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Use of Genome Editing to Characterize Gene Function of *SlIAA9* in Plant Growth, Development and Fruit Quality in Tomato

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Abstract

Genome editing, the CRISPR/CAS9 system, is being hailed as a novel tool to improve quality of crop plants. To test the potential of CRISPR/CAS9 system to improve yield and quality of horticultural crops we have tested its effectiveness in developing knockout mutants in tomato (*Solanum lycopersicum)*, a widely consumed vegetable throughout the world. A CRISPR/CAS9 construct was made containing the guide RNA of *auxin-responsive protein/ IAA9 (SLAUX/IAA9)* gene and used to transform tomato using the *Agrobacterium* based transformation system. *SLAUX/IAA9* encodes a short-lived transcription factor that affects a number of plant growth and developmental processes including leaf architecture. We show that CRISPR/CAS9 system effectively knocked out *SLAUX/IAA9* in tomato. The resulting knockouts exhibited multiple phenotypes including change of leaf architecture from compound leaves to simple leaves. The knockout plants showed reduced plant height; early fruit set and reduced seed number in fruit. The *SLAUX/IAA9* knockout did not altered patterns of ethylene production and lycopene accumulation in ripening fruits but significantly reduced ethylene production during fruit ripening. Taken together, these results show that CRISPR/CAS9 system provides a method of choice to develop desirable mutants not harboring antibiotics resistance genes in the horticultural crops.

INTRODUCTION

Tomato *(Solanum lycopersicum*) a berry fruit belonging to the genus Solanum originated in South America is widely consumed all over the world including the United Stated of America (Kimura and Smith, 2005; Fatima *et al.*, 2008). The *Solanaceae* family is comprised of a number of important agricultural commodities including eggplant, pepper, tobacco and a few others. Tomato fruits, rich in antioxidants, have been implicated in human health by ameliorating many diseases including cancer,

atherosclerosis which leads to cardiovascular diseases, hypertension, diabetes and osteoporosis (Mattoo *et al.*, 2011; Fatima *et al.*, 2013). Over 122.6 million metric tons of tomatoes are produced each year around the globe for human consumption with the United States, Italy, Spain, and the U.A.R. being the leading producer (Fatima *et al.*, 2008). In United States alone on an average 30.8 pounds of tomatoes were reported to be consumed per person in 2013 (Bassett *et al.*, 2013). Tomato fruit quality is controlled by many factors including genetic (genotypes) and epigenetic environmental (temperature, light, rainfall, soil type, nutrient and water supply) and cultural practices (mulching, pruning, thinning, chemical treatments, time and method of harvest). A large number of genes positively and negatively regulate tomato fruit quality (Handa *et al.*, 2014). The available genetic, physiological and biochemical resources in tomato has made tomato an attractive model system to evaluate the mechanisms that regulate plant growth and development, especially fleshy fruit ripening (Handa *et al.*, 2014). Development of new tomato cultivars with increased phytonutrients would greatly contribute to human health (Mattoo *et al.*, 2011; Fatima *et al.*, 2013).

Emergence of new technologies, especially genome editing, is revolutionizing the methods to develop new mutations and investigate gene function in many organisms. (Liang *et al.*, 2014; Gerbach *et al.*, 2015). Its use is rapidly emerging as a method of choice to modify genome in many organisms including plants, animals and human (Cong *et al.*, 2013). Several methods including Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered, Regularly Interspaced, Short, Palindromic Repeats (CRISPR)/CRISPR Associated System (CAS) nuclease, have emerged for the targeted genome editing (Sander and Joung, 2014). However, due to ease of use and high efficiency on mutation, CRISPR/CAS9 system is becoming a method of choice for genome editing in many organisms (Sander and Joung, 2014). To test the efficiency of CRISPR/CAS9 in developing high yield and improved quality of horticultural crops, we have tested this system to knockout *SLAUX/IAA9*, a gene that regulates number of morphological and physiological parameters in tomato (Wang *et al.*, 2005). We report that CRISPR/CAS9 system effectively induced deletion mutations in *SLAUX/IAA9* altering leaf architecture from compound leaves to simple leaves. This mutation also resulted in early flowering and fruit set, reduced seed number and lower ethylene production in ripening fruits. Collectively, our results show that CRISPR/CAS9 provides an efficient tool for the genome editing in tomato.

MATERIAL AND METHOD

Plant Material and Growth Conditions

Agrobacterium-mediated transformation was used to introduce *SLAUX/IAA9*-sgRNA CRISPR/CAS9 construct into tomato (*Solanum lycopersicum cv. Ohio 8245*) as described previously (Tieman *et al.*, 1992; Nambeesan *et al.*, 2010). Briefly, cotyledon segments from 6 to 8 days old seedlings were incubated with *Agrobacterium tumefaciens* strain GV3101 harboring the *SLAUX/IAA9*-sgRNA CRISPR/CAS9 constructs. Putative transgenic plants were selected on a medium containing 20 µg/ml hygromycin in a half strength Murashige and Skoog (MS) culture medium containing 1.5% (w/v) sucrose, 0.6% agar and pH 5.8. Resulting plantlets were rooted and acclimated in a culture chamber room under standard conditions: 14/10 h and 25/20°C for day/night, 250 µmol $m^{-2}s^{-1}$ intense luminosity and 80% humidity. The acclimated plantlets were transferred to 4-inch pots in green house first followed by transfer to soil bags when reached 10-15 cm

height and grown to ripe-fruit maturity under a constant day/ night temperature of 26/23 with a relative humidity 60%. Self-pollinated T0 seeds from each putative transgenic plant were collected and resulting T1 seedlings were characterized to determine the effects of *SLAUX/IAA9* mutation on various growth and development parameters.

Vector Construction

The sgRNA–Cas9 vector was constructed as previously described and pCAMBIA1300 was used for plant expression (Feng *et al.*, 2013; Yanfei *et al.*, 2013; Zhang *et al.*, 2014). The 20 nucleotide sgRNA target sequences for *SlIAA9* were selected from *S. lycopersicum* auxin-responsive protein 9 (*SlAux/IAA9*) representing the nucleotide 380-399 of accession NM_001278959.2. Oligo were synthesized containing *SlIAA9*-sgRNA target and adapters sequences. The double-strand phosphorylated oligos of *SlIAA9*-sgRNA were assembled into the *Bbs*I digested vector with sgRNA backbone and Cas9 protein cassette (Feng *et al.*, 2013). The *SlIAA9*-sgRNA/Cas9 cassette fragment was sub-cloned into *Hind*III and *Eco*RI digested plant expression vector pCAMBIA1300 and resulting chimeric gene was transformed into *Agrobacterium tumefaciens* strain GV3101.

Evaluation of Genome Modifications

Total DNA was extracted from transgenic plants by the 2% CTAB method (Doyle and Doyle, 1987). The genome region associated with s *SlIAA9*-sgRNA region was PCR amplified using *SlIAA9* gene specific primers (SlIAA9-F: taattgtgatgtctccgccgc and SlIAA9-R: ctgctttcacaccggaattcg) and the amplified fragment cloned into TA cloning pGEM®-T Easy Vector (Promega, United States). The resulting plasmids were sequenced to determine the site and the type of mutations caused by CRISPR/CAS9 system in *SlIAA9* coding region.

Quantitative Reverse Transcription-PCR Analyses Of Slaux/ IAA Expression

Leaves harvested from WT and ENT2 mutant were immediately frozen in liquid nitrogen. Total RNA extraction, extraction, cDNA synthesis, and quantitative RT-PCR were performed as previously described (Nambeesan *et al. (*2011). *SlAUX/IAA9* gene-specific primers were used for quantitative RT-PCR with tomato elongation factor-1 (Accession TC98347) as an endogenous reference for normalization. *SlAUX/IAA9* gene-specific forward and reverse primer were ^{777}AGGCCTTCTGCTGTGAATGA^{796} and ^{926}TTTTCCGTCAACCTCTTCGTT^{906}, respectively for accession NM_001278959.2. *SlEF-1 reverse and forward primers for qRTPCR were* TGGCCCTACTGGTTTGACAACTG and CACAGTTCACTTCCCCTTCTTCTG, respectively.

Plant Growth Measurements:

T1 seedling from T0 seeds were grown in green house and their heights were determined throughout the plant growth period until plants reached ripe fruit maturity. The dates of first flowering and fruit set were recorded for each T1 plant. The leaf area of fully expanded leaves was measured by using Ll 310C0 Area Meter. For WT leaves, total area of all leaflets in a compound leaf and for *SLAUX/IAA* mutant, the total area of modified simple leaf was determined.

Ethylene and Lycopene Measurement:

The rate of ethylene production was determined as described previously (Biggs *et al.*, 1988). Briefly, whole single fruit was weighed and sealed in a mason jar fitted with sampling port. Ethylene was allowed to accumulate for two hours and gas sample was withdrawn with a syringe. Ethylene was quantified (nl) using GC-8A (Shimadzu, Japan) equipped with an activated alumina column and flame ionization detector. Rate of

ethylene production was calculated as ul/g/h. Lycopene was determined as described previously (Handa *et al.*, 1985).

RESULTS

Construction of Sliaa9- Sgrnas

We have selected *SlAUX/IAA* to evaluate effectiveness of CRISPR/CAS9 system in tomato as mutation in this gene had been reported to results in an easily visible phenotype of leaf architecture (Wang *et al.*, 2005). *SlAUX/IAA* protein is a short-lived transcription factor and disruption in its expression caused altered leaf architecture from compound to simple leaves (Wang *et al.*, 2005). *SLAUX/IAA* contains multiple domains and we selected an upstream genome region of *SLAUX/IAA* gene as the sgRNA target site. A 20-bp target sequence immediately preceding the 5′-NGG PAM was selected from *Sl*Aux/IAA9 gene (nucleotide 380-399 from accession NM_001278959) (Ran *et al.*, 2013; Shan *et al.*, 2014). BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm the absence of any additional 20-nt sgRNA-binding sequence plus the NGG of the PAM (here it is 5′-CGG) in tomato genome to minimize the off-target effects. CaMV 35S promoter and the *Arabidopsis* U6 promoters were used to drive the codon-optimized *Cas9* gene and sgRNA, respectively, as they have been used previously to facilitate gene editing *by CRISPR/CAS9* in *Arabidopsis* (Yanfei *et al., 2013;* Feng *et al., 2013).*

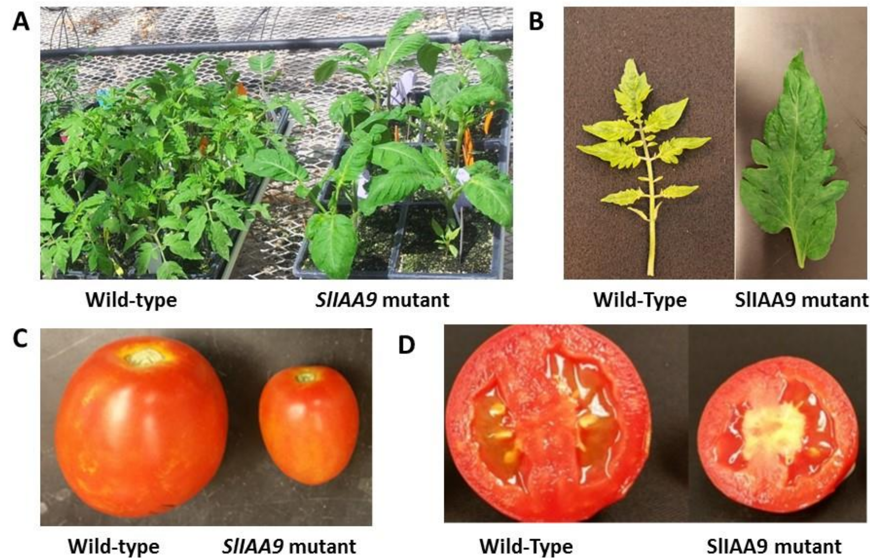

Figure 1: A. Phenotypic effects of CRISPR/CAS9 induced *SLAUX/IAA9* mutation. WT and T1 *SLAUX/IAA9* mutant (A) seedlings; (B) Change of leaf morphology from compound to simple leaf; (C) fruit sizes; (D). Number of seeds.

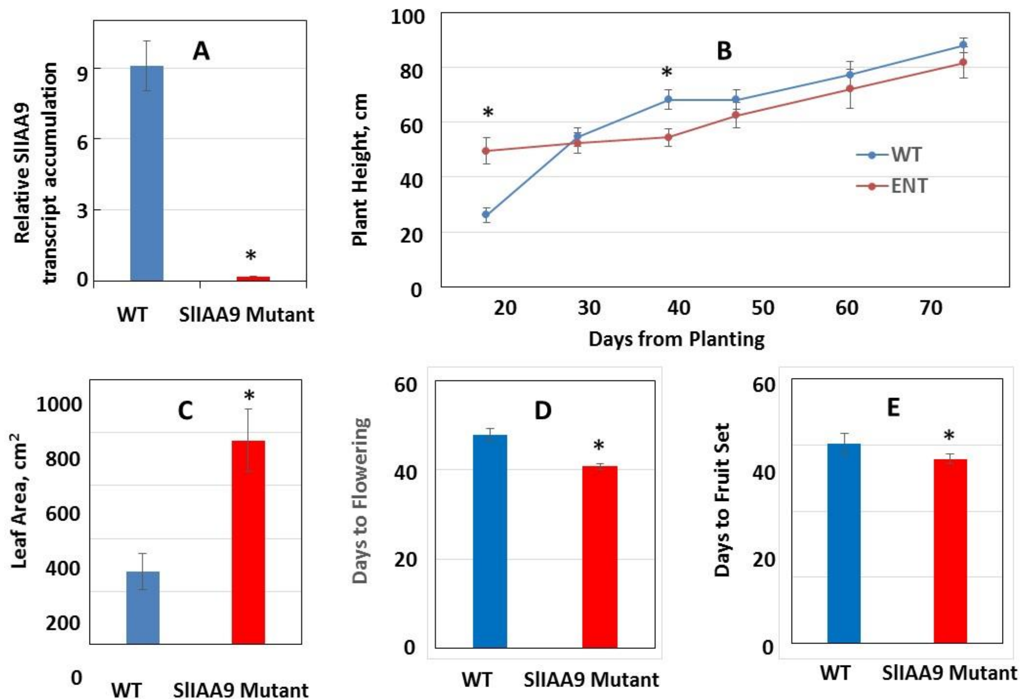

Figure 2: Effects of *SlAUX/IAA9* mutation on (A) *SlAUX/IAA9* transcripts accumulation, (B) plant growth, (C) leaf area, (D) Days to flower and (E) Days to Fruit set. * represents value significantly different from corresponding control

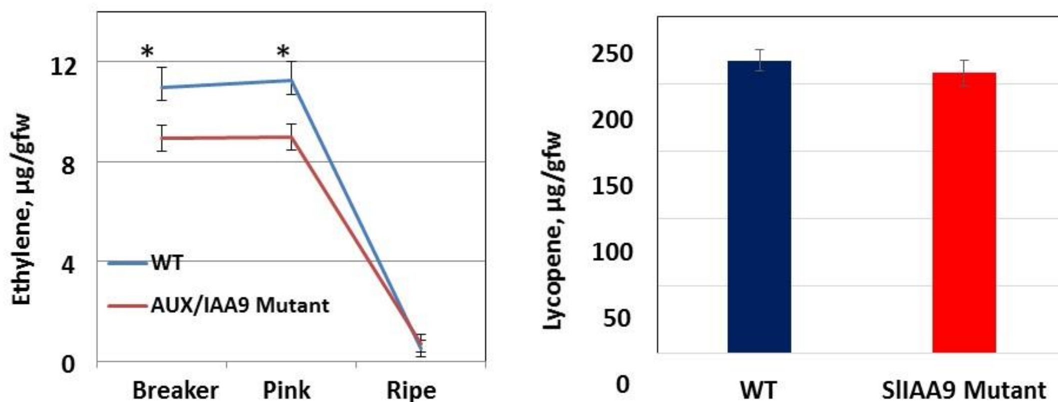

Figure 3: *SLAUX/IAA9 mutation lowered t*he rate of ethylene production during ripening and lycopene contents compared to wild type fruit. * represents value significantly different from corresponding control

Conformation of CRISPR/CAS9 System Induced Target Gene Mutations in Tomato

The PCR amplified fragments of target gene sequence from the putative mutant plants were used to confirm the frame shift mutation generated in *AUX/IAA9* by

CRISPR/CAS9 system. The target site sequences were PCR-amplified from the total DNAs extracted from 4 independent transgenic lines using *SLAUX/IAA9* gene specific primers. The resulting amplified DNA fragments were cloned in a TA vector and sequenced. Results showed that frequency of mutation was about 50% (2 out of 4 plants) and most mutations represented 4 base pairs deletion in the target gene sequence (Data not shown). These results show that the CRISPR/CAS9 system would induce genome editing in the tomato genome with high efficiency.

Quantitative reverse transcription-PCR analyses of *SlAUX/ IAA* transcripts were performed to show efficacy of CRIDPR/CAS9 induced deletion mutation. Leaves from ENT2, CRISPR/CAS9 mutant exhibited several folds reduction in *SlAUX/ IAA* transcript levels (Figure 2A). However, effect of mutation on *SlAUX/ IAA* protein levels remained to be determined.

The CRISPR/CAS9-Induced Sliaa9 Editing Altered Tomato Leaf Architecture from Compound Leaves to Simple Leaves

All self-pollinated T1 seedlings from *SLAUX/IAA9* edited line 2 (ENT-2) exhibited simple leaf phenotype instead of a compound leaf phenotype present in the wild type plants (Figure 1A and B). Although we are yet to systematically determine the homozygosity for the edited gene in ENT-2 mutant line, the simple leaf phenotype of in all T1 mutant seedling of this line suggest that the CRISPR/CAS9-induced *SlIAA9* mutation was either a dominant or resulted in editing of both copies in the genome of tomato. The *SlAUX/IAA9* mutation not only changes the phenotype of tomato compound leaf but also significant increase in total leaf area. The area of mutated simple leaf was 2 to 3-fold larger than the sum of all leaflets present in the compound leaf of the wild-type (WT) parental plant (Figure 2C).

Effect Of SLAUX/IAA9 Editing on Plant Growth, Flowering and Fruit Setting Times, Fruit Size, And Seed Set

In general, the wild type plants had a bigger canopy compared to *SLAUX/IAA9* mutant plants as the *SLAUX/IAA9* mutants exhibited more upright phenotype than WT plants. Although the mutant seedlings exhibited higher height during early stage of growth, the relative plant height gain was lower in the *SLAUX/IAA9* mutant than the wild type plant (Figure 2B). The *SLAUX/IAA9* mutants flowered early compared to WT and exhibited significant early fruit set compared to the WT plants (Figure 2 D, 2 E). *SLAUX/IAA9* mutant ripe fruits were smaller in size than the wild type fruits (Figure 1 C) and contained much less number of seeds compared to the wild type fruits (Figure 1D)

Mutation in Slaux/IAA9 Altered the Rate Ethylene Production and Total Lycopene Accumulation

Figure 3A shows the rates of ethylene production in WT and mutant fruit during ripening. There was significant reduction in the rate of ethylene production in *SlAUX/IAA9* mutant fruits compared to WT fruit at breaker and pink stages of ripening but no difference was observed in fully ripe fruit. *SlAUX/IAA9* mutant fruits also showed decrease in lycopene accumulation compared to WT fruit in spite of their smaller size (Figure 3B).

DISCUSSION

The CRISPR/CAS9 system has been adapted as a powerful tool to create genome modifications in varius organisms (Cong *et al.*, 2013; Feng *et al.*, 2013). It has been used in many plant species including *Arabidopsis* (Feng *et al.*, 2014) *Nicotiana benthomini (*Gao *et al.*, 2014) and rice (Yanfei *et al.*, 2013; Feng *et al.*, 2014; Hsu *et al.*, 2014). Three different types of CAS9 have been used for genome editing in plant and include: human codon-optimized CAS9, plant codon-optimized CAS9 and WT *St. pyogenes* CAS9 (Gao *et al.*, 2014). The human codon-optimized CAS9 protein and sgRNAs under the control of CaMV 35S and the *Arabidopsis* U6 promoters have been shown to be effective in causing genome editing in *Arabidopsis* at high-efficiencies using CRISPR/CAS9 system. In present study, we used the human codon-optimized CAS9 system for target genome editing in tomato using the CRISPR/CAS9 system. We show that CRISPR/CAS9 system provide an effective method to induce target genome editing in tomato. The resulting *SlIAA9* mutants displayed expected altered leaf architecture resulting from the conversion of a compound leaf into a simple leaf in tomato as reported previously (Wang *et al.*, 2005). Sequencing of the *SLIAA9* region in mutants showed that all mutations were frame-shift around the PAM region (data not sown), and likely resulted in termination of correct translational reading frame. All T_1 seedling of ENT-2 mutant exhibited simple leaf morphology showing heritability and stability of CRISPR/CAS9 induced mutations (Figure 1 A.), a result similar to that observed in other plants (Feng *et al.*, 2014).

It has been previously reported that antisense-RNA induced impaired expression of *SLAUX/IAA* caused changes in tomato leaf phenotype from compound to a simple leaves and reduced seed set leading to partial parthenocarpic fruit set (Wang *et al.*, 2005). We obtained these phenotypes in *SlAUX/IAA9* genome edited mutants, confirming the role of SlIAA9 in regulating leaf morphology and seed set (Figure 1). In addition to these phenotypes, the IAA9 frame-shift mutants also exhibited decrease in plant height and change in canopy as mutant plants were more upright than WT plants. Other phenotypes induced by IAA9 frame-shift mutation included early flowering and fruit set and reduced fruit size. Ripening of SlIAA9-mutant fruits showed significant reduction the rate of ethylene production and lycopene accumulation. We have not yet evaluated effects of these multiple effects on fruit quality or the agronomical performance of the IAA9 mutants.

Our results suggest that effects of SlIAA9 frame shift mutation were not lethal for plant growth and development. This may be due to compensation of *SLAUX/IAA9* function by other members of AUX/IAA family. *SLAUX/IAA27*, another member of this family encodes a protein that share common features with AUX/IAA9 protein but has been suggested to regulate tomato fruit initiation and development in a distant manner (Goliber *et al.*, 1999; Hareven *et al.*, 1996; Bassa *et al.*, 2012). A number of genes determine the leaf architecture in plants, including in tomato (Bar *et al.*, 2015). The genes that regulate compound leaf development in tomato include Argonaute1(Bar *et al.*, 2015; Wang *et al.*, 2015), cytokinin and cytokinin regulated gene (Bertoni, 2010; Shani *et al.*, 2010; Shi *et al.*, 2013), of *NAM* (*NO APICAL MERISTEM*) /*CUC (CUP-SHAPED COTYLEDON)* boundary genes (Blein *et al.*, 2008), *PHANTASTICA* (LePHAN) and *YABBY B* (LeYAB B) (Kim *et al.*, 2003), and homeobox gene, *LeT6* (Janssen *et al.*, 1998). It will be of interest to know if expression of these genes is modified in *SLAUX/IAA9* mutation and if *SLAUX/IAA* manifests its effects through regulating expression of one or more these genes.

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