

University of Tartu

FACULTY OF SCIENCE AND TECHNOLOGY

INSTITUTE OF MOLECULAR AND CELL BIOLOGY

Chair of biophysics and plant physiology

Using CRISPR/cas9 to verify the cause of conditional sterility in *Arabidopsis thaliana*
harbouring a mutated MPK12

Bachelor Thesis

Karl Mäll

12 EAP

Supervisor Associate professor Yuh-Shuh
Wang

Co-supervisor Junior research fellow
Chung-Yueh Yeh

Tartu 2021

Info sheet

Using CRISPR/cas9 to verify the cause of conditional sterility in *Arabidopsis thaliana* harbouring a mutated MPK12.

MPK12 is a gene that exists in a number of plants and has many functions, however the development of seeds is not among them. *Arabidopsis thaliana* plants with a specific mutational variant G187R of MPK12 have what is referred to as conditional sterility and are unable to produce seeds at the usual growth temperatures. To confirm whether the mutation MPK12 (G187R) is responsible for the sterility, genetic modifications and DNA sequencing are used. First, a genotype analysis by DNA sequencing was compared to the flowering phenotype to determine whether the G187R mutation was linked to the sterility. Additionally, a CRISPR/cas9 approach was used to knockout the mutated MPK12. If the sterility phenotype is caused by the G187R mutation knock-out of that mutated gene should reverse it. In the paper two constructs with differing methods of replication, are designed and constructed to deliver the CRISPR/cas9 reagents to *Arabidopsis* via *Agrobacterium*-mediated transformation.

Keywords_ MPK12, sterility, CRISPR/cas9, *Arabidopsis thaliana*

CERCS: B225 plant genetics

CRISPR/cas9 kasutamine *Arabidopsis thaliana* muteerunud MPK12-ga tingimusliku steriilsuse põhjustaja kinnitamine.

MPK12 on geen, millel on taimedes mitu funktsiooni, kuid seemnete valmimist ei ole nende hulka loetud. Siiski esineb mutatsiooniga G187R geenis MPK12 *Arabidopsis thaliana* taimel tingimuslik steriilsus ja taim ei ole võimeline seemneid tootma tavalisel kasvutemperatuuril. Kindlaks tegemaks, kas mutatsioon MPK12 (G187R) põhjustab steriilsust, kasutatakse geneetilist muundamist ning DNA sekveneerimist. Sekveneerimise eesmärgiks on kinnitada seos G187R mutatsiooni ja tingimusliku steriilsuse vahel. Geneetilise muundamise käigus geen muudetakse inaktiivseks. Kui steriilsust põhjustab mutatsiooniga geen, siis selle inaktiveerimine taastaks taimede seemnete tootmise võime. Selleks viiakse mutantidesse *Agrobacterium tumefaciens* vahendusel CRISPR/cas9 süsteem, mis peaks viima läbi soovitud muudatused. Töö käigus disainiti kaks vektorit, mis erinevad replikatsiooni meetodite poolest.

Märksõnad: MPK12, steriilsus, CRISPR/cas9, *Arabidopsis thaliana*

CERCS: B225 Taimogeneetika

Table of contents

Info sheet	2
Used abbreviations	5
Introduction	6
Literature overview.....	7
1.1. MPK12.....	7
1.1.1. MPK12 mutant G187R.....	8
1.2. CRISPR/cas9	8
1.2.1. Description of used CRISPR/cas9 system.....	9
1.3. T-DNA vector	9
1.4. Viral vector	10
1.5. Transport of vector to target	10
2. Experimental part	11
2.1. Aims of the study	11
2.2. Methods and materials	11
2.2.1. Cloning protocol.....	11
2.2.2. Bacterial transformation protocol.....	13
2.2.3. DNA extraction protocol	14
2.2.4. Vector transport protocol.....	15
2.2.5. Seed weighing	15
2.2.6. Selection of modified T1 seeds by hygromycin resistance	16
2.2.7. Seedling size comparison	16
2.2.8. Genotyping PCR and gel electrophoresis protocol	17
2.3. Results and discussion	18
2.3.1. Plant examination (leaf size, seeds weight, sterility comparisons)	18
2.3.2. Plant genomic sequencing results.....	19
2.3.3. Designed CRISPR constructs	20
2.3.4. CRISPR mediate knockout results	21

2.3.5. Discussion.....	23
Summary.....	25
CRISPR/cas9 kasutamine <i>Arabidopsis thaliana</i> muteerunud MPK12-ga tingimusliku steriilsuse põhjustaja kinnitamine.....	26
Cited literature	27
Cited websites.....	28
Supplemental part	29
Non-exclusive licence to reproduce thesis and make thesis public.....	39

Used abbreviations

Cas9- CRISPR associated protein 9

CRISPR- clustered regularly interspersed palindromic repeats

gRNA - guide RNA

LIR- long intergenic region

MPK- mitogen activated protein kinase

T-DNA- transfer DNA

sgRNA- single guide RNA

SIR- short intergenic region

Introduction

Arabidopsis thaliana is a standard model organism in plant research, due to its relatively short growth cycle and high seed yield. It has been determined that the *Arabidopsis thaliana* used in the study had a mutated MPK12 gene which resulted in an arginine substitution at the 187-th amino acid (G187R). The plants possessing the mutation also possessed conditional sterility when grown under standard growth conditions at 23°C, but were fertile when grown under a lower temperature at 17°C. However, there is no known link between MPK12 and seed development. The objective of this study is to determine whether the mutation in the MPK12 gene causes the sterile phenotype, or if there are other currently unknown mutations in the plant genome that are responsible for the phenotype. To achieve this objective two methods were used. Firstly, the DNA from F2 plants of the MPK12 (G187R) x wild type plants was sequenced and the genotypes were compared to the phenotypes. Secondly, two different vectors containing CRISPR/cas9 (clustered regularly interspersed palindromic repeats and CRISPR associated protein 9) systems are transferred to the plants via *Agrobacterium tumefaciens* cells to knockout the MPK12 (G187R) gene function.

CRISPR/cas9 is a system that is able to introduce genetic modifications at specific targets within genomes inside living organisms. The two main components of the system are: 1) site specific DNA nuclease (cas9) that cuts both DNA strands, and 2) a single guide RNA (sgRNA), which guides the cas9 to the specific genomic target. The two vectors created during this project both contain the CRISPR/cas9 system, with two sgRNAs targeting different regions of the MPK12 gene. In addition, two selection markers are included to facilitate identification of the gene-edited plants and segregation of transgenes in later generations. The vectors differ by their replication methods- one is based purely on T-DNA (transfer DNA), the other vector combined the yellow dwarf bean viral replication system.

The study's goal is to establish whether there is a link between MPK12 and plant reproduction.

Literature overview

1.1. MPK12

Mitogen-activated protein kinase (MPK) is a widely conserved gene family that is found across most eukaryotic organisms including: animals, fungi, plants and unicellular organisms. The MPK genes constitute a large gene family and are most commonly closely connected to the cells' response to stress. There are 11 genes in the MPK gene family in the human genome alone. The proteins of the MPK genes play a crucial role in cellular and cell to cell signal transfer. The exact roles of the proteins vary by gene and from species to species. Most commonly MPK proteins react to signals and regulate gene transcription connected to mitosis, apoptosis, cell proliferation and cell survival (Teramoto H. and Gutkind J. S., 2013). Proteins in the MPK gene group are the final signal protein in a three-tiered signal transfer system in the larger MAPK cascades. The MPKs are enzymatically inactive until their threonine and tyrosine residues are phosphorylated by MPKKs. It has also been shown that MPK proteins can activate themselves when needed. Once activated, the MPK members are able to move to the nucleus and phosphorylate different substrates, for example transcription factors. (Neupane S., et al, 2019)

In *Arabidopsis thaliana* there are multiple MPK genes, which are connected to a variety of functions as signal transducers. Example genes of the group are MPK1, 3, 6 and 7. These genes are expressed in most plant cells and for most of the *Arabidopsis* developmental stages. MPK1 is connected to physical and environmental stress responses, and it is a component in the auxin signal pathway. MPK3 is also a signal transducer for physical and environmental stressors, and it regulates stomatal and pollen development. MPK6 plays a role in the response to pathogens, and regulates pollen, stomata and root development. MPK7 is not known to be directly connected to stress-related signalling, instead it is a signal transducer for circadian rhythms. (The Arabidopsis Information Resource, 2020)

The gene with the most similar sequence to the MPK12 gene in *Arabidopsis thaliana* is MPK4. MPK4 is expressed in the plant at most stages and in most cells. It is responsive to most of the common plant signal molecules, for example jasmonic acid. MPK4 is part of the signal mediation regarding for example, the stress caused by pathogens and, the changes of the carbon

dioxide level in the plant. Additionally, MPK4 has roles in plant development and the functioning of the stomata. (The Arabidopsis Information Resource, 2020)

MPK12 is a gene specific to Brassicaceae plants. The gene has two main functions. The first is regulating the responses to CO₂ in stomata via stomatal movement. The second function is the regulation of auxin response in the roots. There is no known connection between MPK12 and plant reproduction, and *Arabidopsis thaliana* plants with the complete MPK12 deletion remain fertile (Jakobson L., et al, 2016). MPK12 is similar to MPK4 in both function and nucleotide sequence, and it is believed that MPK12 has evolved from MPK4. (Töldsepp K., et al, 2018)

1.1.1. MPK12 mutant G187R

The mutation MPK12 (G187R) was achieved by Ethyl methanesulfonate (EMS) mutagenesis, a method that randomly creates changes in the genomes of plants that are exposed to it. Due to the innate randomness of this method, it is possible that it caused other mutations in the genome. The MPK12 (G187R) mutant plants are not able to produce seeds at regular laboratory conditions but are fertile in certain conditions. That form of sterility is known as conditional sterility. It is possible to make the plants fertile by growing them at a lower temperature than their normal optimum temperature. The MPK12 (G187R) mutation is located on the fourth exon, and this point mutation has caused the 187th amino acid glycine to be replaced by arginine in the protein.

1.2. CRISPR/cas9

The CRISPR system is a form of an immune system in prokaryotes which facilitates the recording of foreign DNA sequences, like for example those from viruses, in between the repeats of the CRISPR genes (Sander J. D. and Joung J. K., 2014). Based on those archived sequences it is possible to identify and eliminate the same sequence in the future. In prokaryotic cells when the archived foreign DNA is identified, specific crRNA-s are transcribed from the archived sequences, the crRNA is homologously combined at the ends with a different CRISPR system specific RNA (tracrRNA) and additionally combined with cas9 nuclease. Different species possess different CRISPR/cas systems, they are made up of unique tracrRNA and of different conserved sequences at the ends of crRNA. There are also differences in the lengths

and contents of the repeats and spacer-sequences in different species. Thanks to the crRNA-sequences the cas-nuclease complex is able to find specific nucleotide sequences and cut them through RNA-DNA complementary. Since the entire process is based on the specific sequence of nucleotides on the crRNA it is possible in biotechnology to develop exact sequences for specific changes. (Jiang W., et al, 2013).

1.2.1. Description of used CRISPR/cas9 system

To successfully make genetic modifications in molecular biotechnology the CRISPR/cas9 system has been modified in certain ways, which have increased the systems' success and efficiency. For this researchers use a CRISPR/cas9 system where crRNA and tracrRNA are fused to form a single guide RNA (sgRNA) molecule. The sgRNA contains a variable, 20-nucleotide long gRNA (guide RNA) region that is identical to the target site upstream of the protospacer adjacent motif (PAM) in the target genome. After the cas9 cuts the DNA at the desired spot the cell's natural DNA repair mechanisms take place. It can either be homologous, where the complementary region on the other DNA strand is used or non-homologous, where the ends of the cut DNA are simply ligated back together.

1.3. T-DNA vector

The T-DNA vector is based on the genomic integration abilities of T-DNA derived from the *Agrobacterium tumefaciens* Ti plasmid and is commonly used in plant genetic modification. The expression of the T-DNA can start before the T-DNA has been integrated into the cells' genome, as transient expression, and can continue when the T-DNA has successfully integrated into the genome. The modifying sequences must insert themselves into the target cells' genome for replication, because the vectors' replication depend on the target cells' replication processes. "During this step, the T-DNA, which travelled as a single-stranded DNA molecule from the bacterial cell through the host-cell cytoplasm into the nucleus, must covalently attach itself to the host cell's double-stranded genomic DNA. To fulfil its destiny, the T-DNA needs to be directed to its point of integration in the host genome, to be stripped of some, if not all, of its bacterial and host escorting proteins, and to interact with and co-opt the host's DNA-repair proteins and machinery for its complementation into a double-stranded DNA molecule during

its integration into the host genome.” (Ziemienowicz, A., et al, 2017). T-DNA vectors possess a number of benefits compared to other insertional methods. For example, the position of the insertion does not change with time, as compared to transposons, because these vectors are relatively stable and long lasting (Sangwan R., et al, 2012). The negative side of using such vectors is that there is a great chance that one or more genes might be inactivated if the integration occurs within a functional gene or regulatory elements between two genes. Additionally, T-DNA may insert itself into the host’s genome at multiple locations.

1.4. Viral vector

Viral vectors consist of an entire virus genome or only certain components of the virus, which enable the vector to replicate and express genes in the host cell through genome integration or transient expression (Modric T. and Mergia A., 2009). “At the most basic level, viral vectors consist of a viral genome that has been adapted into a plasmid-based technology and modified for safety through the removal of many essential genes and the separation of the viral components” (Patrick M., 2014). There can be a size limit, at which the efficiency of the vector drops, that limits the insert size to the vector. For example, many of the earlier viruses used in biotechnology cannot hold the CRISPR/cas9 system. Additionally, viral components can cause immune reactions in target cells resulting in the destruction of the vectors (Modric T. and Mergia., 2009).

1.5. Transport of vector to target

The method of transporting the CRISPR/cas9 constructs to the plants cells in this study is the *Agrobacterium tumefaciens* floral dip. The use of *Arabidopsis tumefaciens* allows the viral vectors to be constructed only with viral replication and expression genes because, the bacterial cells are able to independently move from cell to cell in the plant, removing the need for the corresponding viral genes. It is known that the cells extract DNA from their plasmids as T-DNA and that the process utilizes bacterial proteins VirD2 and VirE2 (Ziemienowicz, A., et al, 2007). Finally, the vector carrying *A. tumefaciens* is introduced to the flowering plants by the floral dip process. The bacteria enter the cells of the developing seeds and carry with them the modifying vectors. (Sangwan R., et al, 2012).

2. Experimental part

2.1. Aims of the study

The study's goal is to determine if there is a connection between the gene MPK12 in *Arabidopsis thaliana* and the plant's reproduction. To determine this, conditionally sterile plants with the MPK12 gene possessing the G187R mutation are used in the study. The mutated MPK12 gene is attempted to be made non-functioning through CRISPR/cas9 modification. Two different CRISPR/cas9 vectors have been constructed in the study. One is based on T-DNA only and the other also includes viral replication components. The mutant plants are infected with *Agrobacterium* carrying the CRISPR constructs. Additionally, the MPK12 genes of the plants are sequenced, and their genotypes and phenotypes are compared.

2.2. Methods and materials

An overview of the different processes, methods and used materials which were used in the study.

2.2.1. Cloning protocol

The first step was to design suitable constructs that would be able to replicate in the target cells and successfully complete the genetic modification. The constructs were pre-designed by Yuh-Shuh Wang and were constructed during this study. First a plasmid was digested by a restriction enzyme to extract either the insert or plasmid backbone to be used later. Then two separate components were ligated to form a completed plasmid.

The plasmid was digested in a 20 µl mix consisting of 1 µl of the restriction enzyme, 2 µl of the 10X restriction buffer and about 1 µg of plasmid and finally the amount was raised to 20 µl by adding water. The mix was left to digest for 1 hour at 37 °C and then using gel purification the DNA segment was separated out. For the ligation, 0,5 µl of T4 DNA ligase, 1 µl of 10-X ligase buffer, 0,5 µl of the vector and 2 µl of the insert were mixed together and water was

added to bring the final amount of mixture to 10 μ l. the mixture is left at room temperature for 1 hour to complete the ligation.

Firstly, the sgRNA cassettes were constructed. The plasmid AtU6-26long sgRNA/pGEM-GG*BsaI containing all the components of the sgRNA cassette, except for the sgRNA itself. Using the restriction enzymes Eco31I the plasmid was cut between the U626long promoter and sgRNA scaffold_SpCas9. The sgRNA for the first exon of the gene MPK12 was ligated into the cut site. The same process was done with AtU6-29long sgRNA/pGEM-GG*BsaI, only the sgRNA ligated into that plasmid cut site was for the second exon of the gene MPK12. After that the two cassettes are ligated into a single plasmid, AtU6-29long sgRNA/pGEM-GG*BsaI is digested with the restriction enzymes BcuI and XmaJI. The enzymes free the entire sgRNA cassette from the plasmid. AtU6-26long sgRNA/pGEM-GG*BsaI is digested with only BcuI, which creates a cut a few nucleotides away from the U6-26 terminator. The cut AtU6-26long sgRNA/pGEM-GG*BsaI and the sgRNA cassette from AtU6-29long sgRNA/pGEM-GG*BsaI are ligated together, and that forms MPK12-1,2 sgRNA long/pGEM-GG. MPK12-1,2 sgRNA long/pGEM-GG is digested by the restriction enzymes SgsI and BcuI, this releases the two sgRNA cassettes from the plasmid. The constructs are also digested with the restriction enzymes and during ligation the cassettes are ligated to the final construct (table 1).

Table 1. the used constructs, descriptions and the source of those plasmids.

Construct	Description	Source
AtU6-26long sgRNA/pGEM- GG*BsaI	Plasmid containing the sgRNA cassette without the first MPK12 exon gRNA	Built by Yuh-Shuh Wang
AtU6-29long sgRNA/pGEM- GG*BsaI	Plasmid containing the sgRNA cassette without the second MPK12 exon gRNA	Built by Yuh-Shuh Wang
P7-46 Atu6- 26psgRNA/pGEM-GG	Plasmid with AtU6-26 promoter, SpCas9 scaffold and Atu6-26 terminator to be used as a backbone for gRNA insertion	
P7-47 Atu6- 29sgRNA/pGEM-GG	Plasmid with ATu6-29 promoter, SpCas9 scaffold and	Built during this study

	Atu6-26 terminator to be used as a backbone for gRNA	
MPK12-1,2 sgRNA long/pGEM-GG	Plasmid for containing the sgRNA cassettes of MPK12s first and second exon	Built during this study
	Plasmid containing the T-DNA vector without sgRNAs cassettes.	Built by Yuh-Shuh Wang
	Plasmid containing the viral vector without sgRNA cassettes.	Built by Yuh-Shuh Wang
MPK12-1,2 long sgRNA_pFAST-R/pHEE2E	Plasmid containing CRISPR/cas9 reagents, both of the MPK12 targeting sgRNAs for the T-DNA vector.	Built by Yuh-Shuh Wang
MPK12-1,2 long sgRNA_HygR_pFAST-R/pTC217	Plasmid containing CRISPR/cas9 reagents, both of the MPK12 targeting sgRNAs and viral replication system.	Built by Yuh-Shuh Wang

During this process DNA molecules are cut using restriction enzymes and later ligated back together in a separate reaction. This process is used to construct the gene editing constructs that will be transported into the plants.

2.2.2. Bacterial transformation protocol

The transformation's goal is to place the prepared DNA constructs into live bacteria, either *E.coli* or *Agrobacterium*, and to check, via the marker genes and genotyping whether the construct is viable. The transformation used in this study is the heat shock method.

For *E.coli*:

First the frozen competent cells (strain DH10 β), preprepared and stored at -80°C, are placed on ice to thaw slowly. Then 90 μ l of cells are pipetted to 10 μ l of the ligation mix and left on the

ice for 15 min. The water bath is heated to 42°C and the cells are placed in it for 90 seconds. The cells are removed and placed back on the ice for 1 min. 300 µl of LB is added to the cells and they are placed in a 37 °C growth environment for approximately 40 min. Finally, the cells are plated on a kanamycin infused LB plate and placed in 37°C growth environment until visible colonies form.

For *Agrobacterium tumefaciens*:

2 µl of DNA is added to 20 µl of cells (strain GV3101(pMP90)), that have been preprepared and stored at -80°C. The cells are placed in a water bath at 37 °C for 5 min. After the cells are removed from the bath, 300 µl of LB is added and the cells are placed in a shaker for 40 minutes at room temperature. The cells are later plated on a kanamycin infused LB plate until visible colonies have appeared.

2.2.3. DNA extraction protocol

During the experimental part DNA was extracted at two steps in the process. From plants for genotyping PCR of sequencing and as plasmids from *E.coli* to be modified and placed back into bacteria.

Extraction from plants:

Small amounts of plant tissue are removed and added to the centrifuge tubes, 300 µl of Edwards solution (200 mM tris-HCl, 250 mM NaCl, 25 mM EDTA and 0,5% SDS) and around 30 1,3-1,8 mm diameter glass beads are added as well. The tubes are shaken in a Silmat S6 centrifuge tube shaker for 20 seconds each and then placed in a centrifuge for 5 minutes at 12000 rpm (rounds per minute). The extracted DNA is stored at 4 °C.

Extraction from bacterial cells

The extraction follows the guide of the favorgen plasmid extraction mini kit. The cells are first centrifuged into a pellet at 12000 rpm and the supernatant is discarded completely. 200 µl of the solution FAPD-1 is added and the cells were resuspended. 200 µl of the solution FADP-2 is added to the cells and the mixture is inverted 10 times and left for 2 minutes. 300 µl of the solution FAPD-3 is added and the mixture is inverted 10 times. The cell lysate is centrifuged at 12000 rpm for 5 min and the supernatant is placed in a FAPD-column that is in a collection tube. During centrifugation a collection tube is placed on a heating block at 65 °C. The columns

are centrifuged at 12000 rpm for 30 seconds and the flow through is discarded. 400 µl of the W1 buffer is added to the FAPD-column, the mixture is centrifuged at 12000 rpm for 30 seconds and the flowthrough is discarded. 600 µl of wash buffer is added to the FAPD-column, the mixture is centrifuged at 12000 rpm and the flowthrough is discarded. The columns are centrifuged at 12000 rpm for an additional 3 minutes. The FAPD-column is placed into the collection tube in the heated block, 50 µl of elution buffer is added and the column is heated for 1 min. The column is centrifuged at 12000 rpm for 1 min to move the DNA from the FAPD-membrane to the collection tube.

2.2.4. Vector transport protocol

The objective of this part is to introduce the CRISPR/cas9 construct into the plant cells from the bacterial cells. Plants with developing flowers are infected with the construct carrying agrobacteria. The agrobacteria would spread out throughout the plant and even reach the reproduction system, and pass on the gene editing construct to the developing seeds. The construct would in time express the CRISPR/cas9 system genes and cause the desired knockout of MPK12.

This process started when the *Arabidopsis thaliana* plant inflorescent stems have grown to about 15 cm. 40 ml of LB containing transformed *Agrobacterium tumefaciens* is incubated at 28 °C and shaken at 250 rpm overnight. The *Agrobacterium tumefaciens* are centrifuged at 4000 rpm for 10 minutes and the supernatant is discarded. The cells are resuspended in a 50 ml floral dip solution (5% sucrose and 0,05% Silwet-L77). The flowering *Arabidopsis thaliana* plants are dipped into the solution for 2 minutes. Small amounts of the solution are dripped onto smaller flowering stems. The plants are placed in a growth room for seed development, in 1-1,5 months the plants are dried, and the seeds are collected.

2.2.5. Seed weighing

1000 seeds from each plant genotype are placed in pre-weighed micro centrifuge tubes and weighed. This process is done multiple times to find an average weight for each type of seed.

2.2.6. Selection of modified T1 seeds by hygromycin resistance

The objective of this phase is to determine whether, the genetic modification has been successful in the seeds. Because the binary construct contains a hygromycin resistance gene as a marker gene within the T-DNA region, only seeds that have gone through successful T-DNA integration will be able to germinate on the hygromycin-containing plate. This allows the number of T-DNA integrations to be estimated, and also to separate out the resistant seedling for planting into soil.

The seeds are sterilized before being plated. The seeds are suspended in 300µl of 95% ethanol and left for 3 minutes. The ethanol is removed and 300 µl of 2% sodium hypochloride is added and left for 5 minutes. The hypochloride is removed and the seeds are rinsed over with 300 µl of sterile water 3 times.

2.2.7. Seedling size comparison

The overall seedling size of the MPK12 (G187R) mutants was noticeably larger than other plant groups that had grown the same amount of time. As comparisons seeds from the wildtype Col-0, the MPK12 mutant G187E and the MPK12 deletion (mpk12-4) plants, were planted and later measured, alongside the G187R group.

20 seeds were planted from 4 different plant groups on a growth plate and the plate was sealed off. Two types of plates were used, the regular plates (10mM KNO₃, 0.7% phytoagar, 20ug/ml hygromycin) and the ones with added jasmonic acid (½ MS salts with vitamins, 1% sucrose, 1% phytoagar, with or without 50uM MeJA). The plate was then placed vertically in a plant growth chamber for 10 days. After the growing period the plates were scanned by an Epson scanner. The cotyledon from each plant was removed and grouped together with other cotyledons from the same group and scanned by an Epson scanner as well. The scanned images were then analysed using the digimizer program to determine the root lengths of the seedlings and the size of the cotyledons. First, the size markers were measured in the images so the program could make accurate measurements from that reference point. The lengths of the seedling roots were measured by marking the roots with the length measure function. The cotyledons were measured by the area function, the outlines of the individual cotyledons were marked, and the program measure the area inside of them.

2.2.8. Genotyping PCR and gel electrophoresis protocol

Genotyping PCR with gel electrophoresis were used at multiple stages of the study to confirm the genotypes of either plants or bacterial cells. The final reaction amount is 20 µl. Initially, 0,5 µl of the reverse and 0,5 µl of forward primers (table 2) are added to 15,4 µl of purified water. Next 2 µl of 10X PCR buffer, 0,4 µl of 10mM nucleotides and 0,2 µl of DreamTaq polymerase are added to the primer mix. Later 1µl of extracted plant DNA is added. When using bacterial DNA, the amounts used are 16,9 µl of purified water, 0,25 µl each of forward and reverse primers. The bacterial DNA is added as live bacteria that are suspended into the mix.

Table 2. primes used in PCR reaction, with the primer's name, sequence, description of use and the primer's source.

Primer name	Sequence	Application	Source
XII-57	TGTGGTCTCAGGAGTGAG-GTAACAAACAACAAGTTT	Forward primer for genotyping MPK12 CRISPR plants	Integrated DNA Technologies
XII-67	CTCTGTTATAAGCCTAAG-CTGATG	Reverse primer for genotyping MPK12 (G187R) plants	Integrated DNA Technologies
XII-68	GACCAGTGATCAATGCCGT	Forward primer for genotyping MPK12 (G187R) plants	Integrated DNA Technologies
XII-69	TCGGAAGTTGTTCTT-GCGAGC	Reverse primer for genotyping MPK12 CRISPR plants	Integrated DNA Technologies

XIII-31	TCACGGGTGACTCGAGAG	Forward primer for MPK4 genotyping	Integrated DNA Technologies
XIII-32	GAGCGAATAATTTGGT- GGAGATC	Reverse primer for MPK4 genotyping	Integrated DNA Technologies

2.3. Results and discussion

2.3.1. Plant examination (leaf size, seeds weight, sterility comparisons)

The phenotypes of plants that had their genotypes confirmed as possessing the *MPK12* (G187R) mutation through DNA sequencing, were examined and compared to other plant groups. These groups were: wildtype Col-1, *mpk12-4* (plants that have their MPK12 gene removed MPK12 (G187E) (plants with a mutation similar to the MPK12 (G187R), but the amino acid glycine is replaced by a glutamate), and *coi1-16* (plants, that have similar conditional sterility as the MPK12 (G187R) plants and need to be grown at the same temperature as the MPK12 (G187R) plants to produce seeds).

As comparisons seeds from the wildtype Col-0, the mutant MPK12(G187E) and the MPK12 deleted (*mpk12-4*) plants, were also weighed. Because the MPK12 G187R plants produced seeds at a lower temperature than the other plants, seeds from *coi1-16*, which are also were grown at a lower temperature to set seeds, were also weighed, in order to rule out environmental factors. By weighing different seed groups, it was determined that MPK12 (G187R) seeds were on average 50% heavier than the other seeds. The MPK12 (G187R) seeds were also visibly larger than the other groups. This indicates that these mutants produce larger seeds not only by size but biomass (table 3) (table 7).

Using the digimizer program the cotyledon sizes from different plant groups were also measured (image 11). The results showed that the cotyledons of the MPK12 (G187R) seedling were around 20% larger than the cotyledons of the other groups (table 3) (table 8).

The primary root lengths of different plant groups were also measured. The results showed that the root lengths of MPK12 (G187R) and MKP12 (G187E) were similar and were around 35% larger than the other groups (table 3) (table 9) (image 9) (image 10).

Table 3. the *Arabidopsis thaliana* groups (Col-0, MPK12 (G187R), MPK12 (G187E), MPK12-4 and Coi1-16) with their corresponding averaged seed weights, seedling root lengths, cotyledon sizes and calculated confidence intervals. *-student t-test has compared to Col-0 shows that the information is statistically relevant

	Seed wight (mg)	Seedling root length (cm)	Cotyledon size (mm ²)
wildtype Col-0	20,7±2,7	2,48±0,25	5,2±1
MPK12 (G187R)	30,8±7,5	3,38*±0,32	7,4*±0,6
MPK12 (G187E)	24,3±3,4	3,22±0,25	7,8±0,9
MPK12-4	20,9±2,7	2,55±0,23	5,3±0,6
Coi1-16	21,8±2,8	-	-

2.3.2. Plant genomic sequencing results

DNA was extracted from 31 F2 plants of MPK12 (G187R) x Col-0, which are self-pollinated plants of the F1 generation. The MPK12 genomic region was then amplified by PCR. The PCR products were sent for sequencing to the University of Tartu, Institute of Genomics Core Facility and the results were compared to the flowering phenotypes of the plants.

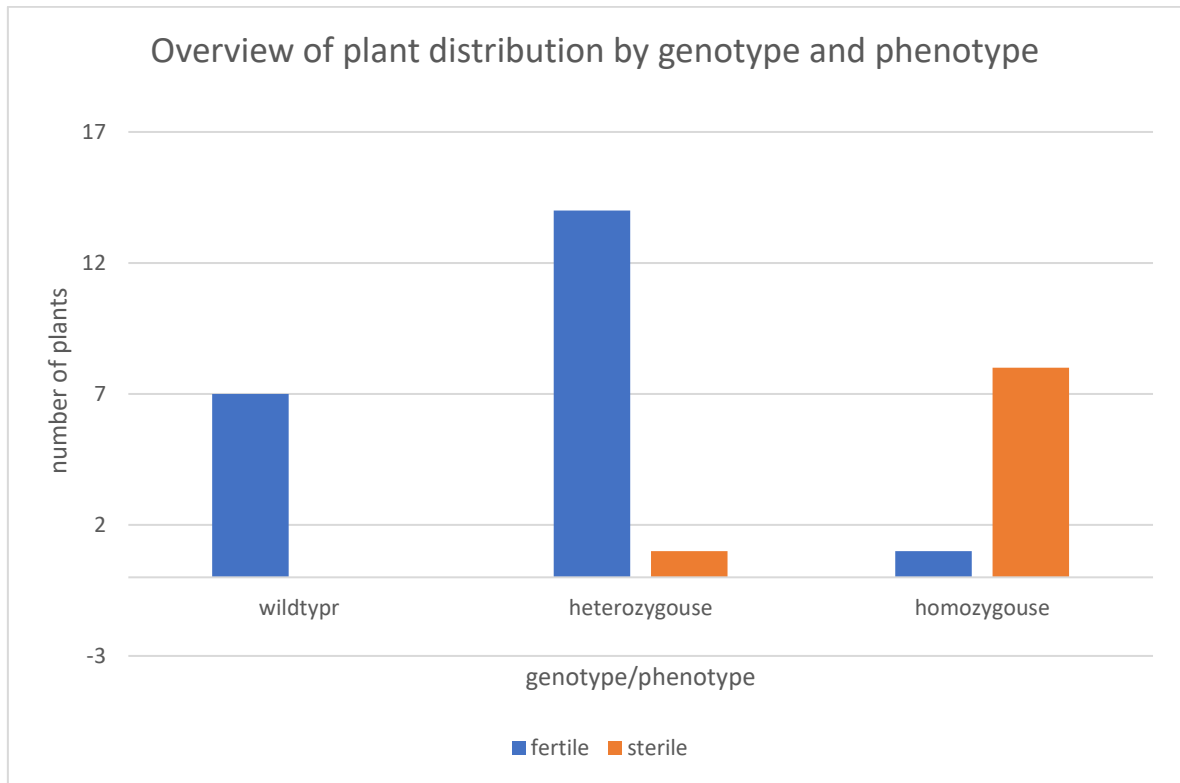


Figure 1. overview of the phenotype and genotype comparison of the 31 plants which had their MPK12 genes sequenced.

Of the 31 plants analysed, there were 7 wild-type plants and all 7 were fertile. There were 15 heterozygous plants, 14 of them were fertile and one (nr 5) was sterile. There were 9 homozygous MPK12 (G187R) plants, 8 of them were sterile and one (nr 22) was fertile (figure 1).

2.3.3. Designed CRISPR constructs

Two different CRISPR/cas9 constructs were designed and built, one based only on T-DNA and the other with additional viral replication components.

Both of the constructs possess two sgRNA cassettes targeting the first two exons of the MPK12 gene. Also, the constructs have the crispr protein coding genes required for the cleavage of DNA. Additionally, the constructs have two marker genes, one is for resistance to the antibiotic

hygromycin, and the second gene expresses a red fluorescent protein in the seeds (pFAST-R) (figure 2).

The designed viral vector has viral components from the yellow dwarf bean virus. The components are DNA sequences of two LIRs (long intergenic region), one SIR (short intergenic region) and one REP replicase gene. These genes are all required for rolling replication (figure 3).

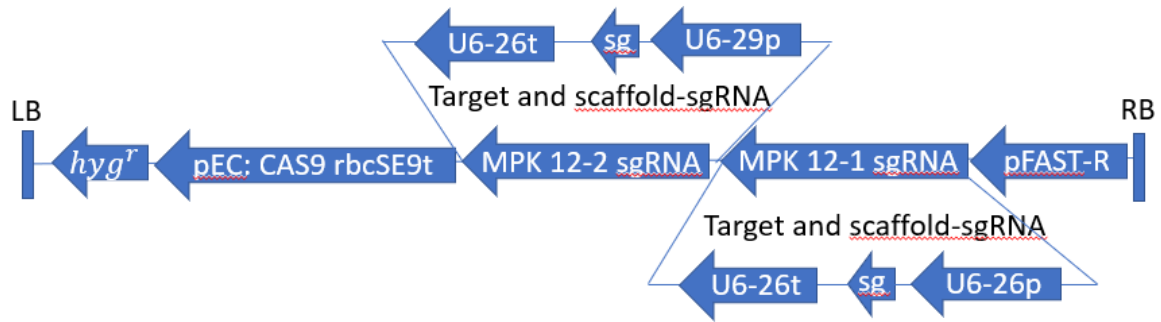


Figure 2. T-DNA construct diagram

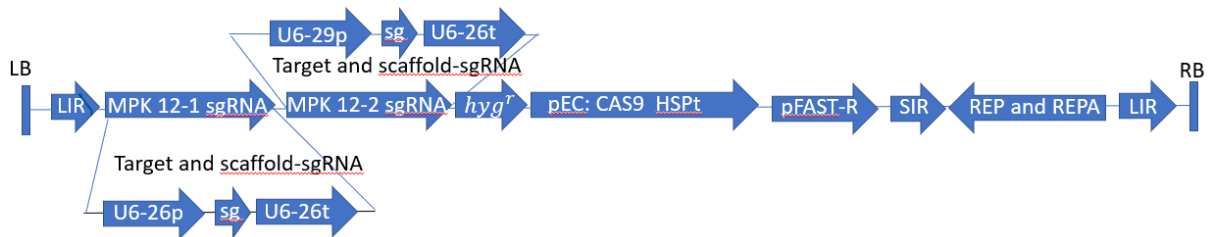


Figure 3. viral construct diagram

2.3.4. CRISPR mediate knockout results

T1 seeds from T0 plants that had been exposed to *Agrobacterium* carrying CRISPR constructs, were selected based on their hygromycine resistance, and grown at standard growth conditions (23°C). If the conditional sterility is caused by the MPK12 (G187R) mutation than the plants that had the gene knocked out by CRISPR would be fertile at standard growth conditions. DNA was extracted from the T1 plants and sent for sequencing to check whether the MPK12 (G187R) genomic sequence had been changed. The sequencing results showed that only 4, out of the 20 plants analysed had their MPK12 (G187R) gene sequences changed.

In plant P8-50-1, the only plant that was successfully modified by the T-DNA construct, sequencing of the MPK12 gene showed, that the first sgRNA (MPK12-1) target site had not been modified (image 1). The second sgRNA (MPK12-2) target site however was modified (image 2). In one copy of the where a 53 nucleotide deletion was found (table 4).

Table 4. The modified sequence in P8-50-1 at the second sgRNA (MPK12-2) target site.

TGCTGCGGTGAACTCAGTGACTGGAGAGAAAGTGGCTATTAAG AAGATCGGTAATGCTTTTGATA	Reference sequence
TGCTGCGGTGAACTCAGTGACTGGAGAGAAAGTGGCTATTAAG AAGATCGGTAATGCTTTTGATA	P8-50-1 copy A
TGCTGCGG-----TGATA	P8-50-1 copy B

MPK12-2 gRNA sequence

There are 3 MPK12-edited plants transformed with the CRISPR construct containing viral replication components (p8-54). In plant P8-54-1, the first sgRNA target site showed modifications when aligned with the MPK12 gene sequence (image 3). Due to the low quality of the sequencing results, it was not possible to determine the exact modification. The second sgRNA target site showed- that one of the sequences had been modified and the other had remained the same, however, the sequencing results were not clear enough to determine the exact modifications (image 4).

In plant P8-54-4, the modifications caused by the CRISPR/cas9 system were the clearest of the modified plants. At the first target sgRNA target site a thymine insertion has occurred at the 614 position in both gene copies (image 5, table 5). At the second sgRNA target site a guanine and thymine have been deleted from the sites 824 and 825 from both gene copies (image 6, table 6). The combination of these modification causes a frame shift within the MPK12 of the P8-54-4 plant, most likely making the gene non-functioning.

Table 5. The modified sequences in P8-54-4, at the first sgRNA (MPK12-1) target site

TGCATCAAAGTTGTACCGACACA_CGGTGGCCGC	Reference sequence
TGCATCAAAGTTGTACCGACACATCGGTGGCCGC	P8-54-4 copy A
TGCATCAAAGTTGTACCGACACATCGGTGGCCGC	P8-54-4 copy B

MPK12-1 gRNA sequence

Table 6. The modified sequences in P8-54-4, at the second sgRNA (MPK12-2) target site.

cagTGCTGCGGTGAACTCAGTGACTGG	Reference sequence
cagTGCTGCGGTGAACTCA__GACTGG	P8-54-4 copy A
cagTGCTGCGGTGAACTCA__GACTGG	P8-54-4 copy B

MPK12-2 gRNA sequence

In plant P8-54-14 the sequencing showed that modifications have occurred at both sgRNA target site. Even though the sequencing results were of low quality it was determined that at the first target site (MPK12-1) a two-nucleotide deletion had occurred (GG), however there were additional modifications that could not be identified (image 7). At the second target site (MPK12-2) it is likely that only one of the copies has been modified, but the exact modifications were not determined (image 8).

Of the 4 plants only 3 grew to the flowering stage (P8-50-1, P8-54-1, P8-54-4) and all 3 remained sterile at 23°C (image 12).

2.3.5. Discussion

The goal of this study was to determine if the gene MPK12 is connected to the fertility of the *Arabidopsis thaliana*.

The data suggests that the mutation G187R in MPK12 is not responsible for the conditional sterile phenotype. This is supported by the genotype and phenotype linkage analysis in F2 plants of MPK12 (G187R) x Col-0. Namely of the 31 tested plants, plant number 5 possessed the conditional sterile phenotype even though it was a heterozygous mutant, and plant number 22 remained fertile even though it possessed two copies of the MPK12 (G187R) mutated gene. Additionally, the 3 CRISPR plants that had their mutated MPK12 modified all remained sterile. These lines of evidence suggest that the G187R mutation is not the cause of the conditional sterility phenotype, which is consistent with our current understanding that MPK12 does not play a role in plant reproduction. In particular, plants with the complete MPK12 gene deleted (*mpk 12-4*) are still fertile under normal growth conditions. However, since the mutation

MPK12 (G187R) is often inherited with the conditionally sterile phenotype, it is likely that the causative gene is close to the MPK12 gene on the *A. thaliana* genome.

One of the CRISPR edited plants (P8-54-14) had stunted growth and was unable to grow to the flowering stage (image 13). A hypothesis was formed that this was due to the similarities between MPK12 and MPK4, such that the gRNA-s could have modified MPK4 instead. Since MPK4 is connected to plant development, that could explain the plants phenotype. This could signal that the CRISPR process is not as exact as assumed. Sequencing however showed that the MPK4 gene remained intact (image 14) (image15). This suggests that the small size of the single plant is caused by a yet unknown mutation or mutations caused either by the gRNA modifying a different MPK gene or from the T-DNA inserting into a crucial gene.

In addition to the conditional sterility, there are noticeable differences in the MPK12 (G187R) plants as compared to other plants. The seeds, cotyledons and seedling roots are considerably larger than wildtype plants. This difference could be due to MPK12 gene's role in auxin response. Both MPK12 (G187R) and MPK12 (G187E) have mutated MPK12 genes and have heavier seeds, larger cotyledons and longer roots than the other plant groups.

The results also show that the viral construct has a higher efficiency rate than the T-DNA construct. As viral construct successfully modified 3 plants and the T-DNA construct only 1. Additionally, the viral construct was more likely to modify both of the target sites and both of the gene copies.

The study should next move on to genes that are connected to plant reproduction and that are situated close to MPK12 on the *A.thaliana* genome. To determine whether a gene is the cause of the conditional sterility, experiments of the same kind: genotyping and CRISPR mediated knockout, if possible, should be used. Seeds from plants number 5, 22 and the plants that had their MPK12 genes successfully modified by CRISPR (P8-50-1, P8-54-1, P8-54-4) should be collected to repeat the experiments. This could yield additional information in the relationship between MPK12 and plant reproduction. Additionally, it should be determined whether the cause of the larger seedling and seed sizes of the MPK12 (G187R) mutants was an impaired auxin response due to the G187R mutation.

Summary

MPK12 has roles in *Arabidopsis thaliana* like auxin sensitivity and carbon dioxide signalling in the stomata. There is no known connection between MPK12 and reproduction, however *A. thaliana* possessing MPK12 with the mutation G187R have conditional sterility. The plants are unable to produce seeds at the standard laboratory temperature (23°C), for successful seed production the plants must be placed in a colder growth environment (17°C). The mutants were created by exposure to methanesulfonate, a random mutagenic process that could have resulted in the creation of multiple mutations. Based on the use of that method the cause of the conditional sterility could be other or multiple mutations.

To confirm whether the G187R mutation and the conditional sterility are connected two methods were used. Firstly, the plant genomes were sequenced and then the MPK12 sequences were studied. If the G187R mutation was the cause of the conditional sterility than it would be in both of the copies of MPK12. Based on the sequencing results the genotypes and the phenotypes of the plants were compared. As the second method two CRISPR/cas9 constructs with different replication systems were designed, built and transported into the conditionally sterile plants. The goal of the constructs was to knock out the mutated MPK12 gene and if the MPK12 (G187R) mutation is the cause of the conditional sterility the plants should become fertile again.

The study's goal is to determine if there is a connection between plant reproduction and MPK12.

Both the sequencing and genetic modification results showed that the G187R mutation is not responsible for the conditional sterile phenotype. The sequencing results showed that there were plants with only one copy of the G187R mutation that were still conditionally sterile and plants with two MPK12 (G187R) copies that remained fertile. Of the plants that were successfully genetically modified by the constructs and grew to the flowering stage all remained conditionally sterile. The results indicate that the MPK12 (G187R) mutation is not connected to plant fertility. It can be assumed that the conditional sterility is caused by a different mutation or mutations that are situated close to the MPK12 gene.

CRISPR/cas9 kasutamine *Arabidopsis thaliana* muteerunud MPK12-ga tingimusliku steriilsuse põhjustaja kinnitamine

Karl Mäll

Resümee

MPK12 geenil on *Arabidopsis thaliana*s kaheks põhiliseks rolliks auksiini tundlikkus ja süsihappegaasi signalisatsioon õhulõhedes. Kuigi geenil ei ole teadaolevalt ühtegi seost taime paljunemisega, esineb *A. thaliana*del mutatsiooniga MPK12 (G187R) tingimuslikku steriilsust. Taimed ei ole võimelised seemneid tootma tavalisel kasvutemperatuuril (23°C), seemnete tootmiseks peavad taimed olema madalamatel temperatuuridel (17°C). Mutandid loodi metanosulfonaadi mutageneesiga, protsessiga, mille käigus võivad tekkida paljud erinevad mutatsioonid ja sellest tulenevalt võib tingimusliku steriilsuse põhjustajaks olla mõni muu või mitu erinevat muteerunud geeni.

Avastamaks, kas MPK12 (G187R) mutatsioonil ja tingimuslikul steriilsusel on omavahel seos, kasutatakse töös kahte meetodit. Esiteks sekveneeritakse taimede genoome ja uuritakse MPK12 järjestusi teades, et mutatsioon avaldaks mõju ainult siis, kui mõlemad geeni koopiad oleksid muteerunud. Lähtudes sekveneerimise andmetest võrreldakse taime genotüüpe fenotüüpidega. Teise meetodina disainiti ja valmistati kaks CRISPR/cas9 modifikatsiooni vektorit, mis kanti üle tingimuslikult steriilsetele taimedele. Modifikatsiooni eesmärgiks on muuta MPK12 geen inaktiivseks. Kui mutatsioon MPK12 (G187R) on tingimusliku steriilsuse põhjustajaks, siis peaks geeni välja lülitamine taimed viljakandvateks muutma.

Töö eesmärgiks on avastada, kas eksisteerib side MPK12 geeni ja taime paljunemise vahel.

Nii sekveneerimise kui ka geenmuundamise tulemused näitasid, et mutatsioon MPK12 (G187R) ei ole tingimusliku steriilsuse põhjustaja. Sekveneerimisel avastati taimi, mille fenotüübid ja genotüübid ei sobinud selle oletusega, et G187R on tingimusliku steriilsuse põhjustaja. Kahe erineva CRISPR/cas9 modifikatsiooni vektoriga nakatatud taimedest toimus edukas MPK12 geeni väljalülitus ja õitsemissstaadiumisse jõudmine ainult kolmel taimel, millest kõigil säilis tingimuslik steriilsus. Tulemuste järgi saab oletada, et MPK12 ei ole seotud taimede paljunemisega. Lisaks saab oletada, et tingimusliku steriilsust põhjustab mõni muu mutatsioon või mutatsioonide kogum, mis paikneb MPK12 geeni lähedal genomis.

Cited literature

1. Jakobson L, Vaahtera L, Töldsepp K, Nuhkat M, Wang C, Wang Y-S, et al. (2016) Natural Variation in *Arabidopsis*.Cvi-0 Accession Reveals an Important Role of MPK12 in Guard Cell CO₂ Signaling. PLoS Biol 14(12): e2000322.
<https://doi.org/10.1371/journal.pbio.2000322>
2. Jiang W., Zhou H., Bi H., Fromm M., Yang B. and Weeks D. P., 2013, Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice, Nucleic Acid Research, 41(20): e188
3. Modric T. and Mergia A., 2009, The Use of Viral Vectors in Introducing Genes into Agricultural Animal Species, Animal biotechnology, 20:4, 216-230.
4. Neupane S., Schweitzer S. E., Neupane A., Andersen E. J., Fennell A., Zhou R. Nepal M. P., 2019, Identification and Characterization of Mitogen-Activated Protein Kinase (MAPK) Genes in Sunflower (*Helianthus annuus* L.), Plants, 8(2), 28
5. Sander J. D. and Joung J. K., 2014, CRISPR-Cas systems for genome editing, regulation and targeting, Natural Biotechnology, 32(4): 347–355.
6. Sangwan R., Ochatt S., Nava-Saucedo J. E., Sangwan B., 2012, T-DNA insertion mutagenesis., Plant Mutation Breeding and Biotechnology, 489-506.
7. Teramoto H. and Gutkind J. S., 2013, Mitogen-activated protein kinase family, „Encyclopedia of biological chemistry, 176-180
8. Töldsepp, K., Zhang, J., Takahashi, Y., Sindarovska, Y., Hörak, H., Ceciliato, P., Koolmeister, K., Wang, Y. S., Vaahtera, L., Jakobson, L., Yeh, C. Y., Park, J., Brosche, M., Kollist, H., & Schroeder, J. I., 2018, Mitogen-activated protein kinases FMPK4 and MPK12 are key components mediating CO₂ -induced stomatal movements, The Plant journal, 96(5), 1018–1035.
9. Ziemienowicz, A., Tzfira, T., Hohn, B., 2007, Mechanisms of T-DNA integration, Agrobacterium: From Biology to Biotechnology, 395-440

Cited websites

1. Patrick M., Addgene blog, Viral Vectors 101: Viral Vector Elements, 07.07.2014, viewed 14.10.2020
(<https://blog.addgene.org/plasmids-101-viral-vector-elements>)
2. The Arabidopsis Information Resource, viewed 10.03.2021,
(<https://www.arabidopsis.org/>)

Supplemental part

Table 4. *A.thaliana* plants with their sequenced genotypes and visually confirmed phenotypes.

Plant number	Phenotype	genotype
1.	Fertile	heterozygous
2.	Sterile	homozygous
3.	Sterile	homozygous
4.	Fertile	heterozygous
5.	Sterile	heterozygous
6.	Fertile	wild type
7.	Fertile	wild type
8.	Fertile	heterozygous
9.	Fertile	wild type
10.	Fertile	heterozygous
11.	Fertile	heterozygous
12.	Fertile	wild type
13.	Sterile	homozygous
14.	Fertile	heterozygous
15.	Fertile	heterozygous
16.	Fertile	heterozygous
17.	Fertile	heterozygous
18.	Fertile	heterozygous
19.	Sterile	homozygous
20.	Fertile	heterozygous
21.	Fertile	heterozygous
22.	Fertile	homozygous
23.	Fertile	wild type
24.	Fertile	wild type
25.	Fertile	wild type
26.	Fertile	heterozygous
27.	Fertile	heterozygous
28.	Sterile	homozygous

29.	Sterile	homozygous
30.	Sterile	homozygous
31.	Sterile	homozygous

Image 1. MPK12 first sgRNA target site of the plant P8-50-1, compared to standard MPK12 gene sequence.



Image 2. MPK12 second sgRNA target site of the plant P8-50-1, compared to the standard MPK12 gene sequence.

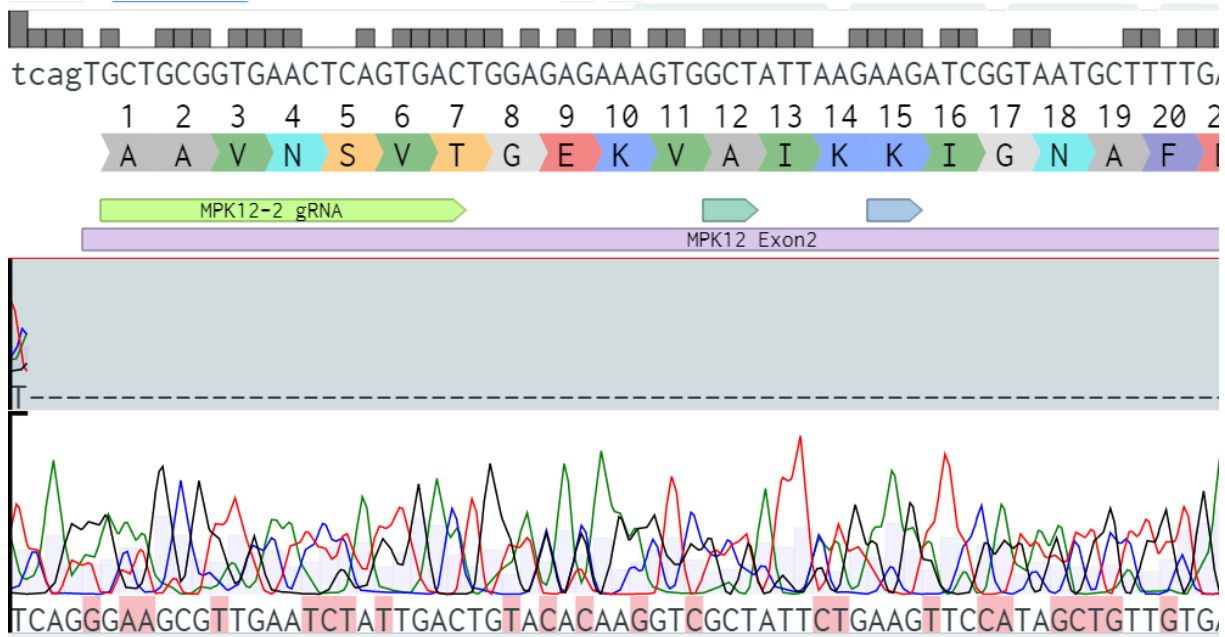


Image 3. MPK12 first target site of the plant P8-54-1, compared to the standard MPK12 gene sequence.

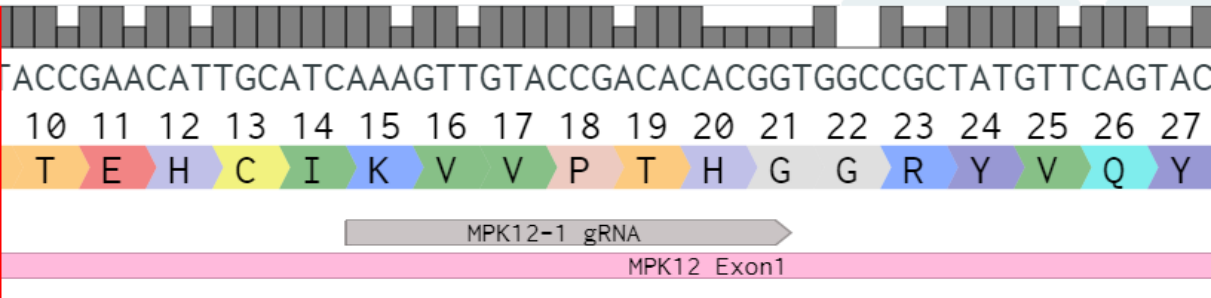


Image 4. MPK12 second target site of the plant P8-54-1, compared to the standard MPK12 gene sequence.

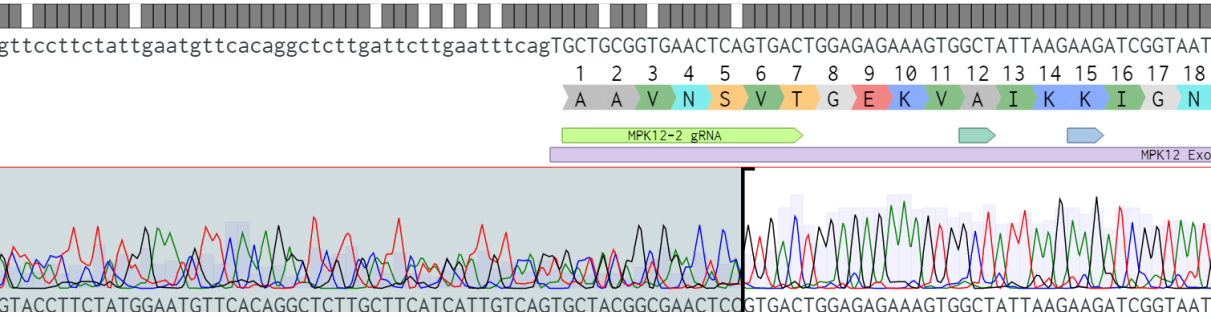


Image 5. MPK12 first target site of the plant P8-54-4, compared to the standard MPK12 gene sequence.



ACCGAACATTGCATCAAAGTTGTACCGACACACGGTGGCCGCTATGTTTCAGTACAACGTTTATGGACAA

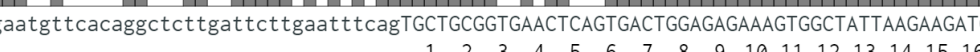
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32

T E H C I K V V P T H G G R Y V Q Y N V Y G Q

MPK12-1 gRNA

MPK12 Exon1

ACCGAACATTGCATCAAAGTTGTACCGACACACTGGGGTCCCGGCTGCTCTGAACCTTCGTGAATCAAGA



 :cttctattgaatgttcacaggctcttgattcttgaatttcagTGTCGGTGAAGTCAAGTCACTGGAGAGAAAGTGGCTATTAAGAAGATCGGTAATGCT

 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

 A A V N S V T G E K V A I K K I G N A

 MPK12-2 gRNA

 MPK12 Exon2

 TCTCTTCTGGAAGCTCACAGGATCTTTCTATAAAGCTTGTACTGCTCATGGCAACCCAGTTGACTGGAGAGAAAGTGGCTATTAAGAAGATCGGTAATGCT

Image 9. Comparison of 10-day old seedlings from the groups: wild-type col-1, MPK12(G187E), MPK12 (G187R) and mpk12-4.

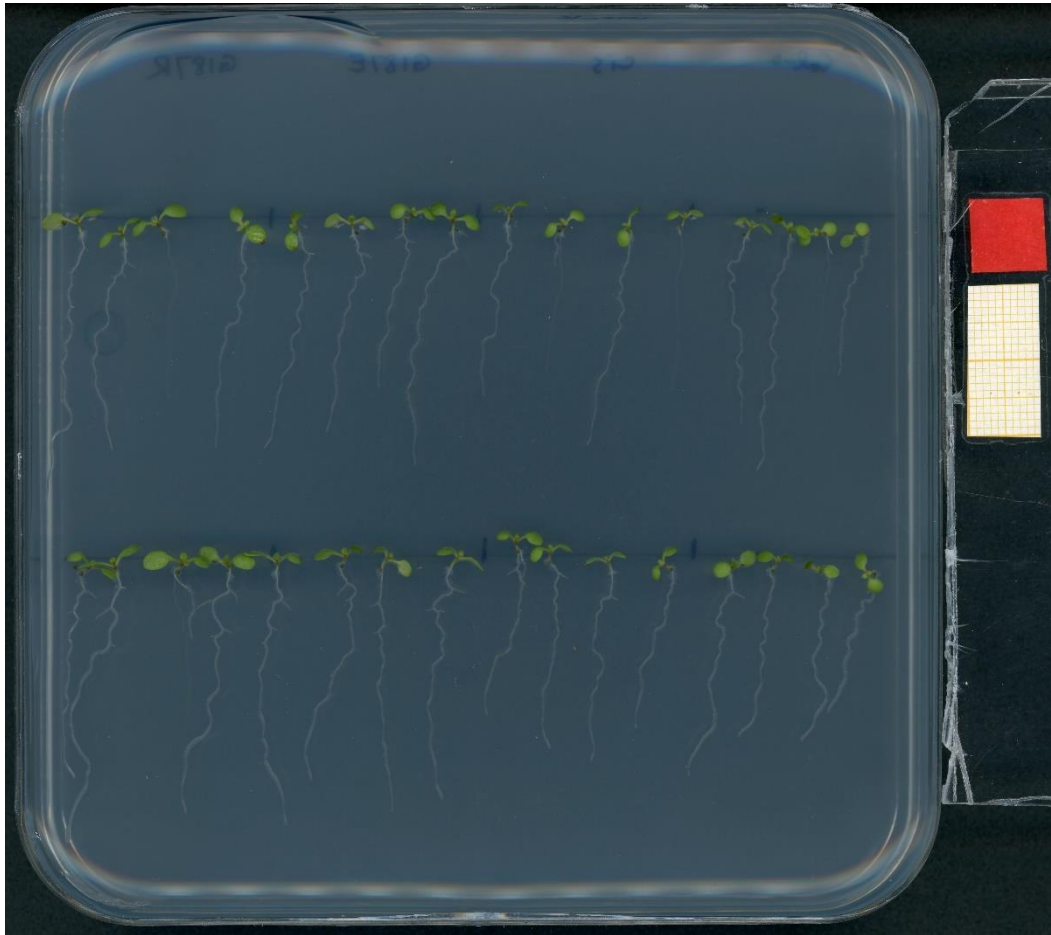


Image 10. Comparison of 10-day old seedlings from the groups: wild-type col-1, MPK12(G187E), MPK12 (G187R) and mpk12-4.

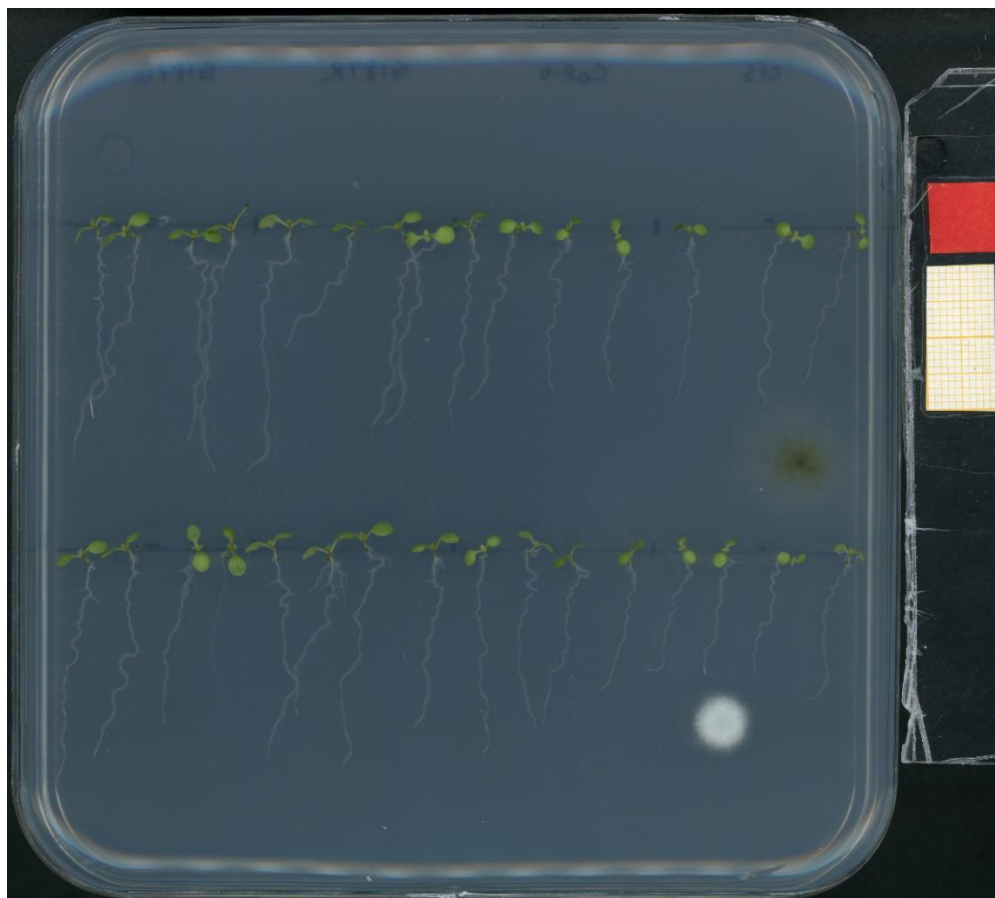


Image 11. Scanned image of the cotyledons of *Arabidopsis thaliana* from the groups wild-type Col-1, MPK2(G187E), MPK12 (G187R) and mpk12-4.

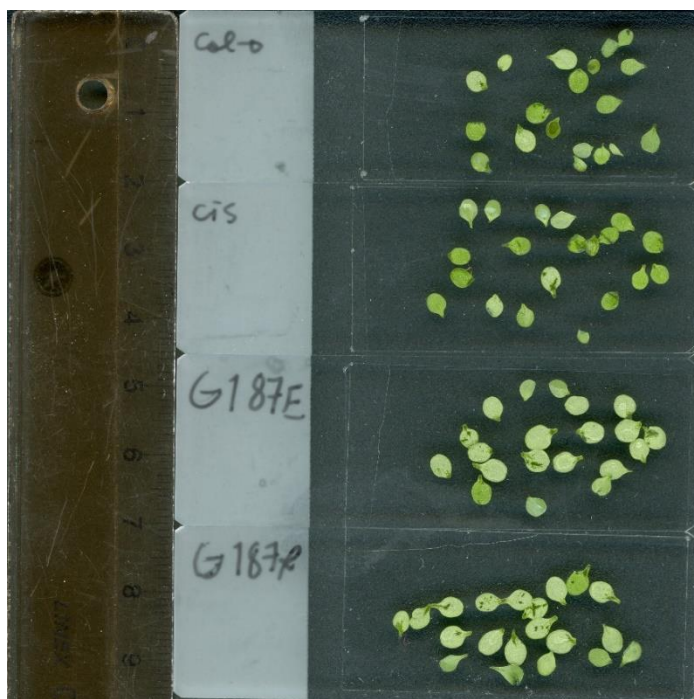


Image 12. Two conditionally sterile *Arabidopsis thaliana* plants (P8-54-1 and P8-54-2).



Image 13. Two *Arabidopsis thaliana* plants (P8-54-13 and P8-54-14) that have been modified by the viral CRISPR construct. Both failed to reach the flowering stage.

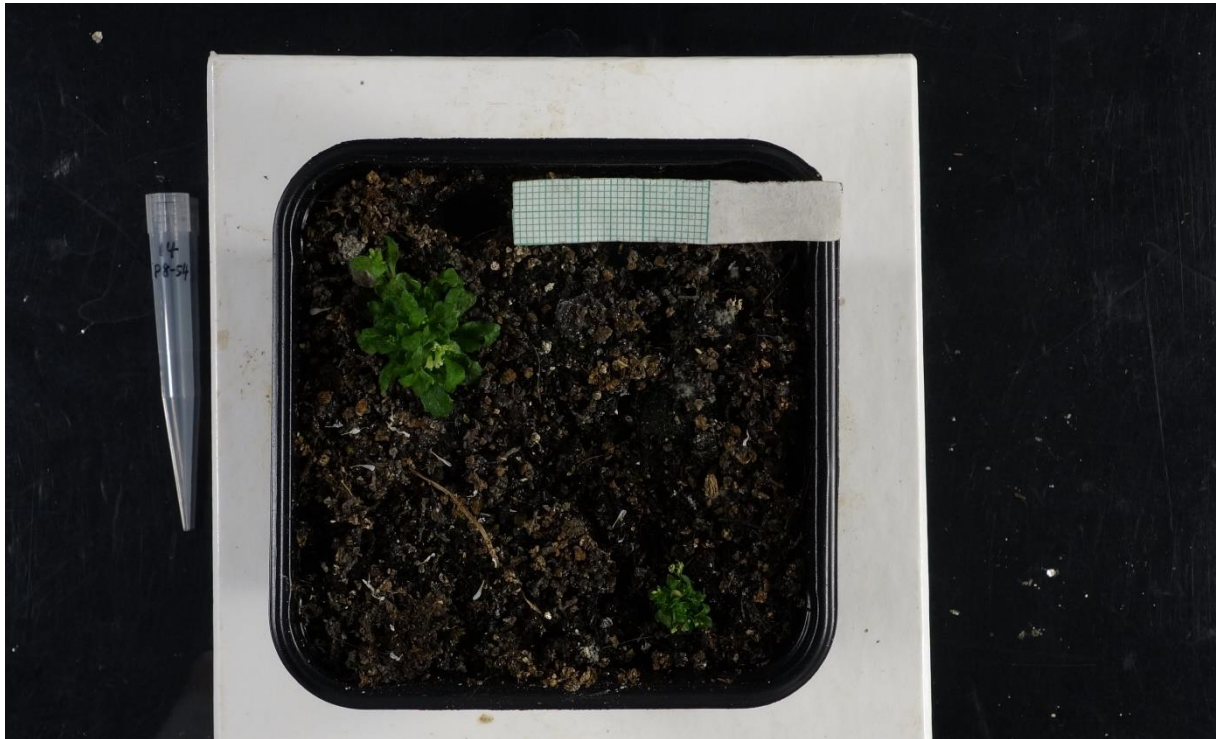


Table 7. The seed groups with their corresponding weights and average weights.

G187R- MPK12 mutation G187R, wildtype- *Arabidopsis thaliana* Col-0 accession,

mpk12-4- MPK12 deletion, G187E- MPK12 mutation G187E, coi1-16- plants grown under same conditions as G187R plants.

	MP12 (G187R)	Col-0	MPK12- 4	MPK12 (G187E)	Coi1- 16
1. weighing (mg)	31,6	20,4	20	24,8	21
2. weighing (mg)	33,3	19,8	20,5	22,8	21,4
3. weighing (mg)	27,4	21,9	22,1	25,4	23,1

average (mg)	30,8	20,7	20,9	24,3	21,8
-----------------	------	------	------	------	------

Table 8. The cotyledon groups with their sizes, wild-type Col-1, MPK12(G187E), MPK12 (G187R) and mpk12-4.

Col-0 (mm ²)	MPK12-4 (mm ²)	MPK12(G187R) (mm ²)	MPK12(G187E) (mm ²)
4,6	5,1	8,1	7,9
4,5	4,5	9,1	5,8
9,1	4,6	9,9	7,1
2,8	6,2	6,8	8,5
4,5	5,8	8	10,2
6,6	4,7	5,6	8,1
3,6	5,7	8,6	3,9
5,9	3,9	7	5,1
5,5	6,2	9,1	7,7
7,5	5,5	8,9	9,3
6,6	7,8	8,3	11,8
6,8	6,4	5,7	9
2,6	6,3	7,5	7,5
3,8	4,7	6,4	9
3,9	3,6	8,3	9,1
3,8	4,1	6,6	8
2,3	2,9	8,6	8
6,7	7,8	5,9	10,5
8,9	4,9	5,2	5,5
-	5,2	6,6	5,4

Table 9. The seedling root lengths, wild-type Col-1, MPK12(G187E), MPK12 (G187R) and mpk12-4.

Col-0 (cm)	MPK12 (G187R) (cm)	MPK12 (G187E) (cm)	MPK12-4 (cm)
2,068	2,624	2,809	2,220
2,123	2,742	3,351	2,434
2,468	1,976	3,007	2,327

2,718	3,720	2,965	2,095
2,631	2,744	4,126	1,407
2,482	3,155	2,644	1,773
1,821	3,206	2,203	1,780
2,601	3,135	2,519	2,015
1,833	2,384	3,183	2,495
1,767	2,050	2,115	2,275
3,173	3,002	2,843	2,374
2,743	3,567	3,087	2,739
3,108	2,677	3,093	2,504
2,319	4,081	2,909	2,897
2,844	2,537	3,252	1,550
1,828	3,763	3,293	-

Image 14. Aligned sequence of MPK4 with MPK12, sgRNA target site 1.

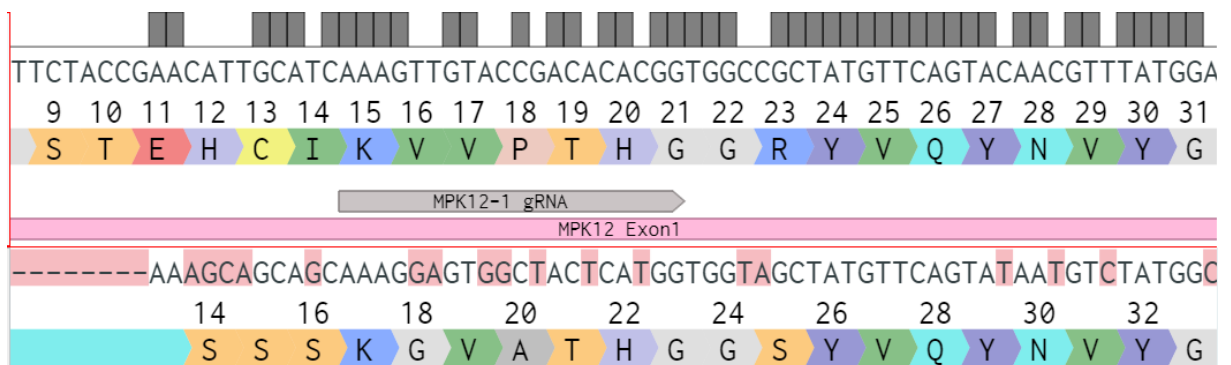
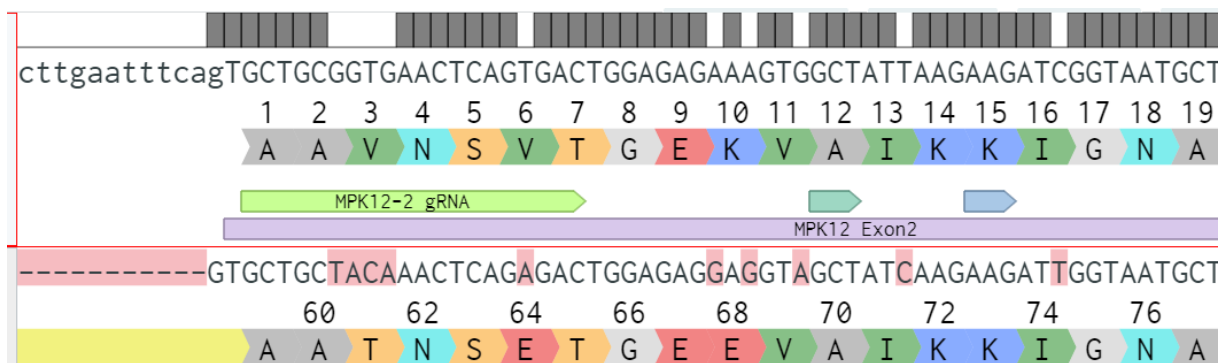


Image 15. Aligned sequence of MPK4 with MPK12, sgRNA target site 2.



Non-exclusive licence to reproduce thesis and make thesis public

I, Karl Mäll,

herewith grant the University of Tartu a free permit (non-exclusive licence) to

reproduce, for the purpose of preservation, including for adding to the DSpace digital archives until the expiry of the term of copyright,

Using CRISPR/cas9 to verify the cause of conditional sterility in *Arabidopsis thaliana*
harbouring a mutated MPK12

supervised by : Associate professor Yuh-Shuh Wang

Junior research fellow Chung-Yueh Yeh

2. I grant the University of Tartu a permit to make the work specified in p. 1 available to the public via the web environment of the University of Tartu, including via the DSpace digital archives, under the Creative Commons licence CC BY NC ND 3.0, which allows, by giving appropriate credit to the author, to reproduce, distribute the work and communicate it to the public, and prohibits the creation of derivative works and any commercial use of the work until the expiry of the term of copyright.
3. I am aware of the fact that the author retains the rights specified in p. 1 and 2.
4. I certify that granting the non-exclusive licence does not infringe other persons' intellectual property rights or rights arising from the personal data protection legislation.

Karl Mäll

29.05.2021