# TEKNOFEST

## AEROSPACE AND TECHNOLOGY FESTIVAL

## BIYOTECHNOLOGY COMPETITION

## PROJECT DETAIL REPORT

### TEAM NAME

**Genetic Reprogrammers**

### PROJECT NAME

**Multiplexed CRISPR-Act2.0 technology in plant**

### APPLICATION ID

464247

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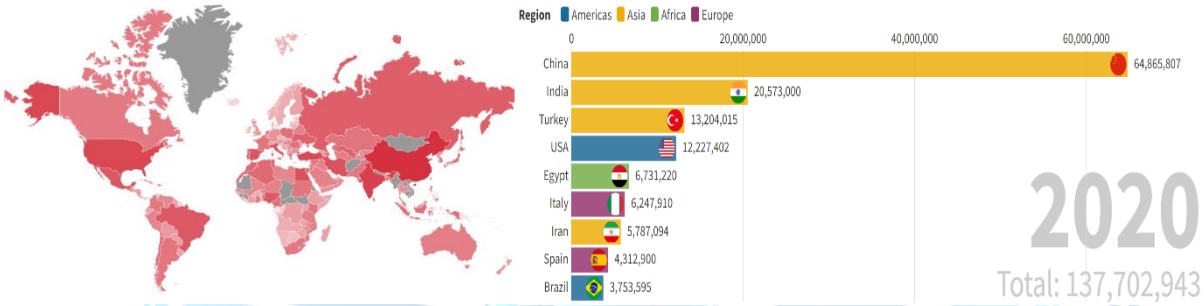
**Project Summary:**

A- The economic value of tomatoes

Tomato (*Solanum Lycopersicon L.*) is among the world's major vegetable crops, and it is grown in almost every country in the world (Figure 1) [1]. In 2020, the worldwide production of tomatoes totaled approximately 186 million tons. Tomato is regarded as one of Turkey's most valuable growing vegetable crops, with a production of over 13 million tons per year in 2020. Turkey ranks in the third-largest tomato producer globally after China and India (Figure 1). Egypt ranks the fifth largest tomato producer globally, with approximately 6 million tons per year (Figure 1). China, India, and Turkey produce together more than 50% of the world's total tomatoes globally [2]. Tomatoes have been linked with numerous medical benefits, including chronic disease prevention [3].

**Figure 1**: Map of Countries by Tomato Production [4] .




B- Tomato crop threats

Talking about tomato challenges is a vast and complex subject; tomato production faces unprecedented global challenges. The absence of high-quality seeds, labor, and understanding of best agronomic procedures, the high cost of agricultural inputs and price variations, weather limits, and the critical problem of insect pests and illnesses are crucial constraints in tomato production [5, 6]. Plant-parasitic nematodes are a critical biological limitation to tomato output among insect pests [7].

C- CRISPR

The crucial to a wealthy agricultural production is to increase genetic variation in plant crops [8]. In the last decade, novel technology has evolved that allows researchers to directly control practically every gene in a wide range of cell types and organisms. The CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats)/CRISPR-associated (Cas) system has lately emerged as a tremendous simple, efficient, and cheap alternative to ZFNs and TALENs, which has been frustrated by several factors [9, 10].

**Problem:**

Farther than 4100 species of Plant-parasitic nematodes (PPNs) are of considerable economic burden on a great diversity of plants and crops worldwide. The activities of phytoparasitic nematodes can harm any crops due to directly targeting plant roots and limiting water and nutrient uptake, resulting in significant economic losses, including tomato, soybean, potatoes, sugar cane, sugar beets, bananas, and coconuts (Figure 2). In addition to the direct damage caused to the plants, nematode infection facilitates subsequent attacks by other plant pathogens such as bacteria and fungi [11]. According to the American Society of Phytopathology (APS) PPNs are counted to reduce crop yield by 14% globally each year, which is nearly $125 billion annually. Other studies illustrated annual economic losses due to nematode infection of crops have been estimated at $173 billion, with $13 billion in the United States [12]. Among them, root-knot and cyst nematodes are the most important ones. We have recently noticed a great interest in research to solve the nematode problem, and the number of articles has remarkably increased over the last 30 years (Figure 3) [13]. This shows us that we are facing a dissimilar problem that could threaten the food security of the entire world.

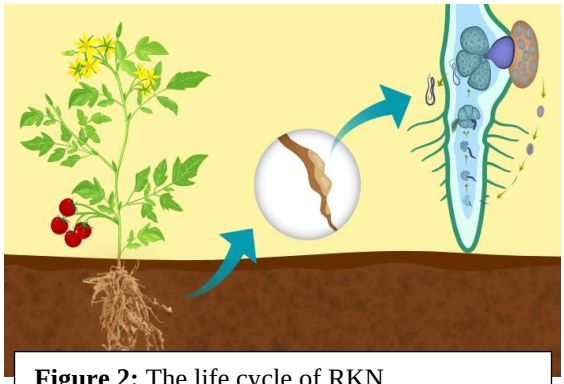
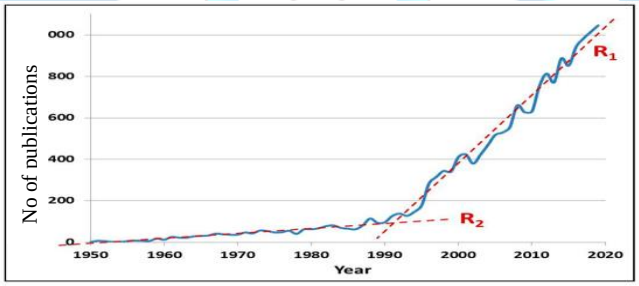


**Figure 2:** The life cycle of RKN.

**Figure 3:** From 1950 to 2019, the annual number of publications on nematodes and plants has expanded [13].




Mesa-Valle, Garrido-Cardenas [13] illustrated that at the 160 most common keywords in nematode and plant articles, the six crops that attract the most attention in these publications appear in these keywords: **tomato,** soybean, potato, Arabidopsis, corn, and wheat. Figure 4 depicts the results of an analysis of all of them based on the country in which the works appeared to be published. **<u>Unfortunately, turkey is not among these countries.</u>**

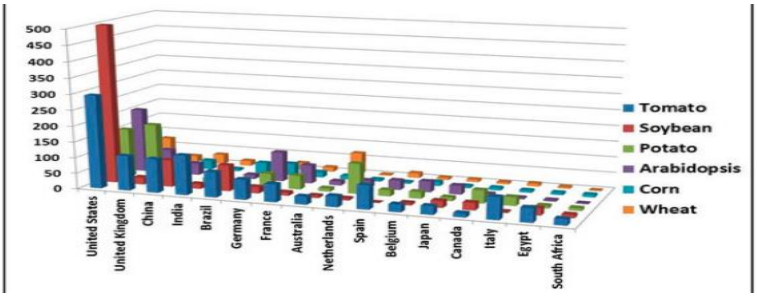


**Figure 4:** Relationship between crops and the countries with the highest scientific production of nematodes + plants [13]**.**

**Solution:**

Plants naturally resistant to PPNs suppress nematode reproduction and reduce M. incognita damage. Plants secrete a complex mixture of water-soluble and volatile organic compounds (VOCs) into the soil, collectively termed plant root exudate. Our study focuses on increasing the amount of other similar derivative fatty acids. Especially hexadecanoic (palmitic acid) in the roots of the tomato plant by using Crispr-dcas9 technology and understanding the mechanical intricacies of how palmitic acid has a crucial role in working as a repellent to root-knot nematode. Acyl-acyl carrier protein (ACP) thioesterases play an essential role in chain termination during *de novo* fatty acid synthesis and channel carbon flux between the two lipid biosynthesis pathways in plants. We discovered two distinct but related thioesterase gene classes in higher plants, FATA and FATB, whose evolutionary divergence appears to be ancient [14]. FATA encodes the already described 18:1-ACP thioesterase. In contrast, FATB representatives encode thioesterases preferring acyl-ACPs having saturated acyl groups. There are FATA (Solyc06g083380,) and FATB (Solyc03g097390 (1), Solyc05g008570 (2), and, Solyc12g006930 (3) genes encoding acyl-carrier enzymes in fatty acid synthesis in tomato plant (https://phytozome.jgi.doe.gov). While FATA takes part in the formation of hexadecenoic, hexadecanoic, octadecanoic and octadecanoic acids, FATB takes part in the formation of octanoic, decanoic, dodecanoic, tetradecanoic acids (https://www.genome.jp). For this purpose, the expression of four genes responsible for synthesizing palmitic acid and its derivatives in the tomato plant will be increased by both the classical overexpression method and CRISPR-dCas9 technology. The Visualization of the assumed a "plant electronic fluorescent pictograph" browsers for exploring the putative tissue expression of *FATA (Solyc06g083380) and FATB (Solyc03g097390, Solyc12g006930, and Solyc05g008570)* genes, based on the expression of tomato genes and protein localization in various tissues and developmental phases Figure 5 [15]

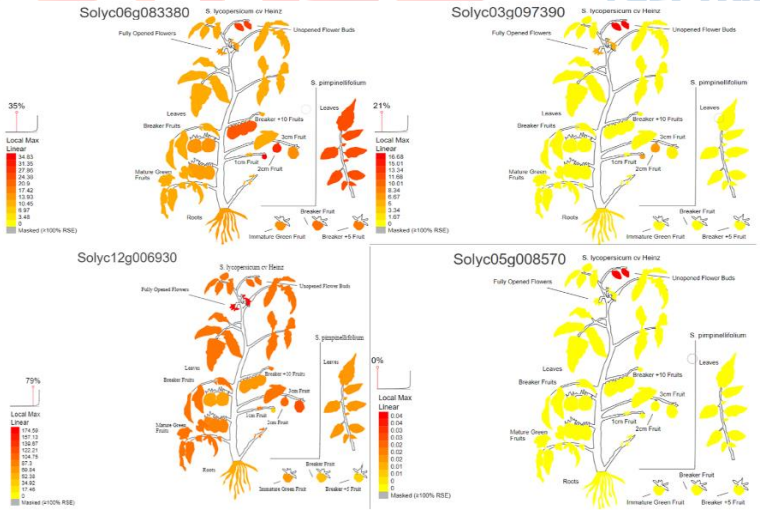



**Figure 5:** Exploring the putative tissue expression for our genes**.**

CRISPR systems have extensively promoted research in genome editing and transcriptional regulation. Several strategies for dCas9-based transcriptional activation are used, and its found that simultaneous recruitment of VP64 by dCas9 and a modified guide RNA scaffold gRNA2.0 (designated CRISPR-Act2.0) yielded more robust transcriptional activation [16]. Finally, determining the effects of transgenic lines with increased palmitic acid content on beneficial bacteria that promote plant growth.

**Material and methods:**

This work to be done; (A) Increasing the expression of four genes responsible for the production of palmitic acid and its derivatives in the fatty acid pathway by using the CRISPR-Act2.0 system and the classical gene overexpression method, (B) Determining the resistance of the obtained transgenic lines in the plant by evaluating the root-knot nematode-removing, lethal and egg-inhibiting effects of the root secretions.

A- Increasing Palmitic Acid Content Using CRISPRAct2.0 System and Classical Overexpression and Gene Transfer Method.

1- Plant material and tissue culture medium:

As plant material, Bobcat tomato variety sensitive to root-knot nematode will be used in gene transfer studies, and this variety will be obtained from Sygenta company (https://www.syngenta.com.tr/Bobcat). The CRISPR/Cas9 system and Agrobacterium-mediated gene transfer method to be used in this study have already been optimized in our laboratory before [17]. In this context, we followed the mentioned protocol for the cloning and agrobacterium mediated transformation in this study.

2- Bacterial strains, media, and plasmids that will be used:

In gene transfer studies, *Escherichia coli* (Dh5α) and *Agrobacterium tumefaciens* (GV3101) bacterial strains and LB long with SOC media will be used as growth media. The plant optimized over-expression vector pIPKb004, will be used in the classical overexpression step of the study, this is available in our laboratory. CRISPRAct2.0 plasmids to be used in Crispr-activation studies will be obtained from Addgene, and vectors containing root-specific promoter *S. lycopersicum RIBOSOMAL PROTEIN L11C* (SlRPL11C) will be obtained from gateway vectors. The plasmids to be used are shown in Table 1.

| Name of the Plasmid | | | | |
|---|---|---|---|---|
| pYPQ131A | pYPQ135A | pYPQ141 | pYPQ145 | pYPQ173 |
| pYPQ132A | pYPQ136A | pYPQ142 | pYPQ146 | modified pIPKb004 |
| pYPQ133A | pYPQ137A | pYPQ143 | pYPQ147 | |
| pYPQ134A | pYPQ138A | pYPQ144 | pYPQ148 | |

**Table 1. Plasmids to be used in the study and obtained from Addgene.**

3- Designing of gRNA

Fatty acyl-ACP thioesterase A (FATA) (Solyc06g083380.2), which encodes the fatty acyl-ACP thioesterase [EC:3.1.2.14] enzyme responsible for producing tetradecanoic acid, hexadecanoic acid and octadecanoic acid, which are involved in fatty acid synthesis metabolism. gRNAs will be designed to increase the expression of four genes Carrier Protein Thioesterase (Solyc12g006930.1), Fatty acyl-ACP thioesterase B (FATB) (Solyc03g097390.2), Fatty Acyl-

ACP thioesterase B (Solyc05g008570.1). The sequence of the relevant genes was obtained from the Phytosome v13.1 database. The -5 to -350 bp upstream region containing the promoter region from the transcriptional start site will be used to design gRNAs. -5 to -350 bp upstream region of each gene obtained from the phytozome database will be designed using the CRISPR-P 2.0 program (http://crispr.hzau.edu.cn/CRISPR2/). For each gene, 4 gRNAs will be created and gRNAs with a length of 19-20 nucleotides will be added to the 5' end of the forward sequences, GATT, and AAAC restriction cut sites will be added to the 5'ends of the reverse sequences and synthesized as primers.

4- Generation of plant expression vectors:

To increase the expression of these target genes in a tomato plant, classical gene transfer is frequently used with Agrobacterium-mediated direct transfer of genes directed by the constitutive promoter 35S. However, undesirable results may occur as the expression of some structural genes due to random placement in the transferred genome. At the same time, it is very difficult to increase the expression of more than one gene using a single plasmid with this method. Therefore, with this project, the CRISPRAct2.0 system will be used to increase the expression of four genes involved in fatty acid synthesis metabolism. To determine the success of the CRISPRAct2.0 system, classical gene transfer will be performed, and the results will be compared.

*4.1 Construction of the Plant Expression Vector (pIPKb004) to be Used in Classical Overexpression Gene Transfer*

4.1.1 Cloning of palmitic acid-encoding genes from tomato plant

To overexpress these genes to be cloned, the tomato plant will be exposed to root-knot nematode and a sample will be taken for RNA isolation. The samples taken will be quickly frozen in Nitrogen and stored at -80 °C.

I. RNA isolation: Total RNA will be isolated using RNeasy Plant Mini Kit from the tomato plant. Total RNAs isolated will be quantified using NanoDrop 2000 UV-VIS spectrophotometer, and quality analyzed using Agilent 2100 Bioanalyzer.

II. cDNA synthesis: cDNA will be synthesized from total RNA treated with 1 µg DNAse I in a reaction volume of 20 µl with the RevertAid™ First Strand cDNA synthesis kit following kit protocol.

III. PCR-assisted cloning of genes: Primers will be designed for each gene using the primer blast program. The CACC region compatible with the entry vector in the Gateway cloning system will be added to the 5' end of the forward primer. The cDNA will be used as a template and amplified by PCR using forward and reverse primers designed specifically for each gene. Before starting the PCR process, the binding temperature (Tm value) of the primers explicitly designed for the genes will be calculated using the NEB Tm Calculator tool. Standard protocol and components recommended by NEB Q5 will be used for PCR studies.

4.1.2 Cloning of Genes into Plant Expression Vector

Since our plant expression vector to be used in the study is suitable for the Gateway cloning method, Gateway cloning method will be used for the cloning of each gene for overexpression of genes. First, competent E. coli cells will be prepared, and then the relevant genes will be individually cloned into the pENTR™/D-TOPO vector to confirm the gene by sequencing and transferred to the plant expression vector.

1- **Cloning of Genes into the Input Vector (pENTR™/D-TOPO):** PCR products of each gene from the agarose gel amplified by PCR will be inserted into the entry vector using the pENTR™/D-TOPO Cloning Kit following manufacturer's instructions (Figure 6). The produced cloning will be transformed into competent E. coli bacteria using heat-shock method. Colony PCR will be performed using gene-specific primers from the colonies obtained after 17 hours. The resulting plasmids will be sent for sequencing with the M13 primer. Fw. Sanger sequencing will be performed by the relevant company using M13 F: GTAAAACGACGGCCAG and M13 R: CAGGAAACAGCTATGAC primers.

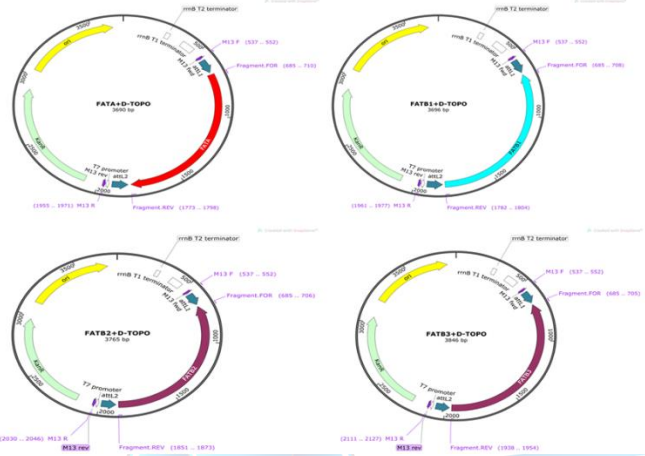



**Figure 6:** pENTR™/D-TOPO entry vectors containing cloned genes (created by snapgene software)**.**

2- **Cloning of genes into plant expression vector (pIPKb004)**: With Gateway® LR Clonase® II enzyme mix kit, they will be transferred to pIPKb004 vector containing two 35S promoters and selective antibiotic gene HPTII (hygromycin). The pIPKb004 plant will be transferred to the overexpression vector and thus, the final vector will be created (Figure 7).

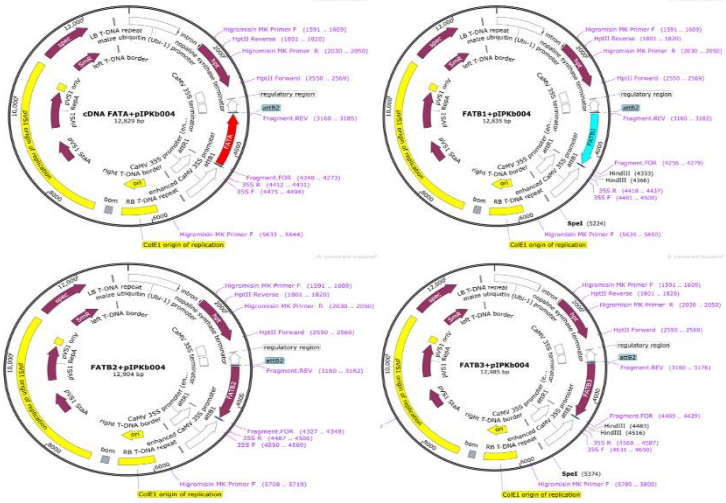



**Figure 7**. Plant expression vector created because of LR clonase enzyme (created by snapgene software).

4.2. Construction of the CRISPR-Act2.0 plant expression vector (Modified-pIPKb004)

In this study, four genes with accession numbers Solyc06g083380.2, Solyc12g006930.1, Solyc03g097390.2 and Solyc05g008570.1, which are responsible for the production of palmitic acid and its derivatives using the CRISPR-Act2.0 system, will be investigated as single, double, triple and four genes simultaneously. Overexpression will be provided in different combinations. For this purpose, firstly, 4 gRNAs will be designed for each gene and these gRNAs will be transformed into tomato plants one by one using the CRISPR-Act2.0 system. Whether there is an increase in the expression levels of these four genes, whose expression is desired to be increased in the obtained genome-processed plants, will be determined by qPCR and two gRNAs that work best will be selected for each gene. Then, using the selected gRNAs, CRISPR-Act2.0 vectors consisting of various combinations in Table 2 will be developed.

**Table 2.** Plasmid constructs to be formed in the CRISPR-Act2.0 system to ensure overexpression of genes in combination**.**

| Vector combinations | Solyc06g083380.2 | Solyc12g006930.1 | Solyc03g097390.2 | Solyc05g008570.1 | Number of gRNAs in vector/2 for each gene (confirmed to be working)) |
|---|---|---|---|---|---|
| Construct 1 | + | | | | 2 |
| Construct 2 | | + | | | 2 |
| Construct 3 | | | + | | 2 |
| Construct 4 | | | | + | 2 |
| Construct 5 | + | + | | | 4 |
| Construct 6 | + | | + | | 4 |
| Construct 7 | + | | | + | 4 |
| Construct 8 | | + | + | | 4 |
| Construct 9 | | + | | + | 4 |
| Construct 10 | | | + | + | 4 |
| Construct 11 | + | + | + | | 6 |
| Construct 12 | + | + | | + | 6 |
| Construct 13 | | + | + | + | 6 |
| Construct 14 | + | | + | + | 6 |
| Construct 15 | + | + | + | + | 8 |

4.2.1. Cloning of the *S. lycopersicum RIBOSOMAL PROTEIN L11C* (*SlRPL11C*) promoter into the pIPKb004 Plant expression vector:

The modified pIPKb004 vector created by Tang, Lowder [18] drives the pco-dCas9-VP64-T2A-MS2-VP64 gene with the AtUBQ10 promoter. This non-tissue-specific promoter sequence will be replaced by the root-specific promoter (*SlRPL11C)* from Tomato's DNA [19]. First, digestion will be performed with the AtUBQ10 promoter *HindIII* and *AscI* restriction enzymes in the modified pIPKb004 vector and the cut plasmid will be isolated from the gel. The In-Fusion® HD Cloning Kit will be used to clone the root-specific promoter (*SlRPL11C)* from Tomato's DNA. First, primers specific to the *SlRPL11C* promoter will be designed using Takara primer design tools. PCR studies using these primers will be performed with CloneAmp HiFi PCR Premix (Takara). The resulting bands will be cut from the gel and isolated with the NucleoSpin® Gel Extraction Kit. The concentration of the cut product will then be measured with the NanoDrop spectrophotometer. In-Fusion® HD Cloning Kit (TAKARA) will be used for ligation.

4.2.2. Construction of the CRISPR-Act2.0 T-DNA vector (pIPKb004) with gRNAs

Single and multiple gRNA cloning will be performed by modifying the Lowder, Zhou [20] (2018) protocol.

I. **Linearization of gRNA expression vectors (pYPQ131A, pYPQ132A, pYPQ133A-pYPQ138A):** gRNA expression vectors will first be digested at 37 °C for 3 hours using *BglII* and *SalI* enzymes. Then, the products will be purified using the

"NucleoSpin® Gel and PCR Clean-up" (MG) kit, and these products will be cut a second time by preparing the reaction. with the 2nd Esp3I (*BsmBI*) enzyme. After cutting at 37 °C, 6 hours, the enzymes will be inactivated at 80 °C for 20 minutes, the products will be purified using the 'NucleoSpin® Gel and PCR Clean-up' (MG) kit, and the DNA concentration will be measured using Nanodrop.

II. **Conversion of single-stranded sgRNAs to double-stranded:**
The gRNAs designed as complementary to the targeted promoter region will be synthesized as a single-stranded primer. These primers must be made double-stranded in order to be cloned into a double-stranded plasmid. The sgRNAs synthesized by the relevant company will be dissolved in 100 µM de-ionized water and 100 µmol/l forward gRNA, reverse gRNA primer and T4 Polynucleotide Kinase to obtain double strand as described [16] . Incubate reactions at room temperature for 1 h to overnight.

III. **Ligation of double-stranded gRNAs to the gRNA expression vector:**
As the sgRNA oligonucleotides (DNA oligos) are being prepared, linearize sgRNA entry plasmids pYPQ141B2.0 for single gene activation in dicots, using BsmBI (Eps3I). Following the recommended protocol for BsmBI as described at [16], digest 2 µg plasmids at 37°C for 3 hr in a heating block. Run agarose gel electrophoresis and clean up digested plasmids with "NucleoSpin® Gel and PCR Clean-up" (MG) kit. The double-stranded gRNAs will be ligated into linearized gRNA expression vectors by creating the reaction mixture [16]. The products will be incubated in the PCR device for 30 minutes at 16 °C and then incubated overnight at 4 °C in the refrigerator. Ligation products will be transferred to competent E.coli Dh5α bacteria by standard heat shock method and plasmids in the obtained colonies will be confirmed by Sanger sequencing.

**IV. Establishment of the golden gate cloning reaction:**
After the gRNAs are cloned individually into the gRNA expression vector, any of the pYPQ14 modules will be selected considering the number of gRNAs to be cloned. For example, if it is desired to clone 2 gRNAs, pYPQ142 will be selected, and if it is desired to clone three, pYPQ143 module will be selected. Once the appropriate vector has been selected, the components and conditions in the golden reaction table 3 will be applied.

**Table 3. Establishment of the golden gate reaction**

| Components | Volume (µl) | Cycle conditions |
|---|---|---|
| pYPQ131-gRNA1 (100 ng/ µl) | 1 | 37°C for 5 m<br>16°C for 10 m<br>20 After cycle<br>80°C for 10 m |
| pYPQ132-gRNA2 (100 ng/ µl) | 1 | |
| pYPQ133-gRNA3 (100 ng/ µl) | 1 | |
| pYPQ143 (100 ng/ µl) | 1 | |
| 10× T4 DNA Ligase Buffer (NEB) | 1.5 | |
| 10× BSA | 1.5 | |
| BsaI (NEB) | 1 | |
| T4 DNA Ligase (HC, NEB) | 1 | |
| ddH2O | 1 | |
| Total | 10 | |

After the cloning phase is completed, the vector obtained will be transformed into competent E.Coli Dh5α bacteria by heat-shock method. Bacteria obtained after transformation will be spread on solid LB medium containing 100 mg/L spectenomycin, and white colonies will be selected from the colonies to be obtained after incubation at 37°C for 17 hours. Colony PCR will be performed using appropriate primers to identify the correct clones. To isolate plasmids from colonies determined by PCR, bacteria will be grown in 10 ml liquid LB nutrient medium to which 100 mg/L Kanamycin has been added, in an incubator at 37 °C 200 rpm for 1 night. GeneJET Plasmid Miniprep kit will be used for plasmid isolation and the protocol of the relevant company will be followed. The presence and concentration of plasmids will be measured with the NanoDrop™ 2000/2000c Spectrophotometer. Then, after checking the plasmids by running on 0.4% agarose gel, the cloning accuracy will be verified by cutting the restriction endonuclease.

V. **Cloning of Multiplex CRISPR-Cas9 system into modified pIPKb004 Plant expression vector:** After gRNAs are cloned into the entry vector pYPQ143 vector, the pIPKb004 vector, which is the plant expression vector and whose promoter has been modified, will be combined with Gateway cloning method. This process will be performed using the Gateway™ LR Clonase™ II Enzyme mix with the protocol recommended by the manufacturer. Thus, the CRISPR-Act2.0 Plant expression vector will be created. After the completion of the LR reaction step, the vector will be transformed into competent E.Coli Dh5α bacteria by heat-shock method. Colony PCR will be performed using the appropriate primers to identify the correct clones. In order to isolate plasmids from colonies determined by PCR, bacteria will be grown in 10 ml liquid LB nutrient medium to which 50 mg/L Kanamycin has been added, in an incubator at 37 °C 200 rpm for 1 night. GeneJET Plasmid Miniprep kit will be used for plasmid isolation and the protocol of the relevant company will be followed. The presence and concentration of plasmids will be measured with the NanoDrop™ 2000/2000c Spectrophotometer. Then, sanger sequencing will be performed after checking the plasmids by running on 0.4% agarose gel.

5- Transfer of plasmids to competent *Agrobacterium tumefaciens* GV3101

A total of 15 CRISPR-Act2.0 plasmids (pIPKb004) shown in table 2 including the *Solyc06g083380.2*, *Solyc12g006930.1*, *Solyc03g097390.2*, and *Solyc05g008570.1* genes confirmed by sequencing, and individual genes that inserted into classical overexpression vector (pIPKb004 + Solyc06g083380.2, pIPKb004 + Solyc12g006930.1, pIPKb004 + Solyc03g097390.2 and pIPKb004 + Solyc05g0085.1) will be transferred into the empty *Agrobacterium tumefaciens* by electroporation method [17].

6- Plant transformation mediated by Agrobacterium tumefaciens

Bobcat tomato variety will be used for gene transfer studies. *Agrobacterium tumefaciens* will be grown in 50 ml liquid LB medium containing gentamicin (30 mg/L), kanamycin (50 mg/L) and rifampicin (10 mg/L) antibiotics at 28°C at 200 rpm for 1 day. Then, 8-day-old tomato cotyledon leaves germinated in MS growth medium (MS (2.2 g/L) + Sucrose (30 g/L) + Phytagel (2.8 g) pH:5.8) in vitro for 10 days will be used for transformation. Plant transformation will be done following our previously optimized protocol [17].

7- Control of hypothetical T1 Transgenic plants

Putative T1 transgenic lines will first be determined whether they are transgenic or not by confirming the presence of the Cas9 and hygromycin genes by PCR. Specific primers for hygromycin and Cas9 genes will be used to control the transgenic lines obtained because of the transformation of 16 CRISPR-Act2.0 constructs. The control of T1 transgenic lines obtained as a result of the transformation of 5 constructs for classical gene transfer will be determined by the presence of the hygromycin gene. Wt plants will be used as a positive control. As a result of germination of T1 plants, genomic DNA will be isolated from plants by using the Favorgen plant genomic DNA isolation kit. Then, the PCR products obtained by performing PCR using the relevant primers will be run on 0.8% Agarose gel and the success of gene transfer will be checked. As a result of evaluating the gel images, at least five transgenic lines will be selected from each construct.

8- Growing and rooting of T1 transgenic plants

Transformation of a total of 21 (19 transgenic, 2 empty vector constructs) constructs will result in 105 hypothetical transgenic lines. A total of 440 plants will be grown, at least 5 transgenic lines for each construct and 4 plants each from WT. Plants will be transplanted into seedlings by planting in plastic pots (18 cm x 18 cm) filled with a steam-sterilized sand and topsoil mixture (1:2). All work will be carried out in a special plant growth room at 25 °C with a 16-hour day/8-hour dark photoperiod. For this study, 4 seedlings from each line and WT control plant will be grown, but then the root samples for qRT-PCR and the root secretions for GC-MS will be combined and analyzed. The seedlings transplanted into the soil will be grown for 4 weeks to obtain root secretion and the roots will be used for root secretion isolation. Root secretion isolation will be done according to Wang, Hu [21] (2005). Briefly, each transgenic line and WT plants will be carefully removed from the pot and gently washed. Then, 500 mL of sterile distilled water will be added to the 500 mL Erlenmeyer flask and incubated for 10 hours at a temperature of 23–25 °C. After 10 hours, distilled water will be taken and filtered with the help of filter paper. This process will be repeated five times, then the filtrate extract will be concentrated with a vacuum rotary evaporator before being transferred to a separatory funnel with an equivalent volume of absolute ether. After the ether fraction has been collected, the previous step will be repeated three times. Finally, the resulting ether fractions will be combined, concentrated with a vacuum rotary evaporator to a volume of 4 ml and then dried. A small amount of pure Na2SO4 will be added and the solutions will be allowed to naturally evaporate up to 2.0 ml in a drying cabinet at 25°C. The resulting root exudates will be used in GS-MS analysis, bacterial analyzes and in-vitro nematode studies. At the same time, root samples will be taken from these plants to be used in gene expression analysis by qRT-PCR. After these root tissues are washed, they will be frozen in liquid nitrogen and stored at -80 °C.

**9-** QPCR analysis and GS/MS analysis of T1 Transgenic plants

The gene expressions and palmitic acid contents of the transgenic lines obtained from different constructs due to qRT-PCR and GS/MS analyses will be compared by taking samples from all the plants mentioned in the above step. Of these transgenic lines generated with a total of 19 constructs (excluding 2 empty vectors), 3 lines with the highest gene expression (at least 2-fold increase relative to control) and the highest palmitic acid content (at least 20% more than control) will be selected. The best three lines (total of 64 lines; 19 transgenic x3, 2 empty vector

lines, 1 WT) chosen from each construct will then be used in in vivo root-knot nematode studies. In cases where qRT-PCR and GC-MS results are inconsistent, the highest GC-MS result will be accepted.

*9.1. qRT-PCR analyses:* High purity RNA samples will be used for qRT-PCR. The cDNA will be synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™), using 1 µg of RNA for each sample as a template. For expression analysis of target genes, primers will be designed to amplify a region of 100-200 bp in length using the Primer Blast program. To make sense of the differences that may occur during the analysis, qRT-PCR will be performed using three biological and two technical replicas of each plant. CFX96 system will be used as qRT-PCR device. The ef-1a (Elongation factor) gene, which was previously stated to be reliable in quantification studies and that there was no change in gene expression level in different stress conditions and periods in tobacco plants, will be used as a reference gene in the study [22]. The qRT-PCR conditions will be applied as follows: initial denaturation at 95°C for 12 minutes, denaturation at 95°C for 10 seconds at 45 cycles, amplification at 60°C for 20 seconds, elongation at 72°C for 20 seconds, and final As a result, the cooling step will be determined as 10 min at 40°C. Since SYBR GreenI technology will be used, the melting curve will be evaluated after amplification and the data in which only a single amplicon has been amplified will be evaluated. Relative expression calculations will be performed with the 2ΔΔCT formula. In expression analysis, WT plants will be used together with transgenic plants created with an empty vector.

*9.2. GC-MS analysis:* Chemical components of root exudates will be determined by 6890-5973N gas chromatography/mass spectrometry (Agilent). This process will be carried out in the central laboratory of our university in the form of service procurement. Samples will be passed through a 30m x 0.25 µm x 250 µm TG-5MS capillary column. The column temperature will be initially held at 50 °C for 4 minutes, then programmed to 250 °C at a 6 °C/min rate with a final hold time of 15 minutes. Helium as carrier gas at a linear rate of 1 ml · min-1; injection volume of 1 µL will be used. In electron impingement (El) mode, the ionization voltage and temperature will be set to 70 eV and 230 °C, respectively. The relative contents of the components, the names to be calculated by the resulting method, will be determined according to the standard map.

**Innovative Direction:**

**For the first time in the literature,** according to the literature reviews, the fact that there is no study carried out this way makes our study unique. The uniqueness of this study comes from using CRISPR activation system 2.0 specified to be expressed in root tissues only because of using root-specific promoter this will guarantee tomato fruits and vegetative parts of the plant be free of any unexpected disruption in their characteristic natural feature. Another exclusivity of our research is using two different combine dCas9 activators VP64 and VPR. Therefore, the potential of the study outputs to be published is high. Insecticides/nematicides are one option for control; however, they are harmful to the environment, and other options such as crop rotation have significant drawbacks. This scenario provides an opportunity to utilize technological advances like CRISPR-dcas9 to engineer resistance plants against these devastating parasites.

**Multiplexing of CRISPR** allows multiple genes to be targeted without significant additional effort. We will use the multiplexing transcriptional activation CRISPR-Act2.0 system to improve tomato plants' defense mechanism against nematodes by activating several genes. Using this system, we will target four different genes (FATA, FATB1, FATB2, and FATB3) at once and in various combinations.

It is noteworthy that CRISPR-Cas9 will lead to the production of non-genetically modified (Non-GMO) labeled crops with the required characteristic, which will help boost yield potential under both biotic and abiotic stress situations accompanied by overcoming international laws to prevent the circulation of genetically modified plants.

**Our study focuses not only on tomatoes or Nematoda, but our idea can be applied to numerous crops for several purposes**.

## Applicability

Even though many countries are working on the development of genome-edited crops for several years, only a few of countries clarified their opinion toward genome editing. In Canada and USA, especially in developing countries such as Pakistan and India, genome edited crops can easily be passed through their regulatory burdens. New Zealand and Europe are strict with their old GMO regulations. In our country, the regulation is same with Europe. With the prediction that genome-edited products will no longer be recognized as GMOs soon. The good example is the UK, after it left the European union, that is planning to change current rules so that gene-edited plants using CRISPR system are treated differently to GMOs.

Since nematodes cause significant crop production losses in our country and throughout the world, the tomato line to be developed as a result of this project will be very important both economically and nutritionally. Due to the GMO regulations in our country, even if our genome edited tomato variety is not commercially available inside our country, we expect it to be commercially sold in the near future, just like in the UK example. Considering the export potential, our genome-edited tomato variety can also be exported to countries where genome-edited crop is not restricted such as USA, Pakistan, India etc.

## Estimated Cost and Project Time Planning

The estimated amount of the project and the consumables to be used in the project are in the table 4 below.

**Table** 4. List of materials and estimated cost

| | **Piece** | **Avg. unit price (TL)** | **Total price (TL incl. KDV)** |
|---|---|---|---|
| **Consumable materials** | | | |
| Restriction enzymes. | 6 | 560 | 3360 |
| Plant material | 1 | 2800 | 2800 |
| Growth medium (plant and bacteria) | 11 | 4809.72 | 52906.92 |
| PCR and RT-qPCR materials | 6 | 1138.5 | 6831 |
| DNA and RNA isolation kits | 2 | 3174 | 6348 |
| Disposable lab equipment | 7 | 376.4 | 2634.8 |
| **Non-consumable materials** | | | 0 |
| Primers and vectors synthesis | 440 | 14 | 6160 |

| | | | |
|---|---|---|---|
| Sequencing services | 85 | 180 | 15300 |
| Plant growth chamber | 1 | 140345 | 140345 |
| Laminar flow cabin | 1 | 132075 | 132075 |
| **Total cost (TL including KDV)** | | | **368760.72** |

At the same time, the project schedule is summarized in figure 8.

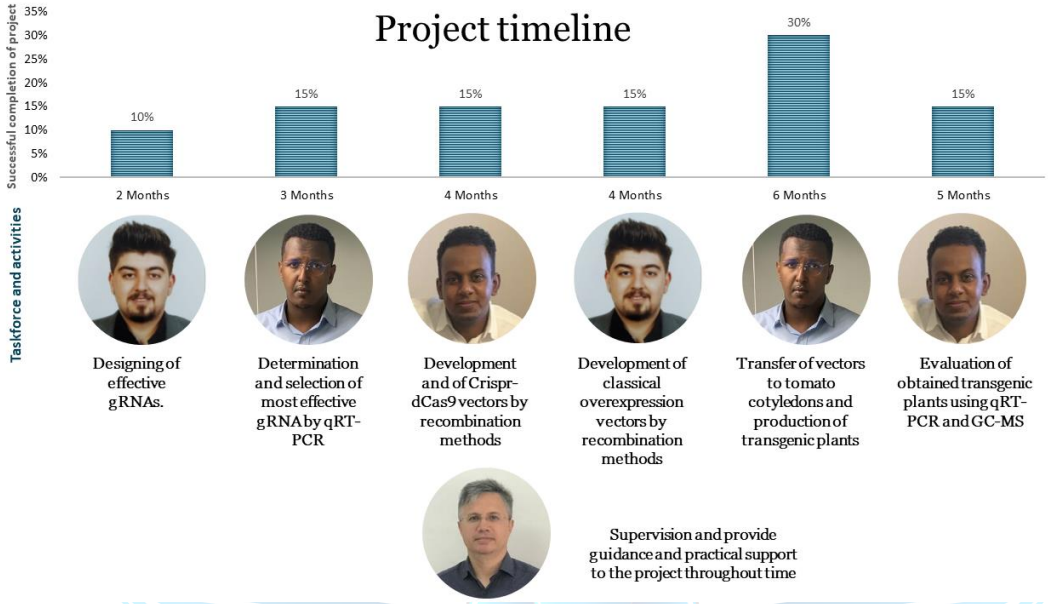



**Figure 8**: Timeline of project schedule and successful completion of project.

**Target Audience of the Project Idea (Users)**

Genome editing with CRISPR-Cas9 can be modified to edit any gene in any plant species. It allows faster genetic modification than other techniques due to its simplicity, efficiency, low cost and possibility of targeting more than one gene. The potential this represents for the development of sustainable agriculture and crop breeding is immeasurable. Genome-edited crops will be alternative genetic resources for breeders and farmers. Therefore, it can be easily applied in their conventional breeding process.

For farmer use, genome-edited crop by CRISPR technology perfectly fit into the enhancement of more sustainable agricultural systems. Through the development of genome edited crops with enhanced water use efficiency or with improved yield, this technology provides new ways to achieve a greater production of foods with a high level of non-renewable resource saving (such as energy, soil and water). Furthermore, numerous successful experiments have been reported to boost the tolerance to drought and heat stresses that undermine food security and reduce crop productivity. Concerning biotic stresses, genome-edited crops offer an opportunity to reduce the use of phytochemicals to protect the environment and save costs for farmers. About this project, our genome-edited tomato variety can be easily applied to nematode-infected soil. It is tough to control the nematode-infected soil with chemical pesticides, and there is a high risk of pesticide residue on the crops and soil. Therefore, our project does not

target breeders and farmers only but also targets both pesticide companies and seed companies as well that's at the local level. Additionally, our study also addresses developed countries and economically developing countries that suffer greatly from nematodes and the cultivation of genetically modified seeds is allowed.

**Risks**

The risks that may adversely affect the success of the project and the measures to be taken to ensure the successful execution of the project when these risks are encountered (Plan B) are outlined in the Risk Management Table 5 below by specifying the relevant work packages. Possible risks related to the research question and/or hypothesis of the project are taken into account. The implementation of plan B should not deviate from the core objectives and original value of the project. If there is a method change in case of switching to plan B, this situation should be detailed. Work packages for which no risk is foreseen are not included in this section.

**Table 5: Risk management table.**

| No | Definition of Risk (s) | Action(s) to be Taken (Plan B) |
|---|---|---|
| 1 | gRNAs not working | If the designed gRNAs do not work, new ones will be designed using other tools such as Crispor and Benchling and the experiment will be repeated. |
| 2 | Failure of regenerations to occur at the desired level | Transformation and regeneration studies of tomato plants have been carried out in our laboratory for about 3 years and we have a well-established regeneration and transformation protocol for the Bobcat variety. However, if the desired result is still not achieved, the Rio Grande variety, which is still being optimized, will be used in the experiments. |
| 3 | Not getting enough lines from each combination. | Transformation will be done again when the target of at least 5 lines from each transformation, which is our success criterion, is not achieved. |
| 4 | Absence of negative morpho- and physiological effects due to increased activation of more than one gene at the same time, although it has not been seen in the literature. | Combinations in which these adverse effects are observed will be excluded from the study. |
| 5 | Primers not working in the qRT-PCR stage<br>Failure of the reference gene used | Primers will be redesigned and synthesized.<br>A new reference gene will be identified. |
| 4 | Failure to provide sufficient fraction in GC-MS analyzes | Studies will be repeated with another column |

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