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A Review on Potential Application of CRISPR/Cas Systems in the Improvement of the Growth Habits and Fruit Quality of Tomato (*Solanum lycopersicum*) in Vietnam

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Abstract

Tomato (*Solanum lycopersicum*) is known as the most important vegetable crop that is widely cultivated throughout the world. Improvements of the growth, development, and productivity have become core strategies for the sustainable development of tomato in many countries. Here, we performed an intensive summary of recent applications of genome editing in customizing the growth habits and fruit quality in tomato plants. First, the advantages of genome editing, particularly CRISPR/Cas systems, were introduced. We then summarized all up-to-date studies related to the genome editing-based functional characterization of genes of interest in tomato with the aim of designing the growth habits and enhancing the fruit quality. Finally, we discussed the potential applications of this promising tool in tomato breeding programs in Vietnam. Taken together, our review has provided a wide view for further studies towards improving the growth, development, and productivity of tomato in Vietnam.

Keywords

Tomato, genome editing, CRISPR/Cas, growth habits, fruit quality

Introduction

Tomato (*Solanum lycopersicum*) has been considered one of the most important crop species that is cultivated throughout the world (Sun *et al.,* 2020). Tomato is used as the major dietary source of the antioxidant lycopene, which has been well-characterized and linked

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to various health benefits for human lives (Miller *et al.*, 2002; Imran *et al.*, 2020). Additionally, tomato has also been noted as a great source of potassium, folate, vitamin C, and vitamin K (Beecher, 1998). However, global tomato production is dramatically affected by adverse environmental conditions, including abiotic (Gerszberg & Hnatuszko-Konka, 2017) and biotic stresses (Scholthof *et al.*, 2011). Thus, many researchers have found it interesting to focus on improving stress tolerances in tomato via biotechnological approaches.

Up till now, great efforts have been made in order to construct improved tomato varieties. Particularly, both conventional and modern approaches have been applied to accelerate the growth, development, and productivity of tomato plants. Among them, genome editing utilizing the CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein) system has been known as one of the newly emerging tools for the improvement of various biological processes, like the growth habits and fruit quality of tomato plants. Thus, the aims of this review were to provide the efficiencies of genome editing tools in the acceleration of breeding programs and to comprehensively summarize the application of genome editing on the improvement of tomato. We first compared conventional and modern tools, including gene transformation and genome editing, to explain the efficiency of genome editing tools in the acceleration of breeding programs. We then summarized the recent achievements of the improvements of the growth habits and fruit quality of tomatoes by CRISPR/Cas systems. Finally, several critical challenges and future directions of the CRISPR/Cas systems were discussed.

The efficiency of genome editing tools in the acceleration of breeding programs

During the domestication process, variations in crop cultivars have been recognized to increase the diversification of the genetic pool. Genetic diversity, which spontaneously appears due to mutations (errors in the DNA replication or DNA damage), is defined as the solid foundation for improving plant characteristics (Sikora *et al.*, 2011), and subsequently plays an important role in the construction of new crop varieties in a breeding program (Chaudhary *et al.*, 2019). Up till now, various induced mutagenesis methods have been applied in crop species. A collection of mutagenic agents, including chemical (like ethyl methane sulphonate, 1-methyl-1-nitrosourea, and 1-ethyl-1-nitrosourea) and physical mutagens (mostly ionizing radiations), have been commonly applied in breeding programs to construct improved crop varieties (Oladosu *et al.*, 2016). One issue that has been reported is that mutational breeding programs based on physical and chemical-induced mutagenesis are still less preferred because of random mutation, and time- and cost-intensiveness. More specifically, both chemical and physical mutagenesis are uneven, and their mutations spectra are not well-characterized (Salava *et al.*, 2021). In order to generate one potential line, they usually require very large populations (at least 10,000 individuals) for the screening of genotypes and phenotypes (Salava *et al.*, 2021). Additionally, chemical and physical mutagenesis also need exclusive facilities and infrastructure to guarantee that the procedures are carried out safely and any bio-hazardous materials are disposed of properly (Oladosu *et al.*, 2016; Salava *et al.*, 2021).

Since the release of the first draft of the tomato genome sequence in 2009 (Mueller *et al.*, 2009), a large number of studies have concentrated on genomic resources by the transgenic approach. Many successful *Agrobacterium*-based transformation protocols of different tomato genotypes have been constructed by using the leaves and cotyledons of tomato plants (Sharma *et al.*, 2009; Honda *et al.*, 2021). One disadvantage of *Agrobacterium*-mediated transformation is that the plant tissue needs to be amenable to infection by the bacterium, but not be adversely affected by the process. The principle of transgenic technology is to create desirable traits by introducing foreign DNA (known as a transgene) into a recipient's genome. Thus, transgenic approaches are more

tedious and the regulatory policies for genetically modified (GM) organisms are still unclear and differ among many countries/regions (Turnbull *et al.*, 2021). Therefore, a huge gap has been raised between traditional breeding programs and global food security.

Of interest to us, genome editing tools enable researchers to precisely replace, delete, or insert a gene in the genome. The big difference between the genome editing and gene transformation approaches is that genome editing is the manipulation of the genome of the organism itself by replacing/knocking out a targeted gene, whereas gene transformation can only insert non-existing foreign genes into the original individuals. Recently, three major genome editing systems, namely ZFN (zinc-finger nucleases), TALEN (transcription activator-like effector nuclease), which are based on sequence-specific nucleases, and CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein) systems such as those using CBE (cytidine base editor), have been regarded as some of the most powerful tools that have been used in the improvements of tomato (**Figure 1**). Due to their efficiency, simplicity, and versatility (Khan *et al.*, 2017), genome editing tools have been successfully applied in various food crops like maize (*Zea mays*) (Liang *et al.*, 2014), sweet orange (*Citrus sinensis*) (Jia & Wang, 2014), rice (*Oryza sativa*) and wheat (*Triticum sativum*) (Shan *et al.*, 2014), soybean (*Glycine max*) (Xu *et al.*, 2020), and especially tomato (Vu *et al.*, 2020). Unfortunately, both ZFN and TALEN have been reported to be rarely used in tomato, whereas the CRISPR/Cas systems have been widely applied to accelerate the majority of tomato breeding programs. Several major breeding goals, such as productivity, stress tolerance, and fruit quality, are increasingly being studied (Rothan *et al.*, 2019) (**Figure 1**). Since 2013, great efforts have been made in order to focus on the feasibility of efficient use of the CRISPR/Cas systems and their potential applicability for studying gene function (Van Eck, 2017).

Improvement of the growth habit by CRISPR/Cas Systems

The favorited targets of genome editing are the customization of tomato cultivars and acceleration of the domestication of wild tomato. Improvement of the growth habit of tomato plants is a putative approach for developing sustainable agriculture due to the loss of arable land. Thus, customization of the growth habit of tomato plants by CRISPR/Cas systems has been considered as the first aim of basic research and breeding. The first publication of CRISPR/Cas9-based tomato genome editing was published in 2014, of which the wiry leaf phenotype was

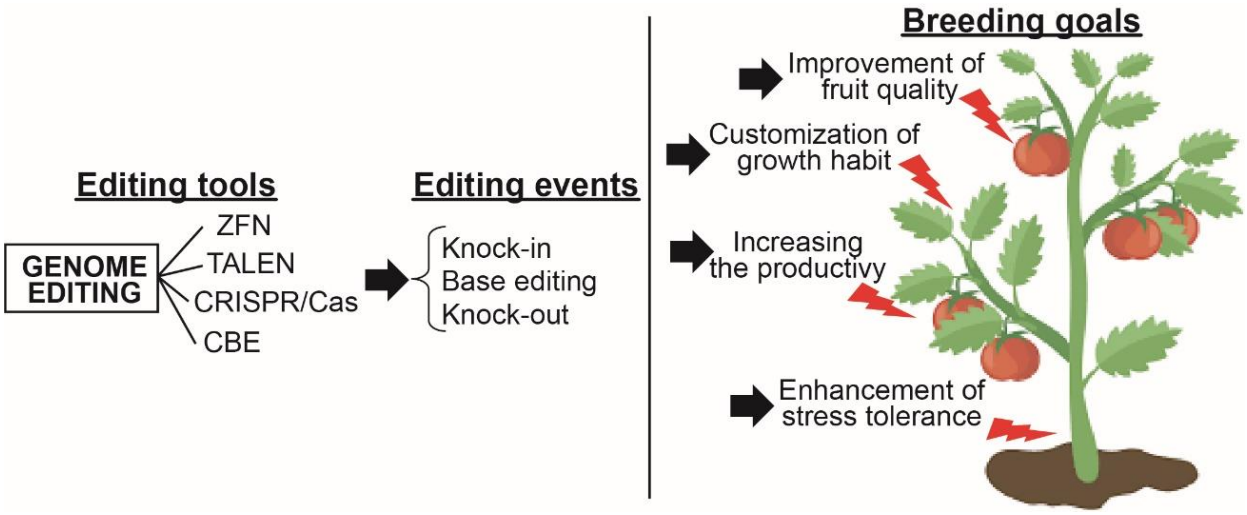

Figure 1. Application of genome editing tools in tomato improvement

observed in mutant lines with an edited *SlAGO7* (Argonaute7, *Solyc01g010970*) gene (Brooks *et* al., 2014). In order to improve the growth and development of tomato plants, CRISPR/Cas9 was successfully applied in the functional characterization of novel genes, like SlCLV1 (Clavata 1, Solyc04g081590), SlCLV2 (Solyc04g056640), and SlCLV3 (Solyc11g071380), which participate in the CLV-WUS (Clavata-Wushel) pathway in the control of shoot meristem size (Xu et al., 2015), providing numerous ideas to customize mutant alleles for further tomato breeding (Rothan et al., 2019; Sun et al., 2020). Next, a number of genes of interest involved in the growth and maturation of flowers, such as the BTB/POZ domain (Broad complex, Tramtrack, and Bric-a-brac/POX virus and zinc finger) transcriptional regulators (Xu et al., 2016), SlMBP21 (a member from the MADS-box family) (Roldan et al., 2017), SlEJ2 (enhancer of jointless 2, Solyc03g114840), and FW3.2 (a minor fruit weight QTL) (Soyk et al., 2017), were functionally characterized via the CRISPR/Cas systems. Additionally, knock-out mutants of BOP (Blade-on-petiole), encoding a transcriptional cofactor linked to inflorescence maturation, exhibited inflorescence defects with only one flower (Xu et al., 2016). Several members of the MADS-box family, such as the SlJ2 (Jointless 2, Solyc12g038510), EJ2, and LIN genes, have been demonstrated to control flower branching by CRISPR/Cas systems (Roldan *et al.*, 2017). These findings suggested that CRISPR/Cas tools might help identify the structures of suitable inflorescences for particular tomato production goals.

Next, several studies have been reported with the aims of designing interesting fruit shapes by CRISPR/Cas systems. Basically, the tomato fruit shape is regulated by the activities of the Ovate family. Mutant lines of Ovate and Suppressor of Ovate1 genes could promote the production of elongated fruits. Furthermore, knockout *TRM5* (Tonneau1 Recruiting Motif 5) could rescue the tomato shape (Wu *et al.*, 2018). Interestingly, CRISPR/Cas-based target mutagenesis of the *Sieno* (Excessive number of floral organs) gene was demonstrated to enhance the floral organ and amount of locules (Yuste-Lisbona *et al.*, 2020). **Table 1** summarizes a number of CRISPS/Cas-based edited genes toward the improvement of growth habits in tomatoes. Taken together, the CRISPR/Cas-based functional characterization of major genes of interest encoding functional and regulatory proteins could provide a foundation for designing the growth habits of tomato plants.

Fruit quality improvement by CRISPR/Cas systems

Recently, fruit quality has been regarded as a factor of increasing interest. Thus, improvement of fruit quality, like seedless fruits and increased freshness, has been highlighted in many breeding programs. It is thought that studying gene functions and their CRISPR/Cas-based mutants could provide comprehensive information in order to customize tomato fruit for better quality. *SlRIN*, encoding a member of the MADS-box TF family, has been known to be involved in the fruit ripening of tomato plants. Knockout *SlRIN* by the CRISPR/Cas9 system caused a disruption of the ripening process, expanded the shelf life, and reduced the accumulation of lycopene in fruits (Ito *et al.*, 2015) (**Table 2**). Interestingly, mutated *alc* (alcobaca) alleles could generate long-shelf-life tomato lines with no significant differences in major agronomical characteristics, such as plant height, stem diameter, fruit soluble solids content, flesh thickness, and fruit firmness, as compared with the control (Yu *et al.*, 2017).

Parthenocarpy is an interesting trait of the tomato, where fruit formation and growth are triggered without fertilization. CRISPR/Cas9-induced targeted mutagenesis of *SlAGL6* (Agamous-like 6) could promote fruit development excluding fertilization in mutant lines (Klap *et al.*, 2017). This study also demonstrated that seeds from the mutant lines are still healthy and developed well, and the fertilization process was performed under normal conditions (Klap *et al.*, 2017). The CRIPSR/Cas-based mutation of *SlIAA9* (Auxin-induced 9), encoding a repressor of fruit development without fertilization, also promoted the construction of parthenocarpic fruits (Ueta *et al.*,

Table 1. Summary of targeted genes and their major CRISPR/Cas-based mutant phenotypes toward the improvement of growth habits in tomato

#	Targeted gene	Gene ID	Annotation	Major characteristics of mutant lines	References
1	SlAGO7	*Solyc01g010970*	Argonaute	Wiry leaves	Brooks *et al.* (2014)
2	SlHPAT homolog	*Solyc08g041770*	Hydroxyproline O-arabinosylatransferases	Altered reproductive development	
3		*Solyc07g021170*			
4		*Solyc12g044760*			
5	SlCLV homolog	*Solyc04g081590*	Clavata	Weak branching and fascinated flowers	Xu *et al.* (2015)
6		*Solyc04g056640*		Weak branching and fascinated flowers	
7		*Solyc11g071380*		Branched inflorescences with fascinated flowers	
8	SlRRA3a	*Solyc04g080080*	Reduced residual arabinose	Branched inflorescences with fascinated flowers	
9	SlPDS	*Solyc03g123760*	Phytoene desaturase	Albino	Pan *et al.* (2016)
10	SlPIF4	*Solyc07g043580*	Phytochrome interacting factor	Less side effects	
11	SlBOP homolog	*Solyc04g040220*	Blade-on-petiole	Flowering defect	Xu *et al.* (2016)
12		*Solyc10g079460*			
13		*Solyc10g079750*			
14	SlJ2	*Solyc12g038510*	Jointless 2	Jointless unbranched inflorescences	Roldan *et al.* (2017); Soyk *et al.* (2017)
15	SlLIN	*Solyc04g005320*	Long inflorescence	Moderately branched inflorescences and enhanced flower production	
16	SlTRM5	*Solyc07g008670*	TONNEAU1 Recruiting Motif	Flatter fruit	Wu *et al.* (2018)
17	SlENO	*Solyc03g117230*	Excessive number of floral organs	Enhanced the formation of floral organs and multilocular fruits	Yuste-Lisbona *et al.* (2020)
18	SlPRO	*Solyc11g011260*	Procera	Dwarf	Tomlinson *et al.* (2019)

2017). Unfortunately, an abnormal leaf phenotype has been observed in these knockout mutants, causing an ultimate effect on tomato productivity (Ueta *et al.*, 2017). These findings together provide important information for the further breeding of parthenocarpic tomatoes.

Next, it is strongly believed that malic acid is an intermediate metabolite of tomato plants, and acts as a regulator in plant growth and fruit quality (Ye *et al.*, 2017). A CRISPR/Cas-based knockout mutant of *TFM6* (tomato fruit malate 6) was recorded to reduce the accumulation of malate in fruit, whereas a 3-bp deletion in the cis-regulatory elements that recognize *SlWRKY42 TF* in the promoter of *TFM6* increased the fruit malate content (Ye *et al.*, 2017). Additionally, with the aim of improving GABA (G-Aminobutyric acid) content in tomato fruits, two major genes are involved in GABA biosynthesis during fruit development, namely *SlGAD2* and *SlGAD3* (glutamate decarboxylase 2 and 3), were targeted by CRISPR/Cas9-based targeted mutagenesis. As expected, the GABA content in the T1 generation from mutant lines of the *SlGAD3* gene harboring a premature stop codon before the auto-inhibitory regions was produced at approximately 7 - 15-fold higher levels than the control (Nonaka *et al.*, 2017). The heterozygous GABA-rich T1 plants introduced fewer effects on the plants and fruit (Lee *et al.*,

Table 2. Summary of targeted genes and their major CRISPR/Cas-based mutant phenotypes toward the improvement of fruit quality in tomato

#	Targeted gene	Gene ID	Annotation	Major characteristics of mutant lines	References
1	SlRIN	*Solyc05g012020*	Ripening inhibitor	Incomplete-ripening fruits, extended shelf life	Ito *et al.* (2015)
2	SlAGL6	*Solyc01g093960*	Agamous-like protein	Seedless	Klap *et al.* (2017)
3	SlIAA9	*Solyc04g076850*	Auxin-responsive protein	Seedless	Ueta *et al.* (2017)
4	SlALC	*FJ404469*	Alcobaca	Long-shelf life	Yu *et al.* (2017)
5	SlGAD homologs	*B1Q3F1*	Glutamate decarboxylase	Increased GABA content in fruits	Nonaka *et al.* (2017)
6		*B1Q3F2*			
7	SlTFM6	*Solyc06g072910*	Aluminum-activated malate transporter-like protein	Reduced malate content in fruits	Ye *et al.* (2017)
8	SlGABA-TP homologs	*AY240229*	pyruvate-dependent γ-aminobutyric acid transaminase	Increased GABA content in fruits	Li *et al.* (2018a)
9		*AY240230*		-	
10		*AY240231*		Increased GABA content in fruits	
11	SlCAT9	*XM_004248503*	Cationic amino acid transporter	No fruit	Vu *et al.* (2020)
12	SlSSADH	*NM_001246912*	Succinate semialdehyde dehydrogenase	No fruit	Vu *et al.* (2020)
13	SlSGR1	*DQ100158*	Stay green	Increased lycopene content in fruits	Li *et al.* (2018b)
14	SlBlc	*XM_010313794*	β-lycopene cyclase	Increased lycopene content in fruits	Vu *et al.* (2020)
15	SlPSY1	*P08196*	Phytoene synthase	Yellow-fleshed fruit	D'ambrosio *et al.* (2018)
16	SlCrtR-b2	*Q9S6Y0*	β-carotene hydroxylase	White-flowers	Vu *et al.* (2020)

2018). Additionally, multiplex CRIPSR/Cas-based mutations of five genes participating in GABA conversions, namely *SlGABA-TP1* (pyruvate-dependent γ-aminobutyric acid transaminase 1), *SlGABA-TP2*, *SlGABA-TP2*, *SlCAT9* (Cationic amino acid transporter 9), and *SlSSADH* (Succinate semialdehyde dehydrogenase), increased the accumulation of GABA in fruits by 3.5-fold as compared with the wild-type (Li *et al.*, 2018a). However, the phenotypes of these GABA-increased mutants tended to differ from the control plants, like reduced growth, prolonged flowering time and changed fruit settings (Li *et al.*, 2018a). These observations were explained by the idea that GABA over-accumulation might affect the expression of genes related to cell elongation in vegetative or flower/fruit tissues (Li *et al.*, 2018a). This phenomenon was also recorded in the *SlGATA-TP1* silenced mutant as previously described (Koike *et al.*, 2013).

Up till now, the lycopene-rich tomato has been regarded as one of the more popular varieties in the market (Imran *et al.*, 2020). Many targeted genes related to lycopene metabolism and cyclization stages, like *SlSGR1* (stay green 1, *DQ100158*), *SlLCY-E* (Lycopene ε-cyclase, *EU533951*), *SlBlc* (β-lycopene cyclase, *XM_010313794*), *SlLCY-B1* (Lycopene β-cyclase 1, *EF650013*), *SlLCY-B2* (Lycopene β-cyclase 2, *AF254793*), *SlPSY1* (Phytoene synthase 1, *P08196*), and *SlCrtR-b2* (Beta-

carotene hydroxylase 2, *Q9S6Y0*), were functionally characterized by CRISPR/Cas systems with the aims of enhancing lycopene production (Vu *et al.,* 2020). A single mutation of *SlSGR1* led to an increase in the lycopene content in fruits by approximately 5.1-fold as compared to that of the control (Li *et al.*, 2018c). Interestingly, the knock-out *SlPSY1* (phytoene synthase 1, *P08196*) by a targeted CRISPR/Cas9 system exhibited yellow-flesh fruits (D'ambrosio *et al.*, 2018), suggesting that control of the carotenoid content might be applied to customize fruit color (Vu *et al.*, 2020). Other CRISPS/Cas-based edited genes toward the improvement of fruit quality in tomatoes were also summerized in **Table 2**.

Challenges and future perspectives in Vietnam

Genome editing tools have been applied in many plants from monocots to dicots (Khan *et al.*, 2017). Among them, CRISPR/Cas systems, particularly CRISPR/Cas9, were widely applied in tomatoes (Vu *et al.*, 2020), whereas other tools, like TALEN and ZFN were rarely used in tomato research (Xia *et al.*, 2021). One of the major reasons for the low efficiency of these precision editing tools in crops is the lack of a stable genetic transformation system. Recently, the latest update of induction of gene-edited meristems and the construction of nanoparticle-mediated plant genome editing were reported in order to reduce the time of tissue culture, proposing a potential protocol for the application of genome editing tools. These achievements could bring new hope for the use of genome editing tools in the functional characterization studies of all plant species. Therefore, it is very important to establish a stable transformation system for cultivated tomato varieties in Vietnam as an initial step for the precision breeding of tomatoes.

Vietnam has been noted as one of the many countries likely to be most affected by climate change. Adverse environmental conditions caused by climate change, including numerous biotic stresses (like bacteria, fungi, viruses, and nematodes) and abiotic stresses (like drought, high salinity in the soil, submergence, and extreme temperatures) will cause significant damage to the production of tomato in Vietnam. Thus, genetic improvement of tomato for resistance to biotic and abiotic stresses is always a major objective for tomato breeders. Up till now, few studies have been performed to characterize candidate genes in tomato by using the CRISPR/Cas systems. For example, gRNAs targeted on *SlIAA9* gene was inserted into CRISPR/Cas9 vectors. The vectors were then successfully transferred into two strains of *A. tumefaciens* (Bui *et al.*, 2020). Recently, gDNAs targeted on *CIF1* gene, a gene related to the sugar synthesis in tomatoes were designed and inserted into CRISPR/Cas9 vectors. The vector was then successfully transferred into the EHA105 *A. tumefaciens* strain (Dao *et al.*, 2021). These findings provided useful information to carry out further functional characterizations of interest genes in tomatoes by using the CRISPR/Cas systems.

Another noticeable point is that a number of the CRISPR/Cas-based mutants are loss-of-function types through the knockout of targeted genes. Most of these mutant lines do not exhibit useful traits for breeding (Zhu & Qian, 2020). On the other hand, CRISPR/Cas-based gain-of-function mutations have not only provided many advantages for functional characterization studies but have also exhibited great potential uses for crop improvement (Zhu & Qian, 2020). As summarized previously, gain-of-function mutations have been reported to enhance abiotic and biotic stress resistance in tomatoes (Vu *et al.*, 2020; Xia *et al.*, 2021). Thus, CRISPR/Cas-based gain-of-function mutations could be a powerful tool for precision breeding by providing broad materials for screening in the fields. Taken together, we strongly believe that genome editing tools, particularly CRISPR/Cas systems, are critical for attaining sustainable farming, from the gene to the field.

Conclusions

Tomato is the largest vegetable crop in the world, and perhaps in Vietnam. Improvements of the growth, development, and productivity of

tomato are some of the major sustainable development strategies. Genome editing, especially CRISPR/Cas-based tools, could be a potential approach for breeding new tomato varieties with rare traits related to growth habits and fruit quality. Numerous studies have reported the functional characterization of genes of interest by CRISPR/Cas systems to customize growth habits. Furthermore, great efforts have been made in order to report the functions of genes related to fruit quality. These findings strongly support that customizing gene structure by the CRISPR/Cas system could be a valuable method for breeding elite innovations in tomato.

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