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יצירת פרתנוקרפיה פקולטטיבית בפלפל באמצעות עריכה גנומית

Generation of facultative parthenocarpy in pepper by genome editing

מוגש לקרן המדען הראשי במשרד החקלאות

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תקציר

הבעיה: חנטת הפרי בפלפל שמהווה גידול חשוב בערבה מוגבלת עקב טמפרטורות חריגות הפוגעות בחיוניות האבקה. בעגבנייה נטרול הגן *SlAGL6* מאפשר חנטה גם ללא הפרייה. *AGL6* שמור גם בפלפל (*CaAGL6*) מה שמעלה את האפשרות שתפקידו בפלפל זהה ושנטרולו יאפשר חנטה ללא הפרייה גם בטמפרטורות חריגות. **מטרות המחקר:** לנטרל את הגן *CaAGL6* ולבחון האם המוטנט מניב פירות פרתנוקרפים וכן להעביר את המוטציה לזן בלוקי. **תוצאות:** ננקטו שתי גישות לייצור מוטנט בגן *CaAGL6*. הוכן ווקטור לעריכה גנומית של גן זה שעבר טרנספורמציה לפלפל מזן קאיין באוניברסיטת UC-DAVIS ובמקביל נסרקה אוכלוסיית מוטנטים בזן בלוקי בשיטת TILLING. למרות קבלת צמחונים רגנרנטים עמידים לאנטיביוטיקה לא הצלחנו לקבל צמחים טרנסגנים המכילים את קונסטרקט העריכה. מאמצי הטרנספורמציה עדיין נמשכים. זוהו שש מוטציות missense ב- *CaAGL6* בשיטת ה-TILLING ובודדו 3 קווים מוטנטים. הקווים המוטנטים רובו והוכלאו לזן הבלוקי מאור על מנת לנקות את המוטציות ב- *CaAGL6* ממוטציות EMS אחרות ולשפר את יכולת חנטת הפרי שהייתה נמוכה ביותר בזן ההורה. יוצרו אוכלוסיות F2 מצמחי המכלוא ופותחו מרקרים מולקולריים לזיהוי המוטציות ואופיינו המוטנטים השונים. המוטנטים הבודדים נבחנו לחנטה ללא הפרייה לאחר סירוס אך לא הראו יכולת ליצירת פרי פרתנוקרפי. **שיטות העבודה:** עריכה גנומית וסריקה של אוכלוסיית מוטנטים בשיטת TILLING. יצירת אוכלוסיות F2 עם הזן הבלוקי מאור וסריקתן לצמחים הומוזיגוטים מוטנטים ושאינם מוטנטים, גידול הצמחים בתנאים מבוקרים, ובחינת יכולתם לחנוט פרי ללא הפרייה (לאחר סירוס). **מסקנות ויישום התוצאות:** המוטציות שנבחנו כנראה שאינן פוגעות מספיק בתפקודו של *CaAGL6* ולכן המוטנטים הרלוונטיים אינם מסוגלים לחנוט פרי ללא הפרייה (פרתנוקרפיה).

הצהרת החוקר הראשי:

הממצאים בדו"ח זה הינם תוצאות ניסויים.

הניסויים מהווים המלצות לחקלאים: לא

חתימת חוקר:

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Introduction:

Pepper is one of the most important vegetable crops in Israel with annual export above 100,000 tons. A major limitation of pepper production in Israel is a short season in which export-grade fruits can be produced. In the Arava region, the main production area, high quality fruits are limited to a narrow window of 3-4 months between October to January. This window is determined by unfavorable temperatures during fruit setting in the summer and winter that severely reduce fruit quality and yield. The use of climate-controlled greenhouses that allow extension of the season is not widespread because of high expenses. One possible way to expand the production window and increase the yield under unfavorable conditions is to use parthenocarpic pepper varieties which are independent of pollination and fertilization. However, large fruited parthenocarpic cultivars are not available today.

A new facultative parthenocarpic tomato mutant capable of fertilization-independent setting of normal, yet seedless fruits was recently characterized (Klap et al., 2017). Mutation mapping followed by CRISPR/Cas9 knockout of candidate genes indicated that a loss-of-function mutation in the *AGAMOUS-Like6* (*SlAGL6*) gene underlies the parthenocarpy and that *Slagl6* is a single recessive source for parthenocarpy, which is not allelic to any of the previously identified parthenocarpic mutations (Sotelo-Silveira et al., 2014). Phenotypic analysis suggests that the main, if not the sole, manifestation of the mutation is the loss of requirement for fertilization as a trigger for fruit set. Moreover, the *Slagl6* mutation does not prevent sexual reproduction, and bears no visible pleotropic effects and does not impose any unacceptable

penalty on yielding potential or fruit characteristics (Klap et al., 2017). The lack of pleotropic effects or adverse effects on fruit weight and shape, the true vegetative nature of the induced parthenocarpy and its facultative manifestation, makes *Slagl6* an attractive single recessive gene for parthenocarpy (Klap et al., 2017). The pepper genome encodes an almost identical AGL6 homolog (*CaAGL6*), strongly suggesting a similar function for *CaAGL6* in pepper and a similar fruit parthenocarpic phenotype upon *CaAGL6* loss-of-function.

Research goals:

Our ultimate goal is to increase pepper yield under fertilization-restrictive conditions by generating improved blocky varieties that are seedless. Specific objectives of the proposal are:

1. To knockout (KO) *CaAGL6* using CRISPR/Cas9.
2. To characterize the *Caagl6* mutants under fertilization favorable and unfavorable conditions.
3. To introgress the generated loss-of-function mutant *Caagl6* alleles into appropriate blocky pepper backgrounds.

Details of the main experiments and the results of the study:

Generation of Caagl6 knockout mutants

To knockout *CaAGL6* using the CRISPR/Cas technology, 3 guide RNAs (gRNAs) were designed to target its first (gRNAs 1 & 2) and second (gRNA 3) exons (Figure 1A). The gRNAs sequences were each incorporated into an sgRNA sequence downstream of the synthetic Arabidopsis U6 promoter and the 3 tandem U6:sgRNA sequences delimited by the *Bsa* I sequences were artificially synthesized and cloned into pMK plasmid (GeneArt). Then the pMK-3xU6:sgRNA plasmid was digested by *Bsa* I and resulting 3x U6:sgRNA fragment was ligated into the corresponding sites of the pYLCRISPR binary plasmid alongside the Cas9 expressed under the control of the Maize ubi1 promoter (Figure 1B). The constructed binary plasmid was transformed into pepper (cv. Cayenne) via *Agrobacterium* mediated transformation. In brief, after transformation of the cotyledons, the cotyledons are placed on callus induction media for 2 months. After the callus get to a certain size, they are transferred to the shoot elongation media (3 months). When shoots with visible main stem are present, they are transferred to rooting

media. When root develop, the plant is ready to be transferred to the greenhouse. The transformation is done in the lab of Prof. Allen Van Deynze located at the Department of Plant Sciences, College of Agricultural and Environmental Sciences, UC-Davis. At the end of the 1st year, we had 12 explants growing in late elongation step and 68 growing calluses. At least 4 out of the 12 explants were ready to go into rooting media. However, during the 2nd year it was reported to us that none of the explants was able to root, probably because they were not true stable transgenic plants. However, in collaboration with the Bocobza lab at the Plant Science Institute, which developed improved pepper transformation protocol, our CRISPR/Cas9 construct is at present being used to transform chili pepper cultivar and we hope to get transformants by the end of 2022.

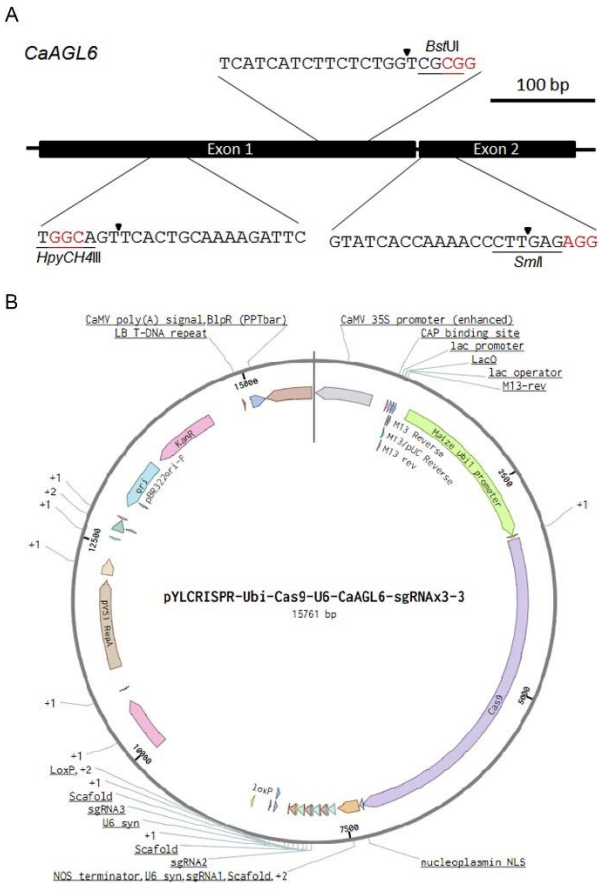


Figure 1. CRISPR/Cas9 knockout of *CaAGL6* gene. (A) Schematic illustration to scale of *CaAGL6* gene region targeted by the 3 gRNAs. Black bars indicate exons. The 1st intron (3252 bp) located between exon 1 and exon 2 is not shown. The gRNAs sequences are shown, the PAM motif sequences are colored in red, black triangles mark the predicted Cas9 cut sites and restriction enzymes recognition sites are underlined. (B) Schematic illustration of the pYLCRISPR-Cas9-3xU6:sgRNA CaAGL6 binary plasmid used for transformation.

Identification of CaAGL6 mutants via TILLING

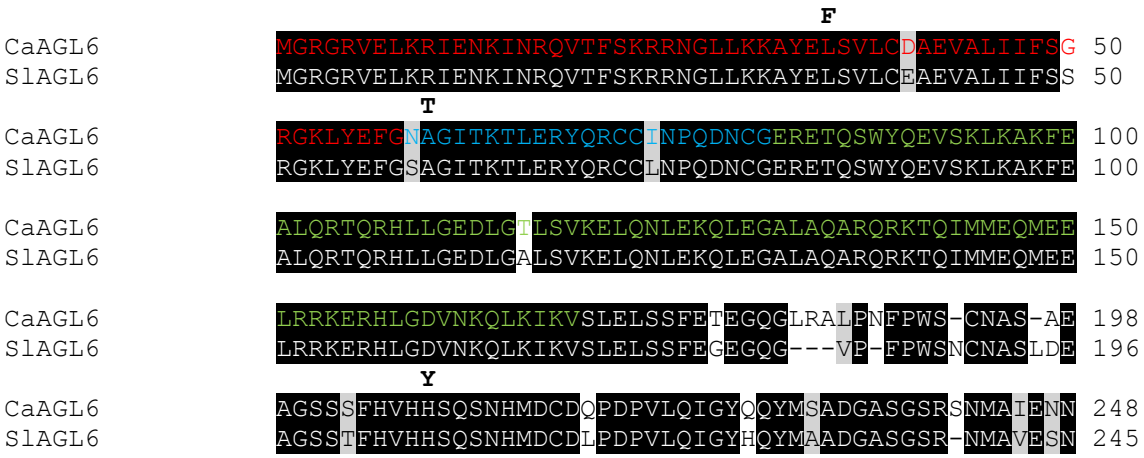
To identify EMS-induced knockout mutants at the *CaAGL6* locus, a pepper TILLING library of 3400 M2 lines (*C. annuum* blocky type cv. Fascinato) was screened by deep sequencing by

Platform Genetics Inc, Canada, following verification of specific mutations by PCR amplification and sequencing the regions containing the putative mutations. Six non-synonymous mutant M2 families were identified, all exhibiting mutations that resulted in changes in respective amino acids (missense mutations). The specific changes in each mutants are listed in Table 1. However, seeds could not recovered by the company for one mutant. In addition, seeds of mutant lines 1539 and 256 did not germinate at the ARO, and since no more seeds were available in the company, these mutant lines were lost. The remaining three mutant lines were further characterized. The 1223 mutant line harbored a single nucleotide substitution that resulted in the replacement of the highly conserved MADS-box DNA-binding domain residue L^{35} for F. The 2718 line harbored a single nucleotide substitution that resulted in the replacement of the intervening (I) domain A^{60} for T. The 357 line harbored a single nucleotide substitution that resulted in the replacement of the C-terminal domain residue H^{208} for Y (Figure 2).

Table 1: List of identified *Caagl6* TILLING mutants

Mutant line name	Position of mutation in *Caagl6* gene	Caagl6 Protein mutation	Restriction enzyme[a]	Status
1223	339	L35F	ApoI	Homozygous mutants available
1539	363	V43I	-	Identified mutant failed to propagate
2718	414	A60T	HpyCH4V	Homozygous mutants available
-	2870	R137K	-	No seeds available.
256	4651	R156H	-	Identified mutant failed to propagate
357	5043	H208Y	FokI	Homozygous mutants available

[a]Restriction enzymes used for the Restriction Fragment Length Polymorphism (RFLP) mutation markers.



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CaAGL6          IIHGWGL 255
SlAGL6          IIHGWGL 252
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Figure 2. Amino acid sequence alignment of tomato AGL6 (SlAGl6) and its pepper homolog (CaAGL6). The alignment was generated with the CLUSTALW program. The amino acid substitutions in 1223, 2718 and 357 mutant lines are indicated above the respective native amino acid residues. The predicted MADS, intervening (I), keratin-like (K) and C-terminal domains in the CaAGL6 protein are indicated by red, blue, green and white, respectively.

Transfer CaAGL6 mutations to Cv. Maor blocky cultivar

In order to characterize the ability of *Caagl6* EMS-mutants to set parthenocarpic fruits, F2 populations from crosses of mutant lines 1223, 2718 and 357 (Table 1) with Cv. Maor were constructed. Transfer of the three mutations to the background of the blocky-fruited Cv. Maor was done because of growth inhibition obtained at the background of the original parent likely as a result of unknown additional mutations in the background of the mutants, as well as poor fruit setting of the original parental line used for mutagenesis. At present an F3 generation was obtained for all single mutants.

Characterization of mutant lines fruits and seeds

The 1223, 2718 and 357 F2 mutant populations, which included 72, 120 and 90 individuals, respectively, were grown in the greenhouse in 5-liter pots in the Volcani Center. RFLP markers were developed for the three mutations based on the respective mutated SNPs and allowed to identify homozygous wild type and mutant progenies (Table 1). Eighteen,16 and 10 homozygous mutants as well as 16, 13 and 20 homozygous wild-types were identified among the 1223, 357 and 2718 F2 individuals, respectively. The mutant and wild-type groups were tested for parthenocarpy as following. For each group, at least 20 flowers were emasculated, by removing the anthers at anthesis before flower opening, thus preventing spontaneous self-pollination. Fruit development was followed starting one week after anthesis. In addition, seedless fruit formation was also monitored.

For populations 1223, 2718 and 357, all the emasculated flowers in both mutant and wild type groups were aborted within one week after pollination. Mutant as well as wild-type fruits that developed from intact non-emasculated flowers had similar morphology, were seeded and reached full size at maturity (Figure 3). Occasionally in both wild-type and mutant plants seedless parthenocarpic fruit were obtained and as expected were smaller and distorted than the seeded fruits (Figure 3). The F3 seeds of both mutants were collected to further multiply and fix the three

mutations. For all mutant populations no difference in seeded fruit weight and morphology were observed between mutant and wild type genotypes (data not shown). However, analysis of their seeds revealed significantly smaller seed area in all mutants (Figure 4A) and accordingly the seed weight of 2718 and 1223 mutant seeds were smaller compared to wild type (Figure 4B). Therefore, although the CaAGL6 mutations did not cause fruit parthenocarpy, seed development seemed to be affected in 2718 and 1223 lines.

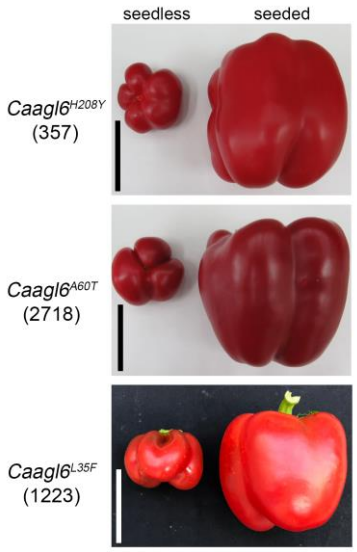

Figure 3. Characterization of *Casgl6* mutants fruits. Pictures of representative seedless and seeded fruits of indicated mutant F2 individuals (line # and name are indicated). The seedless fruits were obtained from unfertilized ovaries, whereas the seeded fruits were obtained following fertilization. Scale bars = 5 cm.

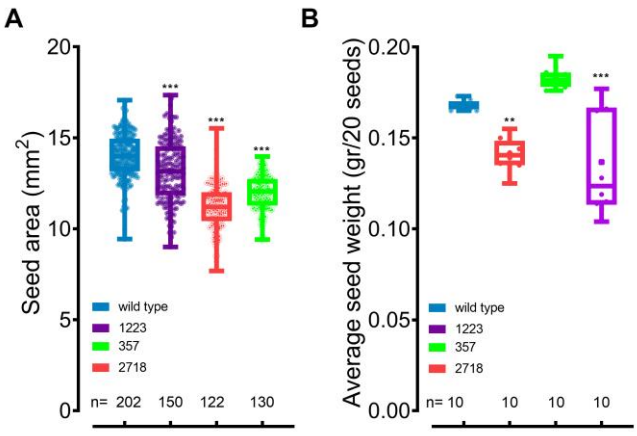

Figure 4. Characterization of *Casgl6* mutants seeds. Measurements of seed area (A) and seed weight (B) in wild-type and indicated mutant seeds. Box center line, median; box limits, upper and lower quartiles; whiskers, min and max values; points, individual values; n = number of individual seeds (A) or groups of randomly selected 20 seeds (B). Asterisks indicate significant difference from wild type (*P*<0.0001 ***, *P*<0.05**) as determined by One-way Anova.

Discussion:

Fruit set, the first step of fruit development, is one of the key determinants of the final yield in all fruit crops and hence of agronomic and economic importance. It was suggested that the development of an unfertilized anthesis ovary into a fruit is actively repressed to prevent parthenocarpic fruit set that is futile and would be a waste of energy to the plant (Vivian-Smith et al., 2001; Medina et al., 2013). Nevertheless, the *slagl6* loss-of-function mutant (*slagl6*$^{CR\text{-}sg1}$) is capable of fertilization-independent setting of normal, yet seedless (parthenocarpic) fruits (Klap et al., 2017; Gupta et al., 2021). This unique parthenocarpic phenotype allows the *slagl6*$^{CR\text{-}sg1}$ plants to set fruits even in conditions that are unfavorable for pollination such as extreme temperatures.

Pepper is an economically important crop in Israel and abroad. To improve pepper fruit set and yield under adverse temperatures, in this study an attempt to generate a loss-of-function mutant of *CaAGL6* gene, that encodes the pepper homolog of SlAGL6, was taken. Pepper species are recalcitrant to genetic transformation by Agrobacterium. Therefore, in addition to taking the transgenic approach to knockout the *CaAGL6* gene via CRISPR/Cas9 gene editing, we have screened a pepper TILLING library to identify *CaAGL6* EMS mutants. At present we report on failure to knockout the *CaAGL6* gene via transgenic expression of CRISPR/Cas9 in pepper. Unfortunately, transformation of pepper remains a major obstacle for use in research and breeding and to date no breakthrough in this field have been reported. Nevertheless, efforts in the Institute of Plant Science led by Dr. Samuel Bocobza are underway to advance this field as part of the genome editing center. At present the Bocobza lab use the CRISPR/Cas9 construct that was generated in this study to transform chili pepper with the hope to isolate *CaAGL6* KO mutants. With regard to the TILLING approach, only missense mutations that cause amino acid replacements rather than stop codon were identified by the TILLING screening. The reason for that is unknown, but notably, screening the same TILLING population for mutations in 4 other genes identified in all missense as well as nonsense mutations.

The *CaAGL6* gene encodes for MIKC^{C} type II MADS-box protein and as such is composed of MADS (M), intervening (I), keratin-like (K) and C-terminal domains (Kaufmann et al., 2005). Seed plants MIKC^{C} group proteins function as developmental master regulators by repressing or activating target genes (Smaczniak et al., 2012; Schilling et al., 2018). This is done by binding target genes regulatory DNA through the highly conserved MADS domain that

recognizes a canonical DNA-binding motif called CArG-box (Aerts et al., 2018). The IKC domains are plant specific and hence are less-conserved than the MADS domain (Kaufmann et al., 2005). Our TILLING screening identified a total of six mutations that lead to amino acid substitutions in the conserved M (2 mutations), I (1 mutation), K (2 mutations) and C-terminal (1 mutation) domains. Out of the six TILLING mutants identified, only three were recovered. The L35F mutation replaced the MADS domain highly conserved hydrophobic L by the hydrophobic aromatic F thus increased the hydrophobicity and volume of the amino acid at that position. The A60T and H208Y lead to changes in the less conserved I and C-terminal domains, respectively. However, both positions are conserved in *SlAGL6* and the replacements changes the amino acid side chain charge. Thus, we hoped that identified mutations will modify the functionality of the CaAGL6 protein, resulting in complete or partial loss of its activity. In the latter case a parthenocarpic phenotype was not expected since even 90% drop in *SlGAL6* expression does not allow parthenocarpy (Yu et al., 2017).

Following the stabilization and cleaning of the TILLING mutants from unknown EMS mutations by crossing to the Cv. MAOR background, F2 populations were generated from all the available mutants and RFLP markers that can identify the respective mutant SNPs were developed (Table 1). In addition, we tested the 1223, 2718 and 357 F2 mutant individuals for facultative parthenocarpy following flower emasculation, namely for the ability to set fruit without fertilization, similar to the tomato *slagl6* mutant (Klap et al., 2017). In all mutant lines, we observed no seedless fruit set following emasculation or among fruits which set from intact flowers, indicating that 1223, 2718 and 357 mutants do not possess an ability to set parthenocarpic fruits. This strongly suggests that the respective Caagl6^{L35F}, Caagl6^{A60T}, Caagl6^{H208Y} mutant proteins most likely did not lose substantial functionality, and therefore are still able to suppress fruit set in the absence of fertilization. Because lack of parthenocarpic fruit set ability in the experiments in the Volcani Center, we decided that testing fruit set in the more extreme environment in the Arava as originally planned will not be carried out. In tomato *SlAGL6* is expressed in young developing seed tissues, suggesting that it is involved in seed development. Our data indicate that 1223 and 2178 mutants have modified seed characteristics suggesting that the CaAGL6 protein may also be involved in seed development and that the respective L35F and A60T mutations may partially compromise its activity (Klap et al., 2017; Gupta et al., 2021).

Appendix:

Publications resulting from the study:

none

Confidentiality report:

Since this is a project aimed at creating agricultural products protected by a patent, we are interested in the confidentiality of the report.

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