precision. This technology does not incorporate transient modifications that become far superior to conventional plant breeding. The recent technique of genome editing is Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated 9 (CRISPR/Cas9) which has greatly advanced the breeding for crop improvement due to its simplicity and high efficiency over other nucleases. CRISPR/Cas9 tool contains a non-specific Cas9 nuclease and a single guide RNA leading Cas9 to the specific genomic location creating double-strand breaks and subsequent repair process creates insertion or deletion mutations. In this paper we aim to focus on the application of CRISPR/Cas9 to increase the sucrose level of tomato fruit (*Solanum lycopersycum* L.). We will review the editing strategy including conversion of vacuolar invertase (TIVI) into cell wall invertases (TIVI1), deletion of sucrose-induced repression of translation (SlbZIP2), and generation active sucrose phosphate synthase (SPS). Tomato cotyledon explant is being targetted with *Agrobacterium* infection. CRISPR/Cas9 enzyme can give novel characters in plant by increasing, decreasing and removing expression of a gene target. The golden gate cloning make addition of the characters in plants can be done by over 10 characters in a single crop. It should be no rejection of genome editing product due to the gene's purity sequence from other organism.

Keywords: CRISPR/Cas9 · Genome editing · Sucrose · Tomato

1 Introduction

Sucrose is the main product of photosynthesis in plants which is resulted from carbon assimilation. In plants, sucrose acts as a regulator of photosynthetic and non-photosynthetic gene expression, such as genes involved in cell division, differentiation,

A. Lelono et al. (Eds.): ICOLIB 2021, ABSR 27, pp. 312–320, 2023.
https://doi.org/10.2991/978-94-6463-062-6_31

and fruit ripening, but also takes roles in providing energy, stimulating growth, and plant development [1]. Sucrose as the end product of carbon assimilation in the photosynthesis process then is translocated to all parts of the plant for plant development [2]. One of the sucrose levels is influenced by the presence of the enzyme Sucrose Phosphate Syntase (SPS). This enzyme functions to catalyze fructose-6-phosphate and UDP-Glucose into sucrose-6-phosphate, and plays a role in sucrose biosynthesis that takes place in the leaf mesophyll [3].

This article will review the editing strategy in order to increase the accumulation of sucrose in the sink compartment. The target is tomato fruit which is well-known vegetable around the world with the CRISPR/Cas9 help's as the vehicle to make sure this strategy precisely work.

1.1 Tomatoes as Model Plant

Tomatoes (*Solanum lycopersycum* L.) belong to the Solanaceae family (nightshade family) along with potato, lettuce and eggplant, which are one of the most important types of vegetables that are cultivated in many countries in the world. Tomato plant (*Solanum lycopersycum* L.) is a perennial plant in the tropics, however tomato plant in the northern area is an annual plant [4]. Tomato plants (*Solanum lycopersycum* L.) air habitus or bushes and pitch into the class of flowering plants (Angiospermae) which originate from the Andes region such as Peru, Bolivia, Ecuador, and Mexico. All tomato varieties in Europe and Asia first came from Latin America brought by the Spaniards and Portuguese in the 16th century. Tomato plants (*Solanum lycopersycum* L.) has an herbaceous stem covered by trichomes, rectangular or round, green, there is a thickening of the segments ba pliers, can have ramifications up to 60–180 cm. The leaves of the tomato plant are oval in shape, the edges are jagged and form pinnate crevices, are green and are odd compound leaves totaling 5–7 strands with a length of up to 45 cm. Tomato leaves are known to have a strong aroma, where the scent of "green fragrance" is produced from the production of 2-hexenal (leaf aldehyde) which accumulates at the tip of the leaf trichome [5].

Tomato plants have long roots that grow penetrate into the soil and fibrous roots that grow to spread sideways but shallow. Flowers bright yellow tomato plants, sized small with a diameter of about 2 cm, with flower green amounted to 5 pieces, arranged laterally with inflorescence be raceme on long flower stalks. Tomato flowers are perfect flowers, because the stamens and pistil are in the same flower. In the middle there is a stamen cone shaped like a bottle [6]. Tomatoes that are still young and light green, when they are ripe, turned red due to the accumulation of lycopene which is a type of carotenoid C-40 that is synthesized via the carotenoid metabolism pathway during fruit ripening. The fruit is round to oval in length, each fruit containing at least 2 seed spaces surrounded by pulp-like jelly [7].

Tomato plants (*Solanum lycopersycum* L.) are often used as research models related to molecular biology studies because of their unique characteristics. Some of these characteristics include diploid plants which are relatively simple diploid (simple diploid genetics), have a large sequence of genomes and genetic and genomic resources both in cultivated species and wild species, genome size is relatively small (DNA size of about 95

picograms), has a short reproduction time, suitable for many transformation methodologies [8]. The development of genetic studies of tomato plants is quite advanced. Qualitative genes and quantitative locus traits (QTLs) for domestication (fruit and growth traits) have been identified. One of the biggest changes due to domestication is the size of the fruit, where the wild type has a smaller fruit size. Locus that encodes a negative repressor for cell division and mutase from the promoter sequence contributes to changes in fruit size to large. Another locus that has been identified is related to the shape of the fruit, where the locus sun and fs 8–1 are responsible for the elongation and formation of boxed tomatoes [9]. Information of tomato genome sequences resulting from a project called the International Solanaceae Genome Project (SOL), coupled with the availability of large genetic and genomic resources can provide researchers in the field of plant biotechnology to implement new methods to obtain odor genotypes that will later be able to answer demands for quality improvement and yield multiplication by consumers, producers, and the food processing industry.

2 Methodology

2.1 Genome Editing

Genome editing is a method of plant breeding that is in its golden age through its ability to be able to accurately modify and manipulate DNA sequences in living cells. Manipulation can be done in the form of insertion, removal, or even editing DNA sequences easily and accurately. Through targetable nuclease researchers can target and theoretically modify each gene in each organism. Targetable nuclease is a type of nuclease programmed with a site-specific DNA binding area that can improve performance, increase the speed of incorporation, and significantly reduce the cost of editing genomes [10]. The CRISPR/Cas system was first discovered in the organism *Streptococcus thermophilus*, which has a role as a specific sequence to develop adaptive immunity against foreign bacterial DNA invasion. This system is an endonuclease guide to cut specific non-host sequences that will protect both bacteria and archaea from viruses and plasmids. A brief form of immunity that occurs is based on small RNA molecules that combine with protein

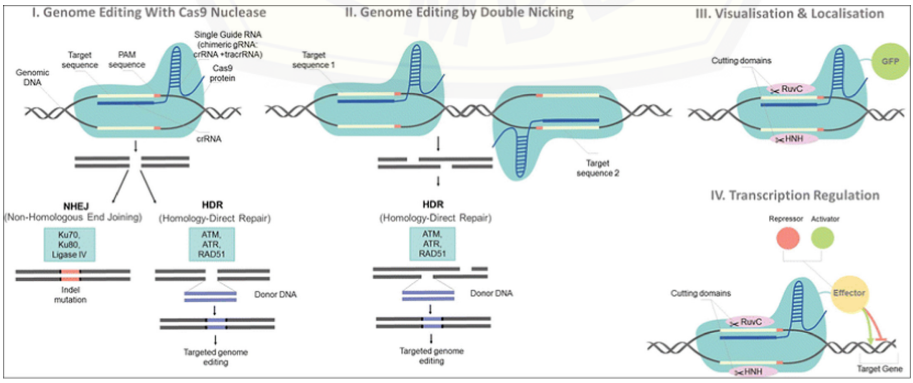

Fig. 1. The mechanism of genome editing using CRISPR/Cas9 [15].

complexes and can specifically target nucleic acids from viruses through base pairing. The defense system by CRISPR/Cas takes place through three stages. First, the injected viral DNA is identified and part of the DNA is inserted into the host CRISPR arrangement as a new non-coding DNA (spacer). These sequences are usually short about 2–5 nucleotides, also known as adjacent photospacer motives (Protospacer Adjacent Motif, PAM). The second stage of the response that occurs is the transcription of the CRISPR pool into a long crRNA (pre-crRNA) precursor. The final step is an interference reaction, in which the mature cr-RNA joins with the larger Cas protein complex to identify and destroy the viral genome [11].

There are three different types of CRISPR Cas systems that have been found in *S. Thermophilus* bacteria, namely type I, II, and III, all of which exhibit the same structural design. The CRISPR cluster can be described as a DNA genomic element, where the first part is a series of short replications, consisting of 24–37 base pairs separated by exclusive spacer sequences of the same length. These sequences make bacteria able to gain adaptive immunity. The second part of the CRISPR Cas system is Cas endonuclease, which has a different type and is tasked to provide immunity against bacteria [12].

The CRISPR Cas type I and III systems use Cas 3 and Cas 6 endonuclease to cleave pre-crRNA. In type I, invading DNA is recognized by the crRNA cascade complex. The PAM motif helps in identifying foreign DNA and the Cas3 nuclease used to cleave the target DNA. Type III systems use the Cas6 nuclease, in which the crRNA binds and recognizes the invading DNA or RNA. The CRISPR Cas type II system uses the Cas 9. Endonuclease. Cas9 is a nuclease enzyme, a protein with the ability to cut DNA strands, and is equipped with two active cutting sites, each on each double-stranded DNA strand [13].

The CRISPR Cas type II system uses a completely different mechanism, where only Cas9 endonuclease is needed to split the target sequence. Cas9 is expressed by two RNAs, namely crRNA (CRISPR RNA) and tracrRNA (trans-activating crRNA), both of which together form endonuclease specific sequences that can cut off foreign genetic sequences to protect host cells. Induction of double-stranded DNA separation is done by splitting DNA at the site that complements with sequences from the guiding RNA, so that for System II to really work, functional Cas on endonuclease and small guiding RNA sequences are needed [14]. Figure 1 shows the mechanism of genome editing using CRISPR/Cas9 system. SgRNA is a combination of the crRNA with tracrRNA and also responsible for recognizing the target DNA. As in the bacterial system, the presence of a PAM directly after the target se- quence is required to hydrolyze DNA via cas9. In mammalian cells, the Cas9 nuclease induces the formation of double- strand breaks (DSB) which can be repaired by two primary mechanisms [15].

CRISPR has some advantages over other systems and tools used in genome editing techniques. CRISPR is considered to have a faster *assembly speed,* higher target efficiency, multi-target potential, relatively lower costs, and simpler method compared to other methods. Simplicity is not separated from the presence of Cas9, where genome engineering requires the production of a protein that has the ability to recognize and bind to specific DNA at the locus. Through Cas9, only a small sequence of RNA is needed to be designed so that it can target almost every part of DNA. The Cas9 protein can be changed its target by replacing sequences from a single RNA guide (gRNA) so that it

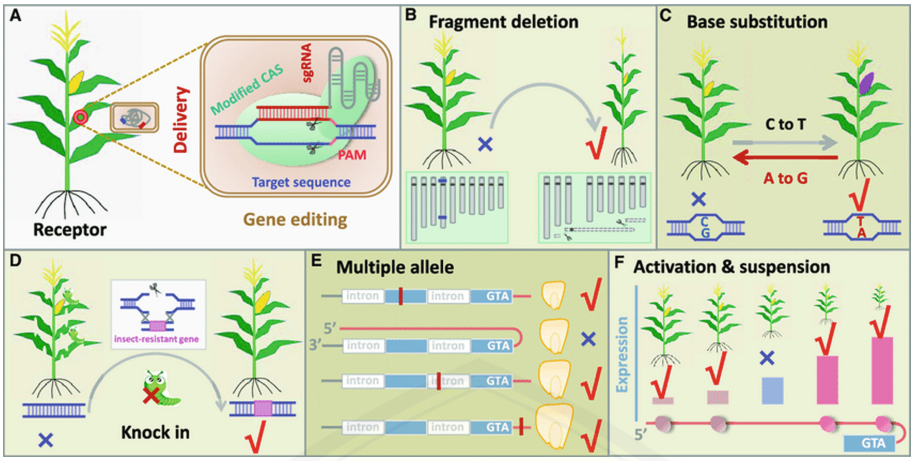

Fig. 2. A Simplified Schematic Representation of Genome-Editing Techniques in Plants and Their Potential Application. (A) Gene-editing model, including editing element delivery and modified model with single guide RNA and different functional Cas protein or protein complex. (B and C) Delete any sequence including large chromosomal fragments or (B) even the entire chromosome via paired sgRNA (C) to achieve any base substitution. (D-F) Add genes that do not exist in the original genome (D), create multiple different alleles of any gene (E), and activate or suspend the function of any gene (F). Ovals represent activator complex (red) and repressor complex (pink) [24].

complements the site on the desired DNA [16]. Figure 2 shows the potential application of GE.

2.2 Annalytical Thinking

Sucrose is a carbon compound which is the end result of the carbon assimilation process in photosynthesis. In addition to sucrose in the process produced starch (starch). Starch is synthesized in chloroplast and is a deposit compound in leaves, while sucrose is synthesized in cytosol and has a major role in carbon distribution throughout plant parts. Most of the assimilated carbon can be allocated to the synthesis of starch or sucrose depending on changes in day and night [17], plant species (genetic factors), environment, and growth stages [18]. The amount of carbon compound that can be allocated to sucrose synthesis during photosynthesis is an important stage that determines plant growth and production. In sucrose biosynthesis, an increase in sucrose synthesis can be done by manipulating starch synthesis or stimulation of sucrose biosynthesis. Inhibition of starch synthesis is done by inhibiting the activity of ADPG-pyrophosphorylase which is a biosynthetic determining enzyme. Arabidopsis mutants and potatoes with reduced ADPG-pyrophosphorylase activity can significantly reduce starch content and increase sucrose content. However in conditions of high light intensity it actually reduces the rate of photosynthesis of sucrose.

SPS enzyme functions to catalyze fructose-6-phosphate and UDP-Glucose into sucrose-6-phosphate, and plays a role in sucrose biosynthesis that takes place in the

leaf mesophyll [3]. This enzyme is the key enzyme for sucrose biosynthesis. To make sucrose, of course the activity of this enzym must be maintained. Logically to make more sucrose, the activity of this enzyme must be upragded. It has to be more active than before. However, another enzyme called invertase which has another role to break down sucrose became glucose and fructose. Molecular approach must be done to modified those enzymes in order to increase the biosynthesis of sucrose without any interferences to breakdown the sucrose.

Another report states that a part of a gene called sucrose-induced repression of translation (SIRT) became a repressor of sucrose translation [19]. This part must be eliminated if higher sucrose level is desired. CRISPR/Cas9 promised a precision cleavage to remove the part needed. Designing the right gRNA is neccessary to make sure this idea work in the future.

3 Results

3.1 Strategies to Increase Tomato Sucrose Levels

Increasing sucrose content in tomato plants through genome editing can be done beginning with Zip2:: Tivi1:: Sps1 gene construct (Fig. 3). SlbZIP1 and SlbZIP2 (basic region leucine zipper) have been identified in tomato plants (*Solanum lycopersicum*). SlbZIP1 and SlbZIP2 contain four and three upstream open reading frames (uORFs). Second uORFs from the 5 ‘end of cDNA are involved in SIRT (Sucrose-Induced Repression of Translaton). SIRT deletion made GMO fruits produced through this strategy have sugar content (sucrose/glucose/fructose) 1.5 times higher than non GMO tomatoes [19]. Furthermore, the conversion from vacuolar invertase (TIV1) to cell wall invertase (TIVI1) contributed to an increase in fruit sucrose content by 40%. Activation of hydrolysis of cell wall invertase from sucrose which is transplanted to the point of dismantling in the sink area causes an increase in gradient between the source organ and the sink organ which triggers the transfer of sugar into the fruit [20].

Meanwhile, overexpression of SPS (Sucrose Phosphate Synthase) can increase the dismantling and sink activity of sucrose into fruit apart from its main role in producing sucrose-6-phosphate [21]. Many studies report that SPS is a key enzyme in sucrose biosynthesis. SPS activity determines the accumulation of sucrose in the leaves and positively correlates with the level of sucrose production in maize, sugarcane, and tomato plants [22]. Overexpression of SPS genes from corn in tomato plants and transgenic Arabidopsis can increase photosynthesis levels, as well as the level of synthesis and storage sucrose and can reduce starch synthesis and storage, on the other hand Arabidopsis and potato plants that are transformed with SPS antisense genes that work to reduce SPS gene expression decrease their sucrose concentration, while their starch levels increase [18]. This is one proof that SPS is the m ain key gene in the synthesis of sucrose. However, SPS N-terminal domain deletion from sugarcane sequence increased the specific activity of SPS [23].

This editing strategy is aiming removal of uORF2 in bZIP2 gene, conversion vacuolar invertase to cell wall invertase and deleting N-terminal domain SPS to increase sucrose level in fruit compartment, which is been paralel-linked in one constract. So, this constract will make 3 changes in the tomato genome at one blow. It can be done

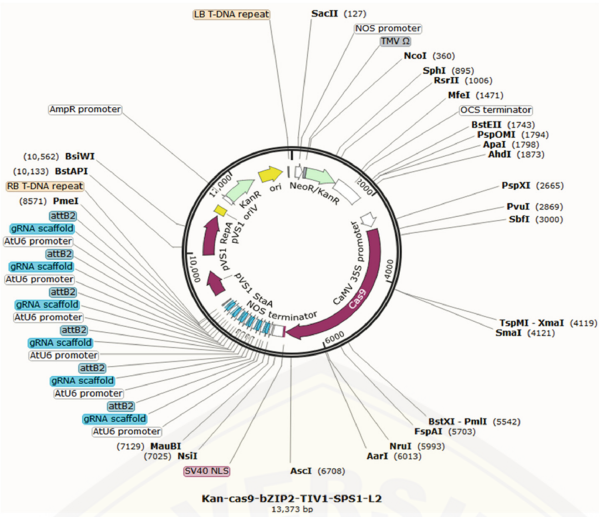

Fig. 3. L2 Plasmid Kan-Cas9-bZIP2-TIV1-SPS1.

using golden gate cloning, putting seperate sequences and joining them together. This construct has been confirm by PCR digest by SacII, PmlI and PmeI. At this time, it is on going transformation gene to tomato on selection phase. Once, they pass the selection, it will continue to confirmation phase by PCR and sequencing.

Acknowledgments. Many thanks to Fundamental Research Project Program which support the research in contract number: 2620/UN25.3.2/LT/2022 (May 22th, 2022).

Authors' Contributions. JYK Conceived the original idea & design gRNA, SA & DP Construct the plasmid DNA & Did engineered plasmid DNA until the 4 plasmids ready in Agrobacterium, WIDF Suggestion in paper revision, MMFU & RF Compose paper writing and seaarch the paper references. All authors read and approved the final manuscript.

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