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Nigel Stadhouders – 29SEP16

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Abstract

Doelgerichte DNA integratie via Homologe Recombinatie (HR) via een toegevoegde template wordt Gene Targeting (GT) genoemd. Dit proces is erg zeldzaam in de meeste planten (10^{-4} tot 10^{-6}). De CRISPR/CAS9 (Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR Associated System) nuclease is succesvol gebruikt als hulpmiddel om dubbel strengs breuken (DSBs) aan te brengen nabij de gewenste recombinatie sequentie om GT frequenties te verhogen.

De twee belangrijkste DSB reparatie systemen zijn HR en Non-Homologous End-Joining (NHEJ), waarbij NHEJ de voornaamste DNA reparatie route is. Hierbij worden de DNA uiteinden rechtstreeks aan elkaar geligeerd en kunnen daarom kleine inserties of deleties bevatten. Huidige kennis over NHEJ toont aan dat er op ze minst twee subroutes zijn genaamd, classic NHEJ (c-NHEJ) en alternative NHEJ (a-NHEJ). Het is bekend dat het eiwit KU80 een cruciale rol speelt in de DSB erkenning in de c-NHEJ route. Één van de a-NHEJ DNA reparatie routes in zoogdiercellen gaat via polymerase- θ (POL-θ). Dit POL-θ gen is ook gevonden in *Arabidopsis thaliana* en wordt het *Tebichi* gen genoemd. Mutanten van dit gen laten morfologische defecten zien.

Eerdere studies tonen aan dat POL-θ nodig is om de a-NHEJ DSB reparatie route te bevorderen in zoogdiercellen ten kosten van HR. Door het creëren van mutanten in het Teb gen, welke codeert voor POL-θ, zou integratie via a-NHEJ geblokkerd moeten worden, waardoor alleen c-NHEJ en HR overblijven als de enige twee reparatie routes. Recente studies tonen aan dat in *teb* mutanten geen T-DNA integratie detecteerbaar was, wat suggereert dat het Teb gen essentieel betrokken is bij integratie van T-DNA.

Fenotypische experimenten zijn uitgevoerd op *teb5*, *teb2*, *ku80* en dubbel mutant *teb5xku80* om het effect te zien van wortel groei in vergelijking met de WT, onder DNA schadende omstandigheden. Vervolgens werd bepaald of reparatie van DSBs geïnduceerd door een artificiële nuclease in *teb5* mutanten gerepareerd kunnen worden via 'Foot Print' analyses.

Enkele studies duiden aan dat in *pol-ϑ* mutanten HR nog steeds optreedt, in sommige gevallen zelfs meer dan in de WT. Om te bepalen of dit ook zo is in planten worden intrachromosomale recombinatie via de GUS assay en integratie van T-DNA via HR in *Arabidopsis thaliana* wortel transformatie experimenten uitgevoerd.

Fenotypische experimenten tonen kortere wortels aan in de enkel mutanten (*teb5* & *ku80)* en nog korte tot nauwelijks wortels in de dubbel mutant *teb5xku80* in vergelijking met de WT. Intrachromosomale HR van het GUS gen is opgetreden in zowel de WT als *teb5*, waarbij een significant hoger HR ratio is gevonden in de *teb5* mutant.Aangezien hier alleen de a-NHEJ en HR reparatie routes beschikbaar waren, hebben er meer HR events plaatsgevonden.

DSB reparatie van *teb5* welke de CRISPR/CAS9-CRU1 nuclease tot expressie laat komen via NHEJ is succesvol volbracht. Footprints zijn gevonden door middel van kleine deleties, ook al waren dit vaak dezelfde. Meer samples zouden getest moeten worden om te bepalen of er verschil zit in de WT en *teb5*. *Agrobacterium* gemedieerde T-DNA integratie in *Arabidopsis thaliana* Root Transformatie is waargenomen. GT events lijken gevonden via specifieke PCRs, maar kan mogelijk ook een vals positieve uitslag zijn omdat de calli van de *teb5* in tegenstelling tot die van de WT, geen scheuten hebben gevormd.

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List of abbreviations

(T)-DNA	Transferred Deoxyribonucleic Acid
(Ti) plasmid	Tumor- inducing plasmid
2,4- D	Dichlorophenoxyacetic acid
2ip	2-isopentenyladenine
a-NHEJ	Alternative Non-Homologous End Joining
AS	Acetosyringone
Cb	Carbencillin
CIM	Callus Induction Medium
c-NHEJ	Classic Non-Homologous End Joining
CRISPR/CAS9	Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR Associated System
CRU	Cruciferine
CTAB	Hexadecacyl Trimethyl Ammonium Bromide
DSB	Double- strand break
DSBs	Double- strand breaks
gRNA	Guide- Ribonucleic Acid
GT	Gene Targeting
GUS	β-glucuronidase
HDR	Homology Directed Repair
HR	Homologous Recombination
Hyg	Hygromycine
IAA	Indole-3-acetic acid
MMS	Methyl Methanesulphonate
MS	Murashige & Skoog
NHEJ	Non- Homologous End Joining
PAM	Protospacer Adjacent Motif
PCR	Polymerase Chain Reaction
POL- θ	Polymerase-Theta
PTT	L-Phosphinothricin
SIM	Shoot Induction Medium
SSB	Single- strand Break
SSBs	Single- strand breaks
Teb	Tebichi
TMEJ	POL-θ Mediated End-Joining
WT	Wild Type

Introduction

Agobacterium mediated transformation

Agrobacterium tumefaciens naturally induces crown gall tumors on various plant species. During its infection *Agrobacterium* replicates a single-stranded copy of the bacterial transferred (T)-DNA that is located on the tumor-inducing (Ti) plasmid and transfers it into the plant host cell where it subsequently integrates into the host genome. The expression of the genes located on the wild-type T-DNA, which contain plant transcription and translation signals, results in overproduction of the plant hormones auxin and cytokinin and hence increased cell division and the tumor phenotype. A set of accessory virulence or effector proteins, encoded by the 'vir' region on the Ti plasmid, is also transported into the transformed cell to confer full virulence (Gelvin, 2003), (Pitzschke & Hirt, 2010).

This T-DNA was adapted for genetic engineering, removing hormone and opine biosynthesis genes , replaced with a selectable marker gene and one or more genes of interest. T-DNA's are engineered in *Escherichia coli* on a binary vector that is introduced into an *Agrobacterium* strain. *Agrobacterium* mediated transformation generally results in random integration of transgenes. However, targeted DNA integration at specific sites in the genome would be more advantageous (Anami, Njuguna, Coussens, Aesaert, & Van Lijsebettens, 2013), (Fauser, Schiml, & Puchta, 2014).

Targeted DNA integration via Homologous Recombination (HR) using an added repair template is called Gene Targeting (GT). This process is very rare in most plants (10^{-4} to 10^{-6}). Introducing a double-strand break (DSB) near the site of the desired recombination event can increase the GT frequency (Baltes & Voytas, 2015).
The CRISPR/CAS9 (Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR Associated System) has successfully been used as a tool to introduce DSBs (Figure1). The CAS9 protein in this system cleaves double-stranded DNA with its two nucleases domains, Ruv C and HNH, each cleaving one of the two strands. After CAS9 cleavage activity the cell will use one of its repair system for DSBs to fix the lesion (Osakabe & Osakabe, 2015), (Belhaj, Chaparro-Garcia, Kamoun, Patron, & Nekrasov, 2015), (Jinek et al., 2012).

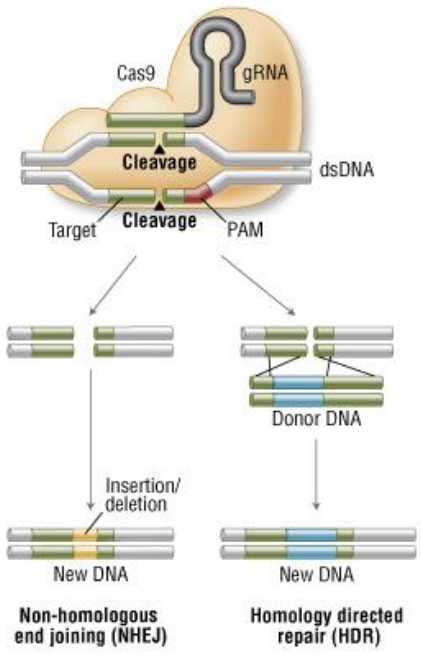

Figure 1. Cas9 nuclease site specifically cleaves double-stranded DNA, activating double-strand break repair machinery. In the absence of a homologous repair template non-homologous end joining can result in insertions/deletions disrupting the target sequence. Alternatively, precise mutations and knock-ins can be made by providing a homologous repair template (Donor DNA) and exploiting the homology directed repair pathway. From: https://www.neb.com/tools-and-resources/featurearticles/crispr-cas9-and-targeted-genome-editing-a-new-era-in-molecular-biology

DSB repair pathways

There are two main DSB repair systems, HR and Non-Homologous End-Joining (NHEJ) (Figure2). HR normally only occurs during the meiosis and the S2-phase of the cell cycle, when the sister chromatids can be used as a repair template. HR is started with the resection of broken DNA ends, homologous sequences are searched for, and the DSB can be repaired, often error-free (Manova & Gruszka, 2015).

NHEJ is one of the major DNA repair pathways, which can function throughout the whole cell cycle. Hereby the DNA ends are directly ligated and repair may therefore be accompanied by a small insertion or deletion. The NHEJ machinery facilitates several genomic rearrangements, some of which can lead to cellular transformations. Current understanding of NHEJ shows that there are at least two sub-pathways described as the classic NHEJ (c-NHEJ) and alternative NHEJ (a-NHEJ) (Manova & Gruszka, 2015). It is know that the protein KU80 play a crucial role in DSB recognition in the c-NHEJ. It forms a heterodimer with protein KU70, which binds to the ends of DSBs (Koike & Koike, 2008).

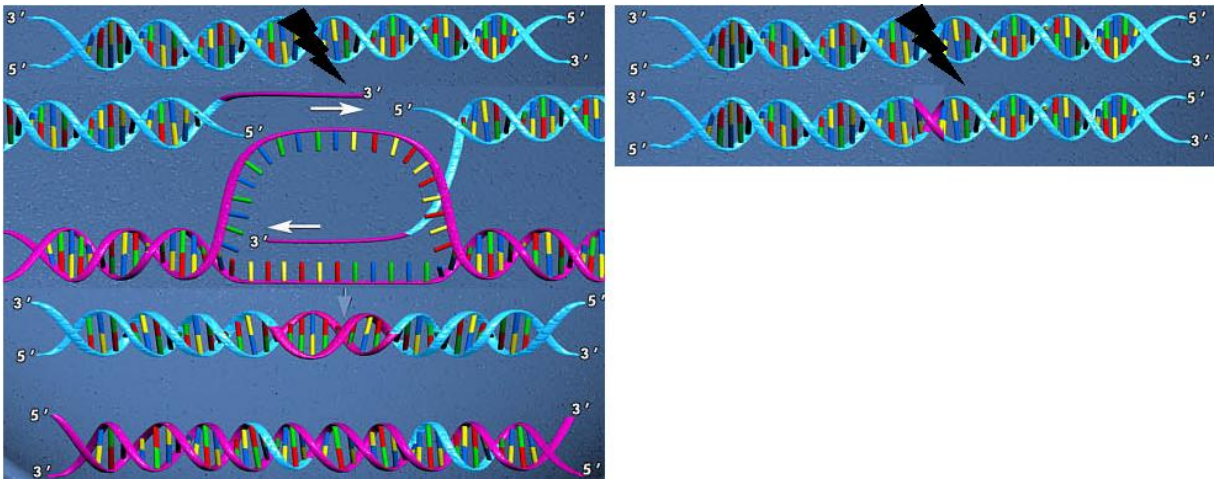

Figure 2.Schematic illustration of the two main DSB repair systems. DNA is damaged and a DSB occurs. During Homologous Recombination, on the left, the broken DNA ends are resectioned and a homologous sequence is used as a repair template to repair the DSB error-free. During Non-Homologous End-Joining, on the right, DSBs are repaired by direct ligation of the DNA ends, in this example with a small insertion. *From : https://www.qiagen.com/us/ shop/genes- and-pathways/pathway-details/?pwid=143*

POL- ϑ & Tebichi gene

One of the a-NHEJ DSB DNA repair pathways in mammalian cells is via polymerase-θ (POL- θ), also called POL-θ Mediated End-Joining (TMEJ). POL-θ prevents spontaneous or DNA damage-induced production of DSB in the DNA (Mateos-Gomez et al., 2015). A POL-θ orthologue is also found in *Arabidopsis thaliana* and is called the *Tebichi* gene. This gene is responsible and required for regulated cell division and differentiation in meristems. Mutants of this Teb gene show morphological defects, such as short roots, serrated leaves, fasciation, as well as defective patterns of cell division and differentiation in the meristems (Inagaki et al., 2006), (Inagaki, Nakamura, & Morikami, 2009).

Earlier studies showed that POL- θ is required to promote a-NHEJ dependent DSB repair in mammalian cells at the expense of HR (Mateos-Gomez et al., 2015), (Ceccaldi et al., 2015). By creating mutants in the Teb gene which encodes POL- θ, integration via the a-NHEJ repair pathway should be blocked, leaving the c-NHEJ and HR pathway as the only two repair pathways.

The aim of this study

Recent studies showed that in *teb* mutants (*teb2* & *teb5* (mutant allele *2* and *5* of the Teb gene)) no T-DNA integration was detectable, meaning that if there still is any T-DNA integration it will be in very low frequencies (Kregten et al., 2016). This suggests that the Teb gene is essentially involved in the integration of T-DNA.

As graduation project I will first perform a phenotypic analysis after DNA damage of Bleomycin and Methyl Methanesulphonate (MMS) of *teb5*, *ku80* and double mutant *teb5ku80* compared to the wild type (WT). The *teb5* mutant has a disrupted a-NHEJ DNA repair pathway, leaving the c-NHEJ and HR pathway as the only two repair pathways. The *ku80* mutant has a disrupted c-NHEJ DNA repair pathway, leaving the a-NHEJ and HR pathway as the only two repair pathways. By creating a double mutant via crossbreeding *teb5* and *ku80* both a-NHEJ as c-NHEJ are inactivated.

Subequently I will determine if repair of DSBs induced by artificial nucleases in *teb5* mutants could be repaired by performing 'Foot print Analyses'. These results will give an indication whether the same repair pathway is used for T-DNA integration and repair of artificially induced DSBs.

Few studies indicate that in *pol-ϑ* mutants HR still occurs, in some cases even more then in WT (Mateos-Gomez et al., 2015), (Ceccaldi et al., 2015). Also as a part of my graduation project I will determine if this is also true in plants by studying intrachromosomal recombination via the GUS assay (Gherbi et al., 2001) and integration of T-DNA via HR in the Arabidopsis thaliana Root Transformation experiments (Vergunst, Lier, Dulk-ras, & Hooykaas, 2003).

Principle of techniques

Phenotypic analysis of teb5, ku80, teb5ku80 mutants and WT

Phenotypic analysis were performed to study what the effect of the *teb* mutation is on root grow and visual appearance of *teb5*, *ku80* and double mutant *teb5ku80* compared to the wild type (WT).

Roots were also exposed to Bleomycin or (MMS). Bleomycin is a nonribosomal peptide that is a hybrid peptide-polyketide natural product obtained from *Streptomyces verticullus* that induces DSBs. While bleomycin induces DSBs, MMS only causes single strand breaks (SSBs). The accumulation of MMS-induced SSBs in the G1 phase will eventually lead to DSBs.

Plants were grown vertically. Roots will grow naturally away from light to the darkest area and plants also use starch granules in root tips to detect the center of gravity. This will stimulate the roots to grow vertically down all in same direction which makes it easy and reliable to compare length with each other.

Nuclease induced DSB and footprint analysis in teb5 and WT

In order to study repair of DSB in *teb* mutants footprint analysis were performed on *teb* mutants expressing artificial nucleases. When such nucleases induced a DSB close to or overlapping a restriction site, repair may result in removing the restriction site, leaving the sequence resistant for this specific restriction enzyme.

Extracted DNA from leaves of the *teb5* mutant and WT expressing the CRISPR/CAS9-CRU1 nuclease, short CRU1 (Figure 3A.), was pre-digested with the restriction enzyme *Pst* I, an endonuclease with recognition sequence 5'CTGCAG 3', resulting in a DSB in the target sequence.

If the artificial nuclease CRU1 successfully made DSBs in the recognition site and the DSBs were ligated together again via NHEJ, which might result in deletions or insertions, the restriction enzyme *Pst* I was no longer able to detect and therefore cut his recognition sequence in the target sequence.

By performing this pre-digestion an increased amount of PCR product without a *Pst* I recognition sequence in the recognition site was formed. A Polymerase Chain Reaction (PCR) of the *Pst* I digested DNA is performed, followed by another *Pst* I digestion. Finally the PCR products were separated by gel electrophoresis.

The 'uncut' DNA fragments were sliced out of the gel and purified via a GeneJET purification column, followed by cloning in pJET1.2 with T4 DNA Ligase and a bacterial transformation in competent DH5α cells. Single transformed colonies were grown and the plasmid DNA was isolated.

The plasmids were digested with *Pst* I and *Bgl* II. *Bgl* II is an endonuclease as well with recognition sequence 5'AGATCT 3'. pJet1.2 contains two *Bgl* II sites surrounding the *EcoRV* site in which the PCR products are cloned (Figure 3B.) Basically if the *Pst* I site was destroyed, the two sequence parts enclosed by *Bgl* II should be ligated together, resulting in three bands when put on gel, two of the original vector and one of the ligated target DNA sequence. To be sure NHEJ of DSB has taken place the plasmids were sequenced to search for small insertions or/and deletions. However if there are four bands visible (two of the original vector and two separated bands of the target DNA sequence, which are not ligated together) NHEJ of DSB could still have taken place without destroying the *Pst* I site.

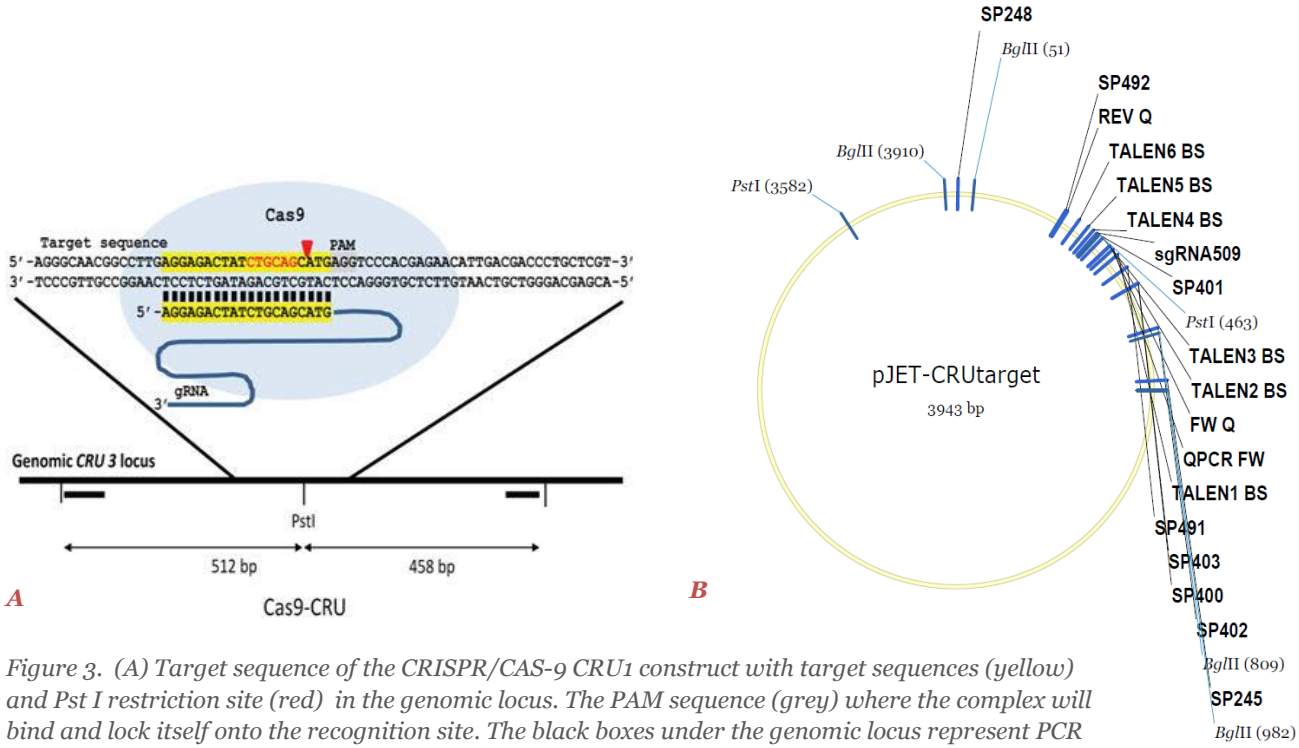

Figure 3. (A) Target sequence of the CRISPR/CAS-9 CRU1 construct with target sequences (yellow) and Pst I restriction site (red) in the genomic locus. The PAM sequence (grey) where the complex will bind and lock itself onto the recognition site. The black boxes under the genomic locus represent PCR primers SP248 and SP245 locations (B) pJET-CRU plasmid (3943 bp) with recognition sites. In total the plasmid contains 2 Pst I (463, 3582) and 4 Bgl II (51, 809, 982, 3910) sites. The CRU1 target sequence is located between Bgl II 51 and Bgl II 809, with Pst I 463 in the middle. Since the Bgl II 3910 and Bgl II 51 have 84 base pairs in between and Bgl II 809 and Bgl II 982 have 173 base pairs in between, these digestion products are too small to be visible on gel. However the Pst I 3582 in the plasmid near Bgl II 3910 with 328 base pairs in between will be visible as a digestion product on gel.

Intrachromosomal recombination of UG-US (GUS) in teb5 mutant and WT

To determine If HR still takes place the GU-US (GUS) experiment was set up with an UG-US gene – Hyg construct (Figure 4B). The antibiotic Hygromicine in the medium functions as a resistance check, so only seeds with the GU-US construct were grown. The main difference in this experiment compared to the Gene Targeting experiments described below is that here intrachromosomal recombination is analyzed. Only if HR occurred the intact reporter gene GUS (β-glucuronidase) will be formed. When incubated with substrate X-Gluc (5-bromo-4-chloro-3-indolyl Glucuronide) the reaction will result in a blue product (chloro-bromoindigo), resulting in blue colored spots if HR did occur. In order to increase the number of GUS spots, the grown plants may be exposed to Bleomycin, which induces DSBs and thus DNA repair.

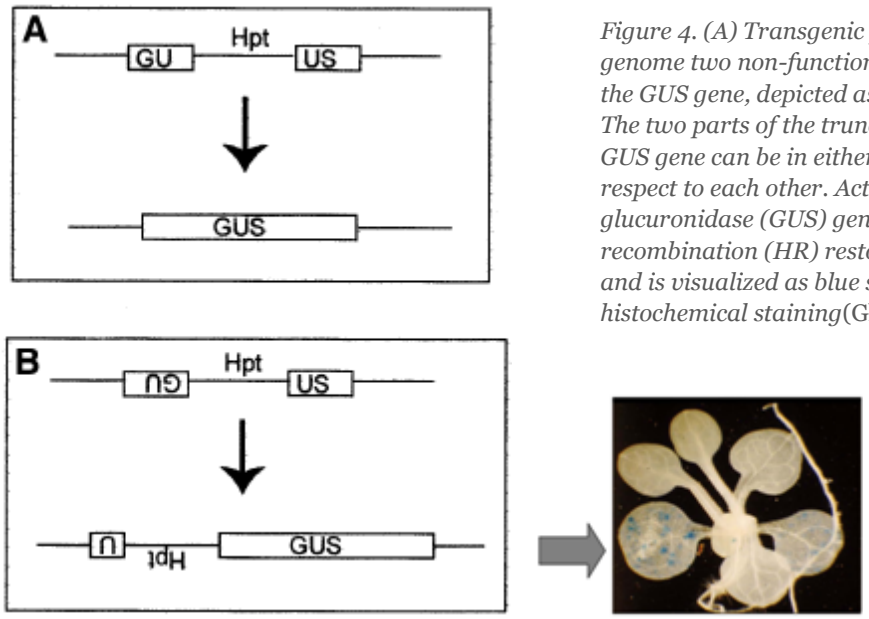

Figure 4. (A) Transgenic plants carry in the genome two non-functional truncated copies of the GUS gene, depicted as "UG" and "US". (B) The two parts of the truncated, overlapping GUS gene can be in either orientation with respect to each other. Activation of the β-glucuronidase (GUS) gene via homologous recombination (HR) restores the gene activity and is visualized as blue spots after histochemical staining(Gherbi et al., 2001).

Gene targeting in teb5 and WT via Arabidopsis thaliana Root Transformation

In Gene Targeting experiments the mutant *teb5* was studied to determine if HR still occurred. Since *teb5* has a blocked random integration system and the HR pathway as the only repair pathway available. Gene Targeting experiments were performed via *Arabidopsis thaliana* Root Transformations.

A CRISPR/CAS9 – CRU – CRU RT – PTT gene construct (short CRU construct) was made with Cruciferin as cleaving target (Figure 5). Cruciferin, a 12-S globulin, is the most abundant storage protein in the seeds of *Arabidopsis thaliana*. During their development, plant seeds accumulate large amounts of CRU proteins. These proteins provide the main source of nitrogen for seed germination and early seeding growth (Nguyen, Cueff, Hegedus, Rajjou, & Bentsink, 2015).

Figure 5. The CRISP/CAS9 – CRU – CRU RT – PTT gene construct. The CAS9-CRU (red box) with Cruciferin as cleaving target, and the repair template CRU-GFP-PTT-CRU. The CRU RT (blue-boxes) with ΔATG as start codon, GFP (green box) with ATG as start codon and PTT (yellow-box) with the 35s promoter sequence.

Teb5 mutant and WT seeds were grown on Callus Induction Medium (CIM) plates, containing auxin 2,4-D and Kinetin to stimulate callus grow (Figure 6). The roots were inoculated with an *Agrobacterium* strain containing the CRU construct.

The roots (explants) were plated on CIM plates containing the same auxins plus Acetosyringone (AS) for cocultivation to induce the bacteria for T-DNA and protein transfer in the roots.

Eventually the explants were transferred to Shoot Induction Medium (SIM) plates, containing high cytokinin 2iP and IAA to stimulate shoot grow, Vancomycin and Timentin to prevent growth of remaining Agrobacterium, and PTT (L-Phosphinothricin) for selection of T-DNA integration.

Roots did eventually form calli and shoots on SIM plates containing PTT. This will confirm that Agrobacterium mediated transformation of the constructs is successfully accomplished, since the roots obtained PTT resistance from the PTT resistance gene found in the construct.

Roots transformed with the CRU construct which eventually formed shoots on PTT plates were identified by performing a PCR to detect possible GT events.

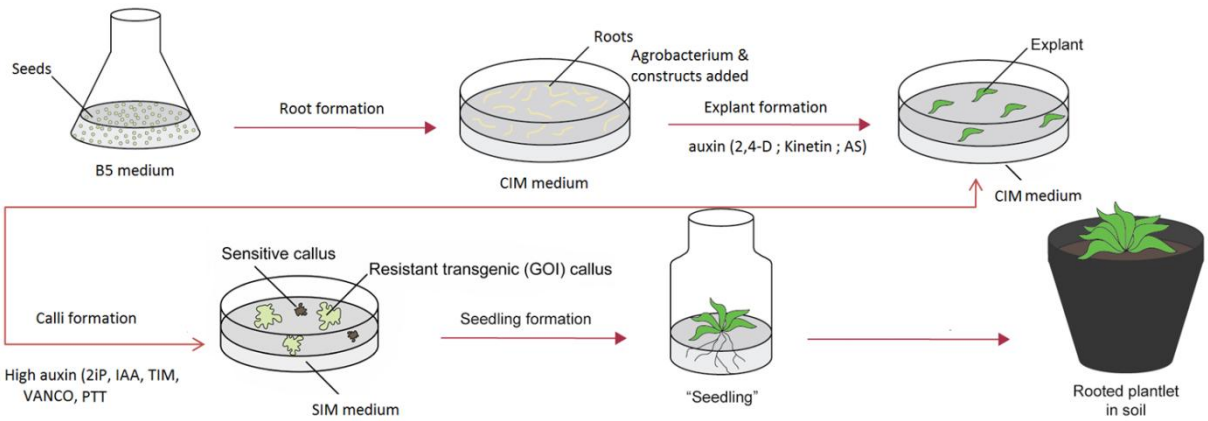

Figure 6. Schematic overview of the Arabidopsis thaliana Root Transformation. Seeds are grown in B5 medium, roots from germinated seeding are grown on CIM plates. Explants are exposed to Agrobacterium containing a binary vector. In the end resistant transgenic callus will be formed if the transformation was successful, which could develop to a fully functioning transgenic plant.

Material and methods

Sterilizing of seeds

Seeds of WT and all mutant lines were sterilized followed by a cold treatment. 3 Micrograms of seeds are weighted in eppendorf tubes (sterile) and sterilized for about 60 seconds in 1 ml 70% ethanol to remove the wax layer of the seeds and 20 minutes in 1 ml 1% hypochlorite solution. Next the seeds were rinsed 3 to 4 times in sterile milli-Q. Finally 1 ml of 0,1% agarose solution was added and the seeds were stored at 4 °C for two to four days.

Phenotypic analysis and root grow

Sterilized seeds were placed on ½ MS medium with and without Bleomycin (0,0250 mg/l), or MMS (0,005%). The plates with seeds were grown vertically in the growth room (21 °C, 16 hours light, 8 hours dark, 2000 lux) for 7 days. Roots were observed with the naked eye or by using a microscope.

GUS assay

Sterilized WT and *teb5* mutant seeds containing the UG-US gene – Hyg construct were plated on ½ Murashige & Skoog (MS) medium with Hygromycine (10mg/l) and grown for five to eight days.

The plants were transferred to 6-wells plates (max 30 plants per well) and 10 ml of liquid B5 medium (sterile) was added to each well. The grown plants were exposed to Bleomycin (0,0250 mg/l). After 24-hours of incubation in the growth room (21 °C, 16 hours light, 8 hours dark, 2000 lux) the treated plants were washed two times and grown in fresh liquid B5 medium (sterile) for five more days.

All plants were then washed with X-gluc buffer (4 ml per well) (Appendix 1) followed by a treatment with X-gluc solution (1 mg/ml, 4 ml per well) and incubated for 24 hours at 37 °C in the dark (plated were wrapped in aluminum foil) on a shaker (80 rpm.).

Finally the plants were washed several times with 70% EtOH until the natural green pigment chlorophyll of the leafs was removed. Blue spots can be observed using a microscope. A T-test was performed on all data to see if there was a significant difference between each WT / *teb5* counts and if there was a significant difference in the number of GUS spots between the WT and *teb5*.

CTAB extraction

A few leaves (2-3) of the *teb5* mutant and WT expressing the CRISPR/CAS9-CRU nuclease were cut of the plants, transferred to eppendorfs with a metal bead and frozen in liquid N_2. The leaves were pulverized by using a TissueLyser 2.0 (frequency 30,0 1/s ; time 0.30 min) followed by DNA extraction using the CTAB (Hexadecacyl Trimethyl Ammonium Bromide) method.

To each frozen sample 500 µl CTAB buffer (CTAB 2%; NaCl 1,4M; EDTA 20 mM; Tris/HCL 100 mM (pH 8,0); RNase 0,2 mg/ml) was added and incubated for 15 minutes at 37 °C, 500 rpm. Next 100 µl chloroform was added to each sample and incubated for 15 minutes at 65 °C, 500rpm. After that 150 µl chloroform and 250 µl buffer saturated phenol was added to each sample, shaken for 1 minute and centrifuged for 1 hour at max rpm at 18 °C.

The waterphase was removed without the interphase and transferred to a new eppendorf, followed by a second phenol/ chloroform extraction. When the waterphase was transferred again a chloroform only extraction was performed by adding 500 µl chloroform to each sample and centrifuge for 5 minutes at max rpm at 18 °C. The final waterphase was transferred again to a new eppendorf where the DNA was precipitated with 10 µl NaAc (pH 5.2) and 1 ml 100 EtOH, centrifuged for 15 minutes at max rpm at 4 °C. Thereafter the supernatant was trashed and the pellets were washed in 500 µl 70% EtOH followed by a 5 minutes centrifuge at max rpm at 4 °C.

Subsequently the supernatant was removed and the pellet was dried by using a Speedvac for 10 to 30 minutes at max rpm and max temperature. The dry pellet was dissolved in 100 µl milli-Q during 15 minutes at 55 °C followed by 16 hr at 4 °C.

Footprint analysis

The DNA (1 µg) was cut overnight at 37 °C with restriction enzyme *Pst* I (20 U). A Polymerase Chain Reaction (PCR) (Appendix 2) of the *Pst* I digested DNA was performed, followed by another *Pst* I digestion. Finally the PCR products were separated by gel electrophoresis.
The resistant DNA fragments were excised out of the gel by using a clean scalpel or razor blade and stored in eppendorfs. 1:1 Volume of binding buffer was added to each sample and the gel mixtures were incubated at 60 °C for 10 minutes or until the gel slice was completely dissolved. The gel mixture was vortexed briefly and loaded on the GeneJET purification column. 800 µl Of the solubilized gel solution was transferred to the column, centrifuged for 1 minute at max rpm and the flow-through was discarded. This process was repeated until the entire volume had been applied to the column membrane.

A 100 µl of Binding Buffer was added to the column membrane, centrifuged for 1 minute at max rpm and flow-through was discarded. Thereafter a washing step was performed by adding 700 µl of Washing Buffer, centrifuging for 1 minute at max rpm and discard the flow-through. The empty column was centrifuged again for another minute to completely remove residual wash buffer.
The column was then transferred into a clean 1,5 ml eppendorf tube where 10 µl of Elution Buffer and 10 µl milli-Q was added to the center of the purification column membrane. Then the column was centrifuged for 1 minute at max rpm and 10 µl of the solution was evaporated by using a Speedvac for 30 minutes at max rpm and max temperature. Finally the pellet was incubated for 15 minutes at 55 °C.

Blunt-End Cloning

A ligation reaction was setup for each sample containing 5 µl 2x Reaction Buffer, 4 µl DNA fragment, 0,5 µl pJET 1.2 / Blunt cloning vector (50 ng/µl) and 0,5 µl T4 DNA ligase. The mixtures were vortexed briefly and incubated for 5 minutes at RT. Next the incubated ligation mix with fragment was added to 200 µl competent cells (DH5α) mixed gently and stored on ice for 10 minutes. A heat shock was performed for precisely 45 seconds at 42 °C and immediately returned to ice where 1 ml of SOB medium was added.

The cells were incubated for 1 hour at 37 °C and 100 µl of each mixture was plated on LC plates containing Carbencillin (Cb) (200mg/ml). The remaining mixture was centrifuged for 1 minutes at 8000 rpm to which the supernatant was almost completely removed, remaining 100 µl in the eppendorf to resuspend the pellet in. This pellet mixture was also plated on LC plates + Cb and grow overnight at 37 °C.

Isolating plasmids

Tubes of 13 ml were filled with 2 ml liquid LC medium + Cb (sterile). One colony per tube was added by using a sterile cocktail stick. The whole cocktail stick was put into the tube. The tubes were incubated in a shaker at 150 rpm at 37 °C and grown overnight

The 2 ml suspensions of each tube/ sample were transferred to 2 ml eppendorfs after which they were centrifuged for 1 minute at max rpm. The supernatant was discarded and 200 µl of Solution 1 was added (50 mM Glucose, 25 mM Tris, 10 mM EDTA). The mixture was vortexed at max speed and 200 µl of Solution 2 was added (1 % SDS, 20 mM NaOH). Followed by gentle mixing until samples become transparent and viscous. Finally 200 µl of 3M KAC (pH 5.2) was added to each sample, which were gently mixed until white precipitation was visible.

The samples were then incubated on ice for 5 minutes and centrifuged for 10 minutes at 4 °C at max rpm. The supernatant (600 µl) was transferred to a new eppendorf, 360 µl of isopropanol was added and the mixture was centrifuged for 10 minutes at 4 °C at max rpm. Thereafter the supernatant was discarded, 500 µl of 70% EtOH was added and centrifuged for 5 minutes at 4 °C at max rpm. The supernatant was discarded again, and the pellet was dried by using a Speed Vac for 10 to 30 minutes. The pellet was dissolved in 50 µl milli-Q by incubating for 15 minutes at 55 °C.

Arabidopsis thaliana Root Transformation

Sterilized seeds were transferred to 250 ml Erlenmeyer's filled with 50 ml liquid B5 medium (sterile)(Appendix 3). The Erlenmeyer's were placed on a shaker (100 rpm.) in a growth room (21 °C, 16 hours light, 8 hours dark, 2000 lux) and incubated for 10 days.

After 10 days the roots were separated from hypocotyls, cotyledons and leaves, and grown on Callus Induction Medium (CIM) plates, containing auxin 2,4-D (0,5 mg/l) and Kinetin (0,05 mg/l) to stimulate plant grow, for 3 days in a growth room (25 °C, 16 hours light, 8 hours dark, 1500-2000 lux).

Agrobacterium strains were grown in 10 ml LC medium + antibiotics from a fresh LC-plate and grown overnight at 29 °C in a shaking water bath. The next day the OD_{600} of the 10-fold diluted overnight Agrobacterium culture were measured. Such an amount of bacteria of the overnight culture in sterile eppendorf tubes was pelleted, resulting in an OD_{600} of 0,1 in 20 ml and that amount was resuspended in 1 ml liquid B5 medium (sterile).

The roots of the CIM plates were transferred to a petridish containing 19 ml liquid B5 medium (sterile) and the 1 ml resuspended bacteria was added. The roots were mixed for at least 2 minutes in order to inoculate the roots with the agrobacterium strain completely. Next the roots were placed in the lid of the petridish, cut into 3-5 mm pieces (explants) using a sharp blade and dried briefly on sterile filterpaper.

The explants were plated on CIM plates containing the same auxins plus Acetosyringone (AS) for cocultivation to induce the bacteria for T-DNA and protein transfer into the roots. Cocultivation was done for three days in a growth room (25 °C, 16 hours light, 8 hours dark, 2000 lux).

The root explants were washed in liquid B5 and transferred to Shoot Induction Medium (SIM) plates, containing high cytokinin 2iP (5mg/l) and IAA (0,15mg/l) to stimulate plant grow, Vancomycin (100mg/l) and Timentin (100mg/l) to prevent growth of remaining Agrobacterium, and PTT (L-Phosphinothricin) (30 mg/l) for selection of T-DNA integration and grown for 2 to 3 weeks. (25 °C, 16 hours light, 8 hours dark, 2000 lux).

Results

Phenotypic analysis of teb5, ku80, teb5ku80 mutants and WT

The *teb5* mutant has a disrupted a-NHEJ DNA repair pathway, leaving the c-NHEJ and HR pathway as the only two repair pathways. The *ku80* mutant has a disrupted c-NHEJ DNA repair pathway, leaving the a-NHEJ and HR pathway as the only two repair pathways. Also double mutants were made by crossbreeding *teb5* and *ku80* to inactivated both a-NHEJ as c-NHEJ.

Phenotypic analysis were performed to study what the effect is on root grow and visual appearance of *teb5*, *ku80* and double mutant *teb5ku80* compared to the WT. Roots were exposed to Bleomycin and MMS to induce DSBs and SSBs (Figure 7).

The roots of the WT looked healthy based on the length of the roots, also the leaves looked normal and were well grown for 7 days of incubation. Compared to the WT, the roots of *ku80* were shorter and the leaves were also less well developed with less green pigment. The roots of the *teb5* were even shorter although the leaves were better developed because of the more intense green pigment. The double mutant *teb5xku80* was barely developed. The roots were 5 to 6 times shorter than the WT and 2 to 3 times shorter then both single mutants.

WT roots grown on ½ MS and the ones grown on Bleomycin were equal in length. The roots of the *ku80* and *teb5* were 2 to 3 times shorter on the Bleomycin plate, while the double mutant had barely grown any roots.

The MMS caused the WT to grow shorter roots than under normal conditions or under influence of Bleomycin. Both double mutant and *teb5* were 2 to 3 times shorter than on Bleomycin. The *ku80* had also shorter roots but two plants were able to grow roots with a similar length as under influence of Bleomycin.

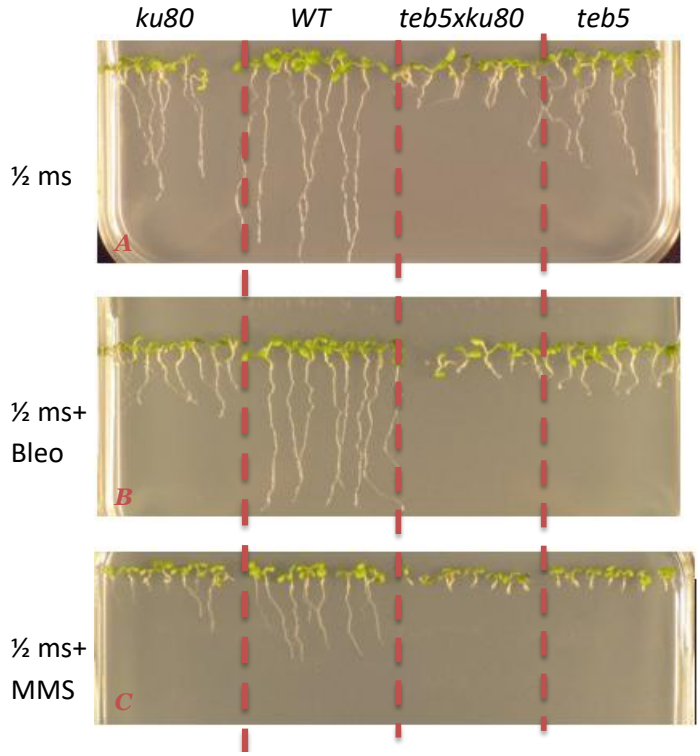

Figure 7. Phenotype of teb5, ku80 and double mutant teb5ku80 on root growth. A) The roots are grown on ½ ms medium without any extra added chemicals to damage the DNA. B) Roots are grown on the same ½ ms medium with added Bleomycin and C) with added MMS. The red dotted line separates the different types of Arabidopsis thaliana with each other. First on the left the ku80, followed by WT, next the teb5xku80 and last the teb5.

Nuclease induced DSB and footprint analysis in teb5 and WT

Because the *teb5* mutant had a inactive a-NHEJ DNA repair pathway, but still an active c-NHEJ and HR DNA repair pathway, *teb5* mutants expressing artificial nucleases were used to induce DSBs and studied to determine if the a-NHEJ pathway is involved in repair of these nuclease induced DSBs, which could lead to so called 'footprints'. When such nucleases induced a DSB close to or overlapping a restriction site, repair may result in removing the restriction site, leaving the sequence resistant for this specific restriction enzyme.

DNA from of leaves of *teb5* and WT expressing the CRISPR/CAS9-CRU nuclease was predigested with *Pst* I, PCR-ed and *Pst* I digested again. Undigested DNA was separated from digested DNA via gel electrophoreses, column purified, cloned in pJET1.2 and transformed in DH5α. Final plasmids were isolated and digested with *Pst* I and *Bgl* II and run on gel (Figure 8).

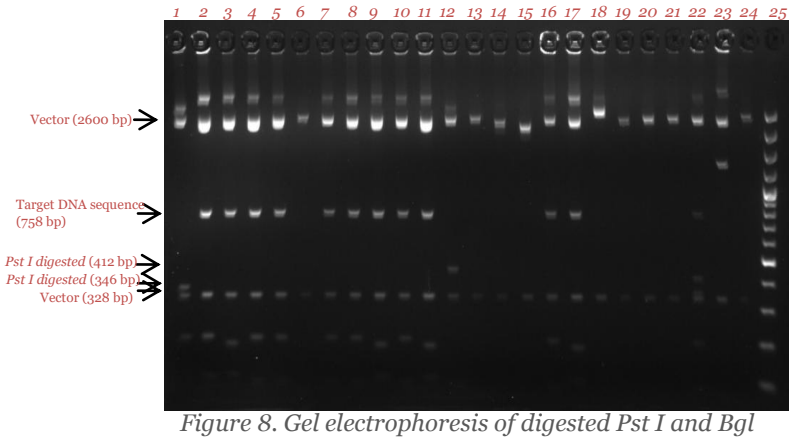

Figure 8. Gel electrophoresis of digested Pst I and Bgl II teb5 and WT plasmid DNA. 1,5% Agarose gel, 100bp ladder. Lanes 1-12 are clones of WT PCR product. Lanes 13-24 are clones of teb5 mutant. Lane 25 is a 1kb ladder.

Basically if the *Pst* I site is destroyed, the two sequence parts enclosed by *Bgl* II should be ligated together, resulting in three bands on gel, two of the original vector and one of the ligated target DNA sequence. However if there were four bands visible (two of the original vector and two separated bands of the target DNA sequence, which are not ligated together) NHEJ of DSB could still have taken place without destroying the *Pst* I site.To be sure NHEJ of DSB has taken place the plasmids were sequenced to search for small insertions or/and deletions (Figure 9).

DNA of lane 2, 3, 7 (*teb5)*

```
Query  447  GGGACCTCATGCTGCAGATAGTCTCCTCAAGGCCGTTGCCCTGTGGGCTACGTGGGTGTC  506
            |||||||||    ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GGGACCTCA---TGCAGATAGTCTCCTCAAGGCCGTTGCCCTGTGGGCTACGTGGGTGTC  417
```

DNA of lane 16, 17 (WT)

```
Query  447  GGGACCTCATGCTGCAGATAGTCTCCTCAAGGCCGTTGCCCTGTGGGCTACGTGGGTGTC  506
            |||||||        ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GGGACCT-------CAGATAGTCTCCTCAAGGCCGTTGCCCTGTGGGCTACGTGGGTGTC  413
```

Figure 9. Footprint analysis of WT and teb5 mutant. Small pieces of comparisons DNA are shown, the marked yellow nucleotides are the places were footprints are found. The Query is the original nucleotide sequence of target DNA, the Sbjct is the sequenced DNA of the samples with the footprints.

In the DNA of lane 2, 3 and 7 which were all from the WT, the same footprint sequences were found. The 'TGC' nucleotides on sequence location 456 – 459 found in these samples were deleted compared to the ones of the original nucleotide sequence. The DNA of lane 16 and 17, which were both from the *teb5* mutant, also contained a single footprint, on sequence location 454-460 'CATGCTG'.

This experiment was performed a second time, in hope to find more different footprint. Also a new sample of *teb5* mutant was used and 8 different clones of *teb5* mutant. Unfortunately the exact same footprints were found 8 times and were exactly the same as in the previous experiment (Figure 10).

DNA of *teb5*

```
Query  421   ACGAGCAGGGTCGTCAATGTTCTCGTGGGACCTCATGCTGCAGATAGTCTCCTCAAGGCC  480
             ||||||||||||||||||||||||||||||||||   ||||||||||||||||||||||          8x
Sbjct  458   ACGAGCAGGGTCGTCAATGTTCTCGTGGGACCTCA---TGCAGATAGTCTCCTCAAGGCC  514
```

Figure 10. Footprint analysis teb5 mutant. Small pieces of sequence DNA are shown, the marked yellow nucleotides are the places were footprints are found. The Query is the original nucleotide sequence of target DNA, the Sbjct is the sequenced DNA of the samples with the footprints.

Intrachromosomal recombination of UG-US (GUS) in teb5 mutant and WT

The GUS experiments were performed to determine if intrachromosomal HR in *teb5* mutants could still occur. Only if HR occurs between the related sequences that are in opposite orientations on the same chromosome, the intact reporter gene GUS will be formed which can be visualized as blue spots after histochemical staining. To boost HR, the plants were exposed to Bleomycin which induced DSBs and thus DNA repair. In this experiment 3 types of plant lines were used: *teb5* heterozygote mutant (+/-), *teb5* homozygote mutant (-/-),and homozygote *teb5* (+/+). Both *teb5* (+/-) and (+/+) are used as WT and were made via crossbreeding and thus originating from the same plant as the *teb5* (-/-) plants.

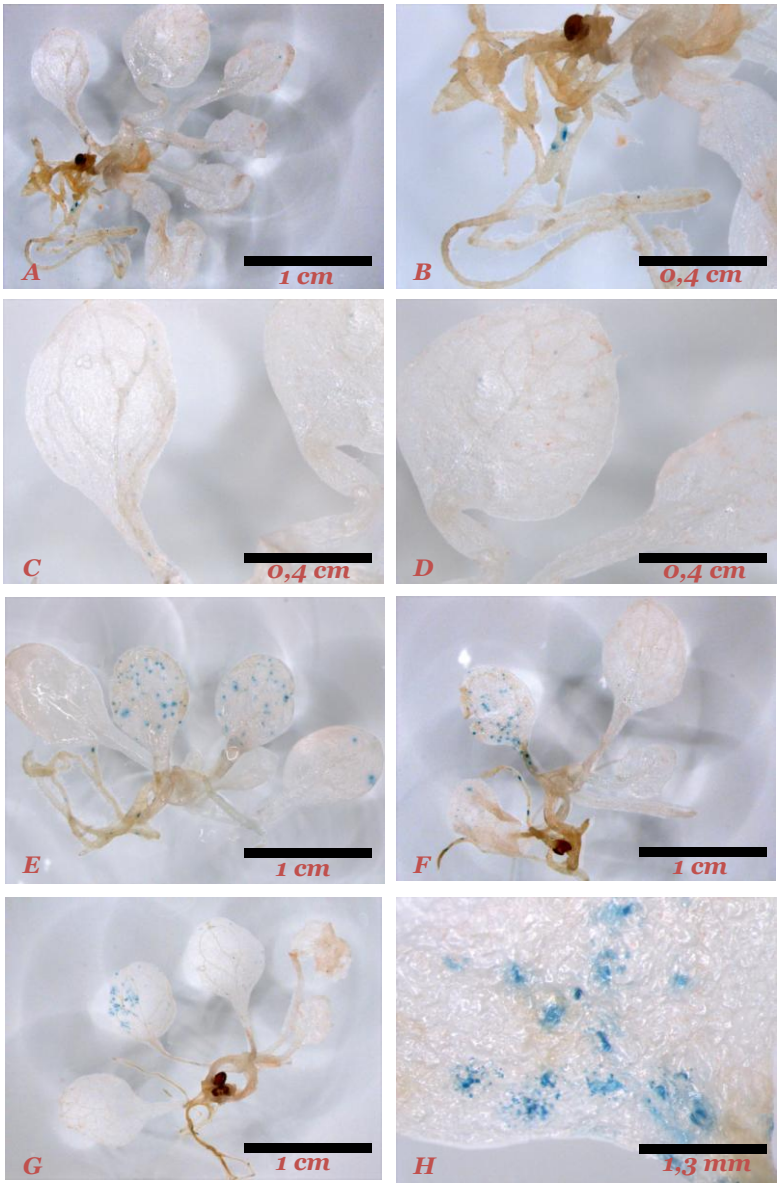

Figure 11. Arabidopsis thaliana with UG-US construct, histochemical stained to visualize gene activity of the complete formed GUS gene. In figure 10.A-D an example of the teb5 (+/+)(WT) is shown, in figure 10.E-H example of teb5 (-/-)(mutant) are shown.

In Figure 11.A-D an example of a *teb5* (+/+)(WT) plant is shown. This plant was exposed to Bleomycin. While the plant was grown well for 15 days and several chemical treatments, only 2 blue spots (11.A-B) appeared on a root after the histochemical staining. All other roots and five leafs (11.C-D) were blue spot free.

Figure 11.E-H shows examples of a *teb5* (-/-)(mutant) plants, also exposed to Bleomycin. The *teb5* plants were grown to a similar length as the WT plant, but contained a tremendously number of GUS spots more compared to the WT. In leaves and roots the GUS construct was successfully recombined as shown in Figure 11.E-H.

These are just a few examples, in total 150 WT and 125 *teb5* plants were tested in the same experiment (Appendix 5).

All these plants were observed under a microscope and all visualized gene activity of the complete formed GUS gene by expressing blue spots were counted.

Of plant line 4 *(*WT (*teb5* +/+)) 80 plants were tested containing in total 568 GUS spots, which makes an average of 7,10 GUS spots per plant (Figure 12).

Next 70 plants of plant line 5 (WT (*teb5* +/-)) were tested and they contained a total of 445 GUS spots with an average of 6,36 GUS spots per plant.

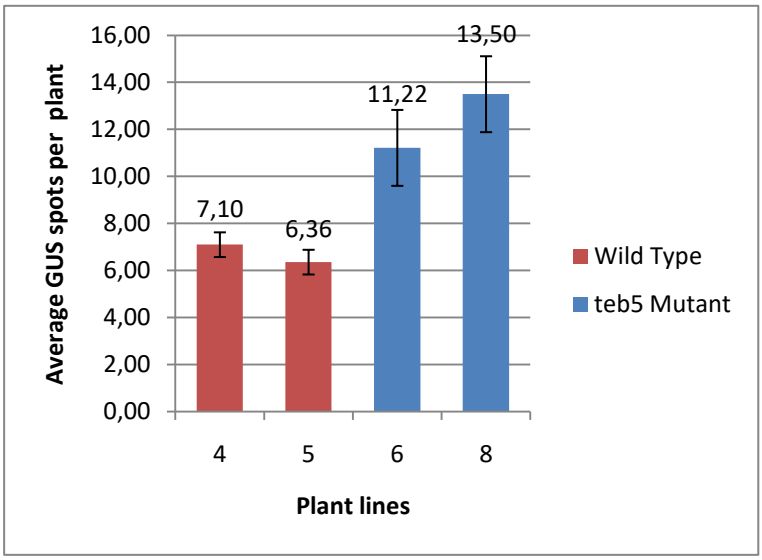

Figure 12. Overview of average intrachromosomal HR of the GUS gene in WT (plant lines 4 and 5) and teb5 (plant lines 6 and 8). On the Y-axis the average number of GUS spots per plant are shown, and on the X-axis the different plant lines are shown. The red colored bars indicate the Wild Type and the blue colored bars the teb5 mutant.

The same was done for plant line 6 (*teb5* (*teb5* -/-)), 83 plants were tested with a total of 931 GUS spots and an average of 11,22 GUS spots per plant. Last 42 plants of plant line 8 (*teb5* (teb5 -/-)) were tested, containing 567 GUS spots in total, creating an average of 13,50 GUS spots per plant.

The total count of all GUS spots of all plants were converted to percentages because an unequal number of plants were tested (Figure 13). For example: In total 1 GUS spot was found in 16,5% of the WT plants and 7,9% of the teb5 plants, while in total 91 GUS spots were found in 1% of the teb5 plant and 0% of the WT plants.

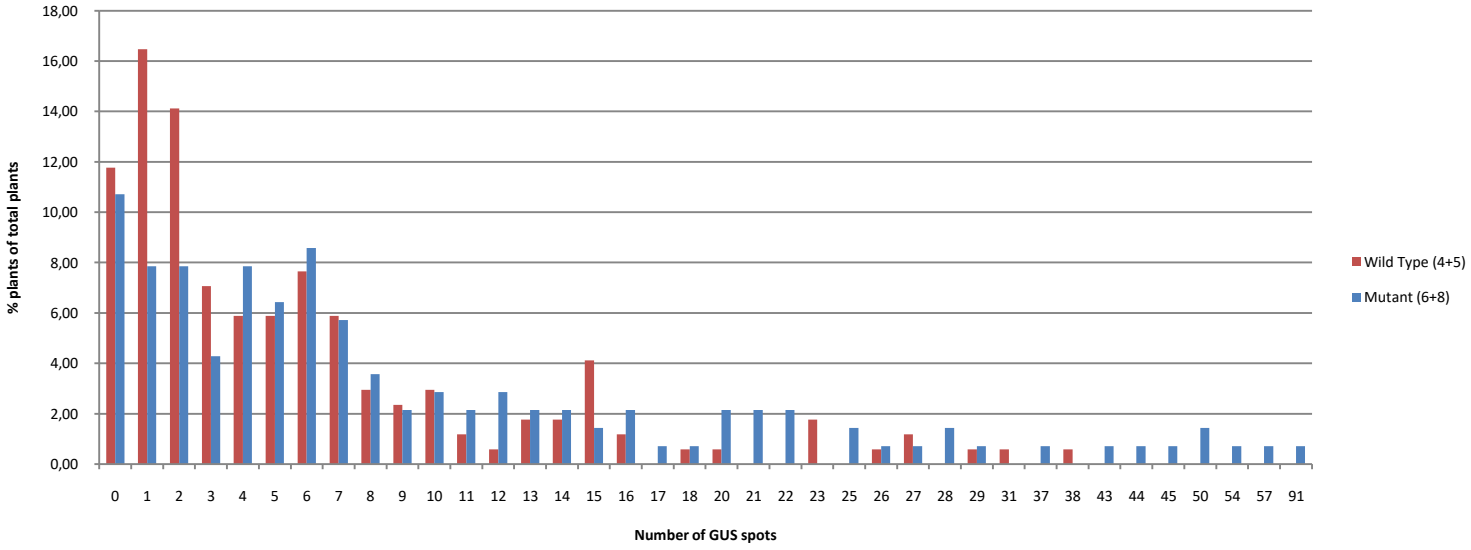

Figure 13. Overview of all intrachromosomal HR of the GUS gene in WT (plant lines 4 & 5) and teb5 (plant lines 6 & 8). On the Y-axis the percentages plants of total plants are shown in how often a specific number of GUS spots appears, X-axis. The red colored bars indicate the Wild Type and the blue colored bars the teb5 mutant.

The GUS experiment was performed a second time. This time plant lines 5 (WT (*teb5* +/-)) and 6 (*teb5* (*teb5* -/-)) were tested for a second time and two new plant lines, 7 *(*WT (*teb5* +/+)) and 12 (*teb5* (*teb5* -/-)) were tested for the first time to see if similar results would be found. Also more plants were tested in this experiment and an equal number of plants.

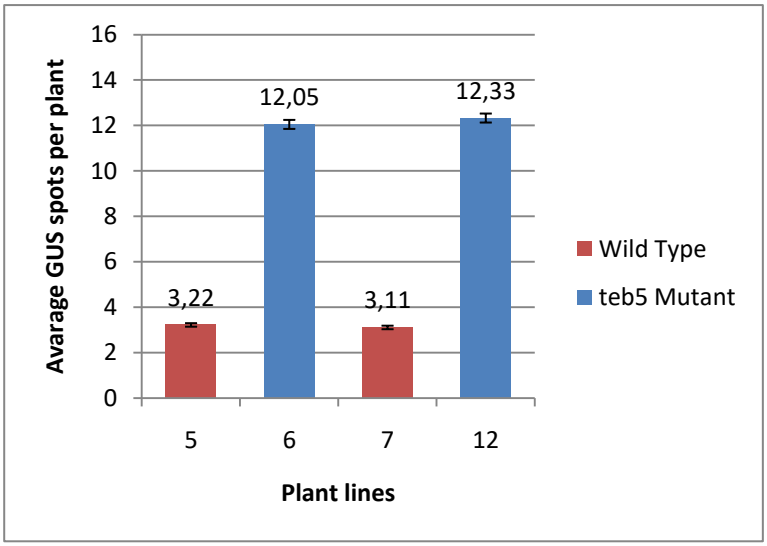

Figure 14. Overview of average intrachromosomal HR of the GUS gene in WT (plant lines 5 & 7) and teb5 (plant lines 6 & 12). On the Y-axis the average amount of GUS spots per plant are shown, and on the X-axis the different plant lines are shown. The red colored bars indicate the Wild Type and the blue colored bars the teb5 mutant.

Of all plant lines a number of 550 plants each were examined. In plant line 5 a total of 1771 GUS spots were found, with an average of 3,22 GUS spots per plant (Figure 14). A similar number of total GUS spots, 1711, were found in plant line 7 with an average of 3,11 GUS spots per plant. Also for both *teb5* plant lines similar results were achieved compared to each other. Plant line 6 had a total of 6630 GUS spots with an average of 12,05 GUS spots per plant and plant line 7 6783 total GUS spots with an average of 12,33 GUS spots per plant. The total number of plants with a certain number of GUS spots are shown in Figure 15. For example: In total 2 GUS spots were found in 90 WT plants and 55 teb5 plants, while in total 26 GUS spots were found in 20 teb5 plants and 0 WT plants.

In both GUS experiments intrachromosomal homologous recombination of the GUS gene occurred in WT & *teb5. A* significant higher HR ratio of the GUS gene was found in *teb5* compared to the WT, resulting in a 2 to 4 times higher GUS spot ratio.

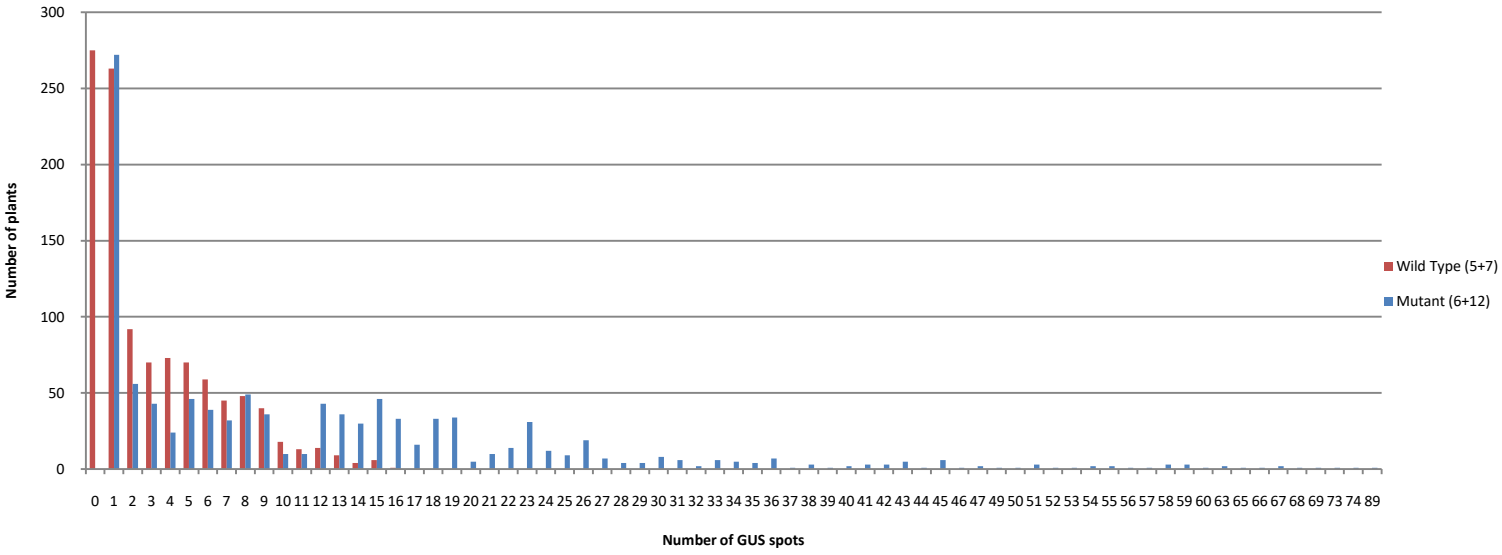

Figure 15. Overview of all intrachromosomal HR of the GUS gene in WT (plant lines 5 & 7) and teb5 (plant lines 6 & 12). On the Y-axis the total number of plants are shown in how often a specific number of GUS spots appears, X-axis. The red colored bars indicate the Wild Type and the blue colored bars the teb5 mutant.

Gene targeting in teb5 and WT via Arabidopsis Thaliana Root Transformation

Because intrachromosomal HR had occurred in the *teb5* mutant, subsequently was tested if GT could occur whereby a homolog T-DNA is build in via HR. In Gene Targeting experiments the mutant *teb5* was studied to determine if HR still occurred. Since *teb5* has a blocked random integration system and the HR pathway as the only repair pathway available. Gene Targeting experiments were performed via *Arabidopsis thaliana* Root Transformations.

A CRISPR/CAS9 – CRU2 – CRU2 RT – PTT gene construct was used with Cruciferin as cleaving target and introduced via *Agrobacterium* T-DNA integration into the roots/ explants of WT and *teb5*. The amount of explants were counted at the beginning of the cocultivation and afterwards when calli were formed. Because random integration was blocked in the *teb5* mutant formed PTT resistant calli would indicate GT. As shown in Figure 16. there is no significant difference in the amount of roots/ explants and formed calli between the *teb5* and WT.

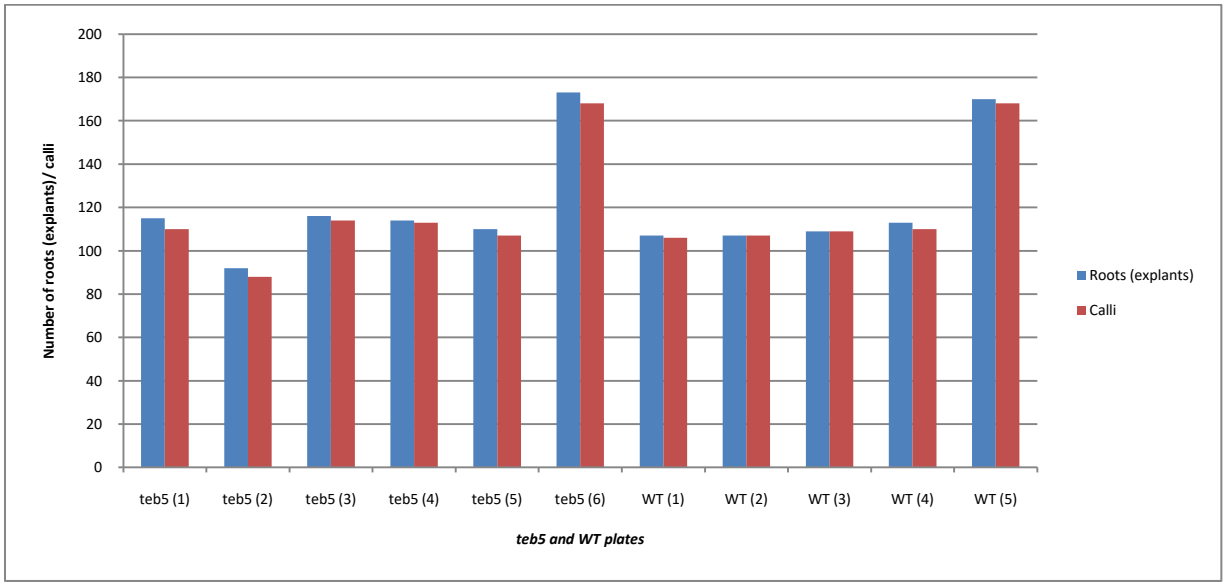

Figure 16. Overview of counted roots (explants) at the beginning of the cocultivation and the formed calli afterwards in WT and teb5. On the Y-axis the number of roots (explants) / calli and on the X-axis the different teb5 and WT plates. The blue colored bars indicate the roots (explants) at the beginning of the inoculation, and the red colored bars the calli formed afterwards.

These calli were transferred and divided to new plates, in total 13 *teb5* and 12 WT plates were obtained. The calli was observed for several weeks and photographs were taken each week to study the development and keep track of the progress of the calli. In figure 17. an example of a WT plate (6) and *teb5* plate (13) is shown.

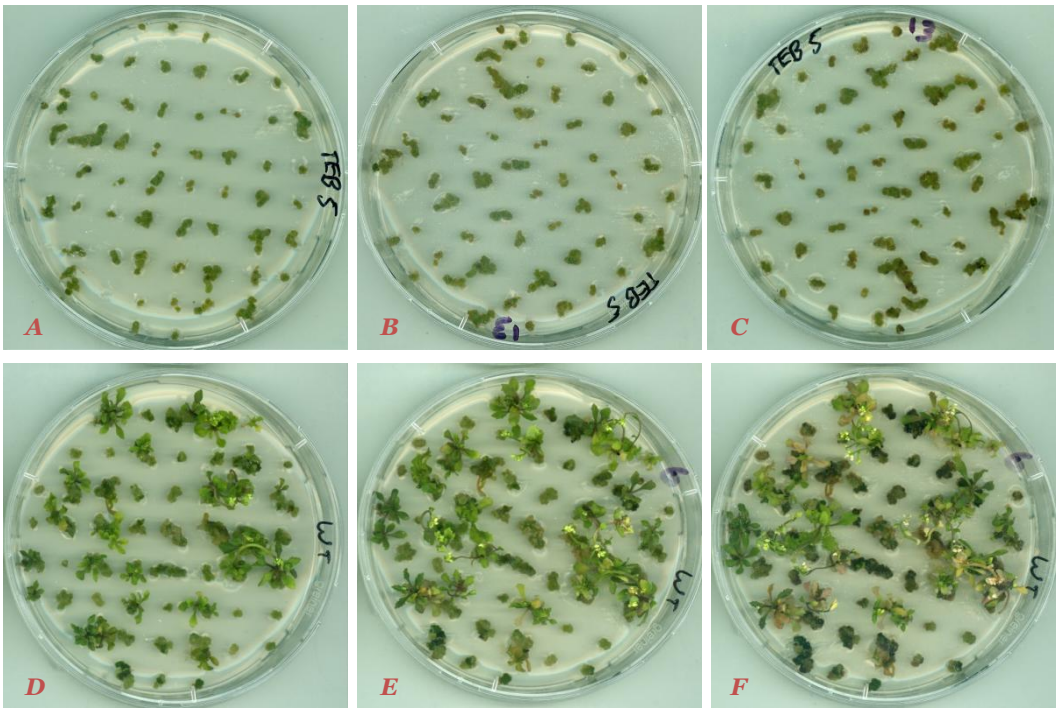

Figure 17. Example of the gene targeting in teb5 and WT via Arabidopsis thaliana Root Transformation. CRU2 construct formed calli. A,D = Day 52 ; B,E = Day 59 ; C,F = Day 5. The upper 3 pictures (A, B, C) is the teb5 plate nr. 13 photographed starting at day 52 of the root transformation (A), day 52 (B) and day 59 (C). The lower 3 pictures (D, E, F) is the WT plate nr. 6 photographed over the same period of time.

The visual appearance of *teb5* calli compared to the WT calli looked different. While the WT calli were developing over time and growing shoots (Figure 17. D-F), the *teb5* calli hardly developed or grew barely (Figure 17. A-C). Despite the fact that no shoots were grown from the *teb5* calli the decision was made to isolate the DNA of both *teb5* and WT, 24 calli from each plate, in order to detect possible GT events.

In total four PCR's were performed with different primer mixes, two for the 5'recombination end and two for the 3'recombination end (Appendix 4). The primer 'GS1' with a recognition site in the 5'-end CRU gene construct was combined with primer 'SP262' with a recognition site in the GFP gene in the same construct, to determine if the construct was present. SP262 was also combined with primer 'SP261', which has a recognition site in the 5'-end targeted gene outside the construct. If the construct is build in correctly and so a GT event has occurred, these primers should be able to generate a product (Figure 18).

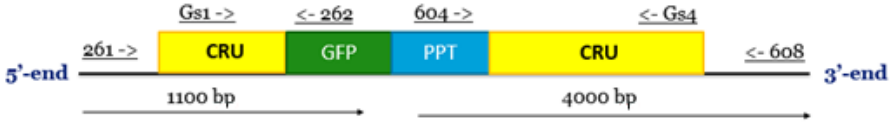

Figure 18. Schematic overview of the CRU construct integrated via HR in the targeted gene. In this overview the primer recognition sites location and the length of the PCR products are shown. The CRU construct does not only contain a PTT gene, which was used in the root transformation assay as a selection of integration on PTT plates and recognition site for primer SP604 , but also a GFP gene, which was later used as a recognition site for primer SP262.

The same was done for the 3'recombination end. The primer 'GS4' has a recognition site in the 3'-end CRU gene construct was combined with primer 'SP604' with a recognition site in the PTT gene in the same construct, again to determine if the construct was even present. Primer 'SP608' with a recognition site in the 3'-end target gene outside the construct was also combined with primer SP604 to determine if a GT event has occurred. All PCR products were runned on a agarose gel (Figure 19).

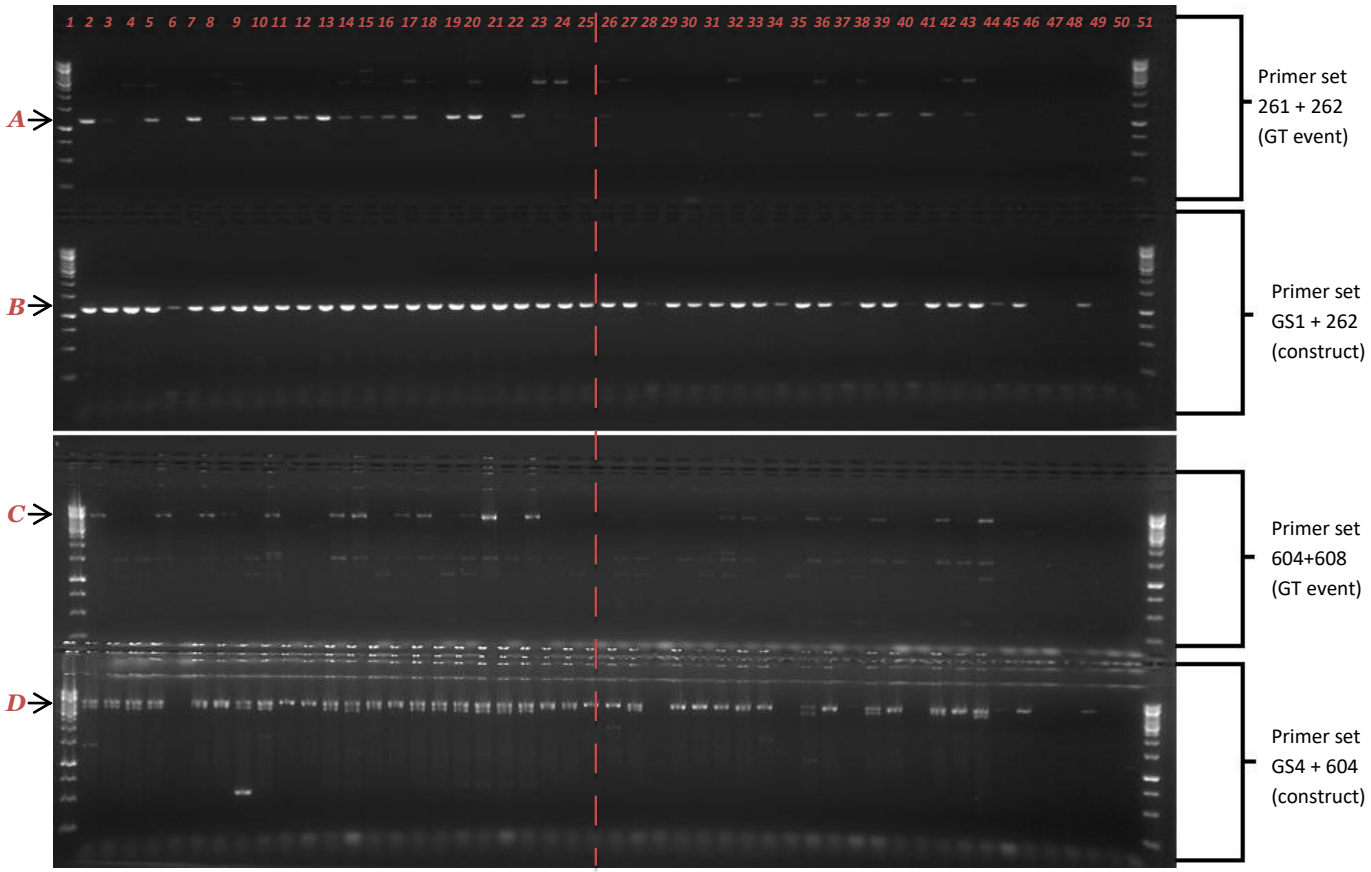

Figure 19 Photographed 1% agarose gels under exposure of UV-light. In both A, B, C, D slot 1 contained a 1kb DNA ladder, slots 2-25 the isolated WT DNA and slots 26-49 the isolated teb5 DNA, followed by a negative water control in slot 50 and a 1kb DNA ladder in slot 51. A-B is the 5'recombination end gel where primers mixes 261+262 (A) for GT events and GS1+262 (B) for construct integration were used. C-D is the 3'recombination end gel where primer mixes 604+608 (C) for GT events and GS4+604 (D) for construct integration were used.

In both B for the 5'recombination end and D for the 3'recombination end with the primer mixes to control if the construct was present, bands were visible in almost every lane. Meaning the CRU2 construct was present in most of the calli. All bands that were also found in both A (5'recombination end) and C (3'recombination end) with primer mixes to control if the construct was integrated into the targeted DNA via HR were possible GT events.

Discussion and conclusions

Phenotypic analysis of *teb5*, *ku80*, *teb5xku80* showed shorter roots compared to the WT, and thus showing morphological defects. The Teb gene which encodes the POL- θ which is required to promote a-NHEJ dependent DSB repair in mammalian cells was blocked in the *teb5* and *teb5xku80* mutant, resulting a less efficient DSB repair system, causing shorter roots. It is know that the protein KU80 forming a hetrodimer in combination with KU70, plays a crucial role in DSB recognition in the c-NHEJ. Roots of the double mutant *teb5xku80* grew even less to barely, since both a-NHEJ and c-NHEJ were inactivated, leaving HR is the only repair pathway left. Under normal conditions both *ku80* as *teb5* and even the double mutant *teb5xku80* could survive and grow, Although the a-NHEJ and/or c-NHEJ repair pathways are inactivated. However when DSB/SSB inducing reagents (Bleo/MMS) were added to create more DNA damage the double mutant *teb5xku80* was not able to develop roots and thus survive. It seems that *ku80* can grow roots and survive under influence of both reagents, while *teb5* can only survive Bleo, and is more sensitive for MMS, suggesting that the a-NHEJ pathway, which is inactivated in the *teb5* mutant, is primarily involved in the repair of SSBs and not DSBs.

Intrachromosomal homologous recombination of the GUS gene occurred in both WT and *teb5. A* significant higher HR ratio of the GUS gene was found in *teb5* compared to the WT. Because this experiment was performed twice, with the same mutants, under the same conditions, and in total more than 2475 plants were tested, the reliability of the results is extremely high. Since the a-NHEJ repair pathway is inactivated in the *teb5*, leaving only the c-NHEJ and HR repair pathways available, more HR events of the GUS gene occurred, resulting in a 2 to 4 times higher GUS spot ratio compared to the WT, where both a-NHEJ and c-NHEJ repair occurred at the expense of HR.

DSB repair of *teb5* expressing the CRISPR/CAS9-CRU1 nuclease via NHEJ was successfully accomplished. Small deletions and thus footprints were found in several samples. Although only a few footprints were found, more samples must be tested to find more and different footprints, in order to determine if footprints are different in *teb5* compared to WT. In future experiments a CRU2 construct with PPO gene will be used since the CRU1 is not that active. This construct is already tested and plants are in development for crossbreeding.

Green *teb5* and WT calli were formed containing a CRISPR/CAS9 – CRU2 – CRU2 gene construct introduced via *Agrobacterium* transformation. Based on the visual appearance, green color, growth and forming of green calli, GT events could have occurred, since the explants/ calli would have died otherwise without the activity of a PTT resistance gene. However the *teb5* never formed shoots like the WT did. Based on the results of the PCR with specific 5'and 3'-end GT primers, GT events did occur in several calli, and the construct was present in almost all.

Despite the fact that GT events were found with these specific primers, it is doubtful that the *teb5* calli never grew any shoots like the WT did. On the other hand as seen in previous phenotype experiments *teb5* suffers from morphological defects, and has always look different from the WT. However it cannot be excluded that maybe PCR artifacts were formed during this process, constructs were transient present instead of built-in and/ or the original *Agrobacterium* DNA was still present. This experiment should at least be repeated once to see and try to obtain shoots from the mutant, maybe by changing the concentration of cytokinins.

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Appendices

1. Reagents preparation list

X-gluc Buffer (Triton/ sacrosyl) 100ml	
-1.0M $NaH_2PO4.2H_2O$ (pH 7.0)	5 ml
-10 % Triton X-100	1 ml
-Na2EDTA	2 ml
-Sarcosyl	2 ml
-K3-Ferricyanide	0,5 ml
-K4-Ferrocanide	0,5 ml
-Milli-Q	89 ml

X-gluc solution (Triton/ sacrosyl) 100ml	
-1.0M $NaH_2PO4.2H_2O$ (pH 7.0)	5 ml
-10 % Triton X-100	1 ml
-Na2EDTA	2 ml
-Sarcosyl	2 ml
-K3-Ferricyanide	0,5 ml
-K4-Ferrocanide	0,5 ml
-Milli-Q	88,5 ml
-1mg/ 5µl X-Gluc in DMSO	0,5 ml

2. PCR primers for footprints

Primer name	Target sequence	TM	PCR program
SP 245	TGCCAACACTCCAGGCTCTG	68.9	A
SP 248	CCGTTGTCGTTGACCACTTG	66.5	A

**PCR program A:* 4 min 98C; 35 cycli [15s 98C, 15s 65C, 30s 72C]; 3 min 72C

3. Medium preparation list

1.1) B5 medium liquid	per liter:
-Gamborg B5 medium, including vitamins Prod# G0210.0050	3,16 g
-glucose	20 g
-MES	0.5 g
(0.8 % Daishin agar (Brunschwig Chemie)	8 g)

pH 5.7 Sterilize by autoclaving 20 minutes 110 °C

1.2) CIM (Callus induction medium)

-B5 medium + 0.8 % agar

Add to sterilized medium (cooled down):

-0.5 mg/l 2,4-D (stock 10 mg/ml in DMSO, store at -20°C)

-0.05 mg/l kinetin (stock 5 mg/ml in DMSO, store at -20 °C)

For cocultivation add: 100 µM acetosyringone (AS) (stock 0.2 M in DMSO, store at 4 °C)

1.3) SIM (shoot induction medium)

-B5 medium + 0.8 % agar

Add to sterilized medium:

-5 mg/l 2iP (stock 20 mg/ml in DMSO, store at -20°C)

-0.15 mg/l IAA (stock 1.5 mg/ml in DMSO, store at -20°C)
To prevent bacterial growth add:
-100 mg/l vancomycin (stock 100 mg/ml in H_2O, filtersterile)
in combination with phosphinothricin or
-100 mg/l timentin (stock 100 mg/ml in H_2O, filtersterile)
For selection:
- 20 mg/l hygromycin (Calbiochem!) (stock 50 mg/ml)
- 30 mg/l L-phosphinothricin (PTT) (combined with vancomycin and carbenicillin)

1.4) 1/2 MS medium per liter:

-Murashige and skoog medium mod no. 1B Micro and ½ macro elements including vitamins. Prod# M0233.0050	2,29 g
-2 % sucrose	20 g
-MES	0.5 g
-0.7 % Daishin agar	7 g

pH 5.8 Sterilize by autoclaving 20 minutes 110 °C

4. PCR primers for HR & GT events

Primer name	Target sequence	TM	PCR program
SP 261	CTCAGCAATCTCCTCGTTG	61.2	A
SP 262	TCGCCCTTGCTCACCAT	65.7	A
SP 604	ATGAGCCCAGAACGACGCCC	72.8	B
SP 608	CAGAAACAGAGCACCAAATGGG	67.7	B
GS 1	TAGGTTCTCCTCGTCCTTAACGG	63.0	A
GS 4	AAACTACTTGGATCATGCACGAG	64.0	B

*PCR program A: 4 min 98C; 35 cycli [15s 98C, 15s 63C, 40s 72C]; 5 min 72C
*PCR program B: 4 min 98C; 35 cycli [15s 98C, 15s 66C, 150s 72C]; 5 min 72C

5. *Raw data GUS* experiment

Experiment 1

4)	
4.1)	spots
1	18
2	1
3	1
4	9
5	5
6	10
7	9
8	7
9	3
10	2
11	16
12	3
13	5
14	8
15	5
16	2
17	1
18	3
19	23
20	2
21	7
22	3
23	14
24	1
25	7
26	2
27	2
28	4
Total	173
Avarge	6,18

4)	
4.2)	spots
1	2
2	2
3	2
4	4
5	6
6	2
7	38
8	7
9	2
10	10
11	9
12	23
13	4
14	8
15	6
16	15
17	2
18	6
19	31
20	1
21	15
22	3
23	3
24	7
25	4
Total	212
Avarge	8,48

4)	
4.3)	spots
1	7
2	13
3	1
4	6
5	3
6	5
7	2
8	6
9	1
10	1
11	2
12	11
13	6
14	6
15	5
16	5
17	15
18	2
19	1
20	4
21	4
22	4
23	1
24	7
25	15
26	27
27	23
Total	183
Avarge	6,78

5)	
5.1)	spots
1	2
2	2
3	13
4	3
5	10
6	29
7	2
8	6
9	10
10	6
11	4
12	27
13	2
14	8
15	8
16	2
17	1
18	7
19	13
Total	155
Avarge	8,16

5)	
5.2)	spots
1	7
2	8
3	1
4	10
5	15
6	1
7	4
8	2
9	2
10	20
11	1
12	15
13	2
14	1
15	3
16	1
17	1
18	9
19	1
20	1
21	5
22	2
23	1
24	16
Total	129
Avarge	5,38

5)	
5.3)	spots
1	1
2	3
3	14
4	1
5	5
6	3
7	11
8	7
9	26
10	6
11	1
12	1
13	1
14	2
15	1
16	3
17	14
18	6
19	4
20	5
21	6
22	5
23	15
24	6
25	1
26	12
27	1
Total	161
Avarge	5,96

All spots 4.	568
All plants 4.	80
All average 4.	7,10
All spots 5.	445
All plants 5.	70
All average 5.	6,36
All spots 4.+5.	1013
All plants 4.+5.	150
All average 4.+5.	6,75
STD	0,525279

6)	
6.1)	spots
1	22
2	21
3	12
4	4
5	21
6	50
7	5
8	4
9	9
10	4
11	20
12	1
13	43
14	37
15	4
16	4
17	29
18	5
19	14
20	12
21	8
22	4
23	14
24	6
25	16
26	6
27	5
Total	380
Avarge	14,07

6)	
6.2)	spots
1	7
2	3
3	6
4	7
5	27
6	12
7	1
8	16
9	5
10	5
11	6
12	9
13	7
14	1
15	8
16	7
17	2
18	8
19	2
20	4
21	3
22	11
23	5
Total	163
Avarge	7,04

6)	
6.3)	spots
1	28
2	6
3	13
4	11
5	3
6	1
7	6
8	22
9	45
10	2
11	11
12	1
13	4
14	26
15	1
16	6
17	8
18	14
19	4
20	15
21	1
22	25
23	3
24	16
25	6
26	10
27	6
28	7
29	20
30	7
31	57
32	1
33	3
Total	389
Avarge	11,79

8)	
8.1)	spots
1	91
2	6
3	8
4	2
5	2
6	50
7	10
8	2
9	13
10	1
11	21
12	4
13	10
14	5
15	22
16	1
17	5
Total	253
Avarge	14,88

8)	
8.2)	spots
1	2
2	18
3	6
4	1
5	2
6	7
7	10
8	7
9	5
10	20
11	15
12	44
13	25
14	17
Total	179
Avarge	12,79

8)	
8.3)	spots
1	9
2	2
3	6
4	2
5	28
6	13
7	3
8	2
9	12
10	54
11	4
Total	135
Avarge	12,27

All spots 6.	931
All plants 6.	83
All average 6.	11,22
All spots 8.	567
All plants 8.	42
All average 8.	13,50
All spots 6.+8.	1498
All plants 6.+8.	125
All average 6.+8.	11,98
STD	1,614418

Experiment 2

5)		5)		5)		5)		5)		5)		5)		5)		5)		5)	
5.1)	Spots	5.2)	Spots	5.3)	Spots	5.4)	Spots	5.5)	Spots	5.6)	Spots	5.7)	Spots	5.8)	Spots	5.9)	Spots	5.10)	Spots	5.11)	Spots
1	2	1	5	1	2	1	1	1	1	1	12	1	8	1	9	1	4	1	2	1	4
2	3	2	9	2	1	2	1	2	1	2	4	2	4	2	8	2	7	2	1	2	3
3	1	3	0	3	6	3	1	3	1	3	0	3	6	3	8	3	2	3	6	3	5
4	4	4	8	4	2	4	2	4	1	4	0	4	1	4	0	4	2	4	3	4	1
5	2	5	10	5	0	5	9	5	0	5	0	5	1	5	0	5	1	5	1	5	2
6	4	6	2	6	9	6	2	6	8	6	8	6	4	6	1	6	4	6	0	6	4
7	1	7	2	7	0	7	0	7	2	7	0	7	1	7	3	7	0	7	4	7	4
8	0	8	2	8	3	8	1	8	3	8	2	8	9	8	1	8	0	8	1	8	0
9	3	9	1	9	8	9	2	9	5	9	2	9	0	9	0	9	0	9	1	9	2
10	2	10	0	10	7	10	0	10	0	10	0	10	12	10	6	10	5	10	1	10	2
11	15	11	2	11	2	11	8	11	0	11	3	11	0	11	4	11	7	11	1	11	0
12	0	12	5	12	2	12	2	12	12	12	5	12	12	12	4	12	5	12	7	12	1
13	1	13	1	13	5	13	6	13	6	13	0	13	1	13	0	13	14	13	15	13	0
14	5	14	4	14	0	14	7	14	0	14	2	14	1	14	3	14	2	14	3	14	6
15	1	15	3	15	0	15	0	15	2	15	11	15	3	15	8	15	2	15	1	15	7
16	5	16	6	16	10	16	0	16	1	16	4	16	4	16	1	16	6	16	9	16	8
17	1	17	2	17	0	17	3	17	4	17	1	17	2	17	0	17	0	17	2	17	3
18	1	18	0	18	1	18	0	18	3	18	3	18	6	18	6	18	0	18	0	18	0
19	0	19	1	19	10	19	2	19	8	19	10	19	6	19	0	19	1	19	4	19	2
20	7	20	9	20	1	20	2	20	0	20	6	20	4	20	1	20	0	20	1	20	0
21	2	21	4	21	3	21	1	21	1	21	1	21	3	21	0	21	3	21	4	21	2
22	0	22	0	22	0	22	14	22	0	22	4	22	1	22	2	22	0	22	4	22	1
23	0	23	0	23	7	23	8	23	0	23	0	23	0	23	2	23	4	23	0	23	0
24	15	24	0	24	0	24	2	24	6	24	0	24	8	24	6	24	0	24	10	24	4
25	1	25	9	25	6	25	4	25	0	25	10	25	5	25	1	25	5	25	0	25	1
Total	76	Total	85	Total	85	Total	78	Total	65	Total	88	Total	102	Total	74	Total	74	Total	81	Total	62
Avarage	3,04	Avarage	3,4	Avarage	3,4	Avarage	3,12	Avarage	2,6	Avarage	3,52	Avarage	4,08	Avarage	2,96	Avarage	2,96	Avarage	3,24	Avarage	2,48

5)		5)		5)		5)		5)		5)		5)		5)		5)		5)		5)	
5.12)	Spots	5.13)	Spots	5.14)	Spots	5.15)	Spots	5.16)	Spots	5.17	Spots	5.18)	Spots	5.19)	Spots	5.20)	Spots	5.21)	Spots	5.22)	Spots
1	0	1	1	1	11	1	5	1	6	1	15	1	2	1	2	1	1	1	1	1	1
2	2	2	2	2	1	2	1	2	2	2	5	2	10	2	4	2	0	2	1	2	8
3	2	3	0	3	1	3	0	3	0	3	0	3	3	3	0	3	5	3	0	3	1
4	9	4	1	4	1	4	4	4	1	4	1	4	9	4	0	4	6	4	0	4	0
5	9	5	0	5	2	5	3	5	3	5	8	5	3	5	11	5	3	5	11	5	4
6	2	6	0	6	1	6	8	6	10	6	7	6	0	6	3	6	3	6	0	6	6
7	4	7	2	7	0	7	13	7	13	7	5	7	1	7	0	7	7	7	1	7	0
8	15	8	6	8	0	8	3	8	4	8	2	8	6	8	4	8	1	8	9	8	1
9	5	9	12	9	6	9	0	9	3	9	1	9	2	9	7	9	1	9	4	9	5
10	5	10	10	10	2	10	12	10	2	10	2	10	1	10	1	10	6	10	9	10	6
11	6	11	0	11	1	11	3	11	15	11	0	11	5	11	2	11	8	11	0	11	6
12	1	12	1	12	3	12	6	12	2	12	10	12	0	12	1	12	1	12	0	12	0
13	0	13	0	13	1	13	3	13	0	13	1	13	2	13	1	13	2	13	6	13	0
14	0	14	0	14	7	14	2	14	1	14	7	14	5	14	3	14	2	14	0	14	0
15	7	15	0	15	1	15	1	15	1	15	10	15	4	15	4	15	1	15	0	15	1
16	3	16	1	16	8	16	7	16	0	16	2	16	1	16	1	16	2	16	2	16	3
17	6	17	2	17	4	17	2	17	0	17	7	17	0	17	0	17	1	17	6	17	6
18	1	18	2	18	3	18	0	18	5	18	8	18	3	18	1	18	4	18	0	18	0
19	1	19	0	19	4	19	1	19	3	19	3	19	1	19	1	19	1	19	3	19	7
20	1	20	3	20	5	20	0	20	3	20	0	20	0	20	5	20	6	20	5	20	7
21	8	21	1	21	11	21	1	21	3	21	10	21	4	21	13	21	4	21	1	21	0
22	1	22	13	22	16	22	2	22	1	22	2	22	14	22	0	22	6	22	8	22	2
23	1	23	2	23	9	23	0	23	0	23	2	23	1	23	1	23	7	23	1	23	14
24	1	24	1	24	1	24	1	24	5	24	4	24	3	24	0	24	0	24	0	24	1
25	0	25	1	25	0	25	1	25	0	25	0	25	3	25	1	25	3	25	0	25	0
Total	90	Total	61	Total	99	Total	79	Total	83	Total	112	Total	83	Total	66	Total	81	Total	68	Total	79
Avarage	3,6	Avarage	2,44	Avarage	3,96	Avarage	3,16	Avarage	3,32	Avarage	4,48	Avarage	3,32	Avarage	2,64	Avarage	3,24	Avarage	2,72	Avarage	3,16

6)		6)		6)		6)		6)		6)		6)		6)		6)		6)	
6.1)	Spots	6.2)	Spots	6.3)	Spots	6.4)	Spots	6.5)	Spots	6.6)	Spots	6.7)	Spots	6.8)	Spots	6.9)	Spots	6.10)	Spots	6.11)	Spots
1	24	1	7	1	18	1	8	1	2	1	6	1	14	1	9	1	74	1	1	1	5
2	3	2	4	2	9	2	8	2	26	2	4	2	5	2	8	2	13	2	13	2	6
3	30	3	2	3	2	3	5	3	4	3	12	3	17	3	23	3	7	3	15	3	15
4	4	4	21	4	67	4	6	4	19	4	18	4	11	4	21	4	6	4	19	4	2
5	8	5	1	5	33	5	2	5	15	5	17	5	15	5	31	5	2	5	7	5	9
6	1	6	3	6	10	6	8	6	6	6	36	6	1	6	7	6	16	6	9	6	16
7	4	7	45	7	1	7	12	7	19	7	52	7	7	7	3	7	42	7	2	7	23
8	1	8	65	8	1	8	2	8	10	8	3	8	6	8	1	8	35	8	36	8	13
9	1	9	1	9	11	9	50	9	8	9	9	9	6	9	2	9	3	9	9	9	1
10	73	10	2	10	14	10	1	10	13	10	1	10	38	10	6	10	1	10	1	10	2
11	1	11	14	11	13	11	55	11	11	11	1	11	15	11	49	11	15	11	12	11	1
12	3	12	11	12	1	12	18	12	14	12	3	12	6	12	15	12	2	12	5	12	6
13	1	13	31	13	1	13	8	13	5	13	5	13	13	13	2	13	8	13	7	13	9
14	3	14	1	14	6	14	9	14	22	14	6	14	8	14	12	14	5	14	4	14	19
15	8	15	1	15	7	15	10	15	58	15	25	15	45	15	19	15	15	15	58	15	34
16	1	16	13	16	2	16	6	16	36	16	9	16	1	16	1	16	15	16	18	16	15
17	14	17	1	17	1	17	5	17	13	17	13	17	30	17	10	17	2	17	17	17	15
18	1	18	25	18	1	18	26	18	1	18	13	18	21	18	14	18	8	18	2	18	1
19	1	19	1	19	4	19	5	19	8	19	13	19	25	19	35	19	13	19	1	19	2
20	2	20	22	20	67	20	4	20	20	20	5	20	8	20	30	20	2	20	12	20	15
21	1	21	2	21	1	21	7	21	6	21	1	21	9	21	20	21	1	21	19	21	2
22	3	22	2	22	6	22	24	22	3	22	54	22	6	22	1	22	9	22	23	22	6
23	2	23	1	23	1	23	6	23	3	23	8	23	1	23	1	23	5	23	5	23	7
24	15	24	2	24	4	24	18	24	2	24	13	24	6	24	3	24	21	24	1	24	23
25	3	25	1	25	8	25	2	25	10	25	12	25	2	25	1	25	2	25	1	25	5
Total	208	Total	279	Total	289	Total	305	Total	334	Total	339	Total	316	Total	324	Total	322	Total	297	Total	252
Avarage	8,32	Avarage	11,16	Avarage	11,56	Avarage	12,2	Avarage	13,36	Avarage	13,56	Avarage	12,64	Avarage	12,96	Avarage	12,88	Avarage	11,88	Avarage	10,08

6)		6)		6)		6)		6)		6)		6)		6)		6)		6)		6)	
6.12)	Spots	6.13)	Spots	6.14)	Spots	6.15)	Spots	6.16)	Spots	6.17)	Spots	6.18)	Spots	6.19)	Spots	6.20)	Spots	6.21)	Spots	6.22)	Spots
1	59	1	5	1	1	1	23	1	69	1	15	1	1	1	15	1	1	1	51	1	7
2	1	2	10	2	3	2	5	2	1	2	1	2	1	2	1	2	1	2	17	2	21
3	1	3	1	3	5	3	8	3	1	3	17	3	53	3	19	3	19	3	1	3	15
4	13	4	1	4	1	4	1	4	23	4	18	4	9	4	19	4	1	4	15	4	58
5	23	5	55	5	39	5	19	5	1	5	89	5	8	5	5	5	63	5	1	5	3
6	1	6	9	6	2	6	17	6	1	6	45	6	23	6	7	6	14	6	13	6	5
7	9	7	8	7	21	7	26	7	59	7	1	7	15	7	66	7	1	7	19	7	6
8	16	8	6	8	30	8	1	8	1	8	1	8	6	8	1	8	23	8	5	8	12
9	1	9	7	9	4	9	1	9	1	9	6	9	8	9	3	9	1	9	23	9	19
10	34	10	8	10	14	10	1	10	15	10	7	10	11	10	14	10	22	10	14	10	9
11	23	11	1	11	32	11	36	11	23	11	9	11	14	11	16	11	14	11	16	11	33
12	1	12	1	12	16	12	45	12	9	12	1	12	19	12	27	12	5	12	19	12	8
13	3	13	23	13	26	13	3	13	14	13	1	13	33	13	5	13	9	13	1	13	12
14	9	14	22	14	59	14	8	14	2	14	23	14	42	14	3	14	7	14	1	14	19
15	1	15	17	15	5	15	8	15	3	15	17	15	1	15	22	15	4	15	43	15	1
16	8	16	20	16	3	16	29	16	1	16	14	16	1	16	14	16	1	16	8	16	1
17	23	17	1	17	7	17	13	17	1	17	19	17	12	17	17	17	31	17	16	17	23
18	1	18	3	18	18	18	1	18	29	18	18	18	18	18	1	18	18	18	1	18	14
19	12	19	9	19	6	19	5	19	14	19	1	19	1	19	1	19	5	19	19	19	1
20	19	20	34	20	15	20	4	20	2	20	3	20	2	20	13	20	1	20	14	20	8
21	10	21	3	21	15	21	19	21	31	21	5	21	2	21	12	21	20	21	12	21	9
22	43	22	8	22	1	22	2	22	3	22	11	22	8	22	13	22	1	22	12	22	1
23	5	23	15	23	9	23	1	23	1	23	2	23	9	23	8	23	8	23	1	23	1
24	1	24	14	24	1	24	1	24	2	24	3	24	1	24	9	24	6	24	1	24	1
25	1	25	1	25	1	25	1	25	2	25	3	25	1	25	1	25	4	25	12	25	1
Total	318	Total	282	Total	334	Total	278	Total	309	Total	330	Total	299	Total	312	Total	280	Total	335	Total	288
Avarage	12,72	Avarage	11,28	Avarage	13,36	Avarage	11,12	Avarage	12,36	Avarage	13,2	Avarage	11,96	Avarage	12,48	Avarage	11,2	Avarage	13,4	Avarage	11,52

7)		7)		7)		7)		7)		7)		7)		7)		7)		7)	
7.1)	Spots	7.2)	Spots	7.3)	Spots	7.4)	Spots	7.5)	Spots	7.6)	Spots	7.7)	Spots	7.8)	Spots	7.9)	Spots	7.10)	Spots	7.11)	Spots
1	0	1	1	1	0	1	1	1	12	1	4	1	0	1	1	1	1	1	1	1	1
2	0	2	0	2	0	2	0	2	0	2	5	2	1	2	0	2	0	2	0	2	1
3	1	3	0	3	3	3	6	3	1	3	1	3	4	3	1	3	0	3	0	3	9
4	4	4	0	4	2	4	4	4	0	4	0	4	9	4	1	4	1	4	1	4	5
5	9	5	3	5	9	5	0	5	10	5	0	5	7	5	5	5	1	5	1	5	6
6	0	6	0	6	7	6	6	6	1	6	11	6	1	6	0	6	9	6	1	6	4
7	1	7	1	7	1	7	1	7	0	7	0	7	0	7	6	7	5	7	13	7	7
8	3	8	4	8	0	8	1	8	0	8	1	8	0	8	12	8	7	8	12	8	0
9	8	9	9	9	0	9	2	9	6	9	5	9	1	9	0	9	0	9	1	9	1
10	1	10	2	10	1	10	0	10	7	10	7	10	5	10	1	10	1	10	5	10	0
11	6	11	8	11	1	11	3	11	3	11	9	11	8	11	1	11	6	11	4	11	1
12	7	12	7	12	1	12	1	12	6	12	1	12	7	12	1	12	0	12	0	12	6
13	3	13	0	13	3	13	8	13	0	13	3	13	6	13	1	13	1	13	0	13	7
14	0	14	1	14	2	14	0	14	1	14	5	14	0	14	5	14	2	14	0	14	2
15	0	15	3	15	8	15	1	15	8	15	0	15	0	15	4	15	8	15	6	15	4
16	5	16	4	16	0	16	1	16	9	16	1	16	1	16	1	16	4	16	9	16	3
17	9	17	2	17	0	17	6	17	0	17	1	17	4	17	0	17	5	17	6	17	3
18	10	18	0	18	4	18	7	18	1	18	8	18	11	18	1	18	1	18	0	18	0
19	0	19	5	19	3	19	3	19	1	19	6	19	1	19	5	19	0	19	5	19	0
20	0	20	6	20	8	20	2	20	1	20	0	20	0	20	6	20	9	20	0	20	1
21	1	21	7	21	0	21	6	21	0	21	12	21	8	21	1	21	7	21	7	21	12
22	1	22	0	22	1	22	1	22	6	22	0	22	1	22	9	22	8	22	8	22	0
23	3	23	1	23	5	23	2	23	4	23	0	23	1	23	10	23	1	23	0	23	13
24	1	24	1	24	3	24	0	24	0	24	1	24	0	24	1	24	0	24	0	24	1
25	2	25	3	25	9	25	5	25	0	25	2	25	0	25	0	25	1	25	0	25	0
Total	75	Total	68	Total	71	Total	67	Total	77	Total	83	Total	76	Total	73	Total	78	Total	80	Total	87
Avarage	3	Avarage	2,72	Avarage	2,84	Avarage	2,68	Avarage	3,08	Avarage	3,32	Avarage	3,04	Avarage	2,92	Avarage	3,12	Avarage	3,2	Avarage	3,48

7)		7)		7)		7)		7)		7)		7)		7)		7)		7)		7)	
7.12)	Spots	7.13)	Spots	7.14)	Spots	7.15)	Spots	7.16)	Spots	7.17)	Spots	7.18)	Spots	7.19)	Spots	7.20)	Spots	7.21)	Spots	7.22)	Spots
1	1	1	1	1	1	1	12	1	1	1	0	1	0	1	1	1	1	1	13	1	0
2	1	2	0	2	0	2	0	2	2	2	1	2	0	2	2	2	2	2	1	2	0
3	0	3	1	3	1	3	1	3	5	3	1	3	1	3	0	3	0	3	0	3	0
4	0	4	5	4	5	4	0	4	0	4	0	4	7	4	1	4	0	4	0	4	8
5	5	5	9	5	5	5	3	5	0	5	5	5	8	5	0	5	4	5	4	5	1
6	11	6	13	6	8	6	1	6	12	6	4	6	5	6	0	6	3	6	3	6	7
7	8	7	1	7	1	7	5	7	0	7	0	7	0	7	9	7	7	7	0	7	0
8	9	8	1	8	0	8	4	8	1	8	7	8	0	8	11	8	8	8	1	8	1
9	1	9	5	9	0	9	11	9	5	9	1	9	5	9	0	9	4	9	1	9	1
10	0	10	6	10	10	10	5	10	6	10	5	10	0	10	3	10	0	10	9	10	13
11	0	11	7	11	1	11	9	11	1	11	7	11	1	11	1	11	1	11	9	11	4
12	1	12	0	12	1	12	1	12	0	12	1	12	2	12	0	12	1	12	1	12	8
13	5	13	0	13	5	13	0	13	10	13	0	13	5	13	0	13	0	13	9	13	9
14	4	14	1	14	6	14	0	14	1	14	0	14	1	14	8	14	5	14	0	14	0
15	0	15	1	15	1	15	1	15	9	15	2	15	0	15	4	15	8	15	0	15	1
16	7	16	2	16	1	16	1	16	4	16	8	16	11	16	7	16	4	16	1	16	5
17	0	17	5	17	0	17	5	17	5	17	0	17	8	17	6	17	9	17	4	17	5
18	8	18	7	18	4	18	6	18	1	18	4	18	1	18	0	18	11	18	6	18	1
19	0	19	8	19	3	19	1	19	0	19	5	19	7	19	1	19	0	19	8	19	6
20	1	20	9	20	2	20	2	20	2	20	3	20	0	20	8	20	12	20	0	20	1
21	0	21	0	21	1	21	3	21	4	21	11	21	1	21	0	21	1	21	1	21	4
22	3	22	0	22	5	22	0	22	5	22	0	22	5	22	4	22	0	22	1	22	1
23	2	23	5	23	7	23	1	23	1	23	7	23	4	23	0	23	0	23	4	23	6
24	8	24	1	24	9	24	1	24	1	24	1	24	1	24	9	24	1	24	3	24	1
25	0	25	5	25	10	25	0	25	0	25	1	25	1	25	1	25	1	25	2	25	1
Total	75	Total	93	Total	87	Total	73	Total	76	Total	74	Total	74	Total	76	Total	83	Total	81	Total	84
Avarage	3	Avarage	3,72	Avarage	3,48	Avarage	2,92	Avarage	3,04	Avarage	2,96	Avarage	2,96	Avarage	3,04	Avarage	3,32	Avarage	3,24	Avarage	3,36

12)		12)		12)		12)		12)		12)		12)		12)		12)		12)		12)	
12.1)	Spots	12.2)	Spots	12.3)	Spots	12.4)	Spots	12.5)	Spots	12.6)	Spots	12.7)	Spots	12.8)	Spots	12.9)	Spots	12.10)	Spots	12.11)	Spots
1	45	1	19	1	13	1	16	1	23	1	12	1	13	1	1	1	22	1	16	1	1
2	19	2	8	2	27	2	1	2	7	2	1	2	1	2	28	2	1	2	7	2	1
3	12	3	1	3	24	3	24	3	8	3	14	3	26	3	15	3	15	3	1	3	1
4	1	4	21	4	1	4	18	4	1	4	25	4	17	4	26	4	14	4	68	4	12
5	13	5	43	5	19	5	12	5	35	5	16	5	1	5	1	5	25	5	7	5	15
6	1	6	1	6	18	6	1	6	1	6	1	6	6	6	2	6	1	6	2	6	34
7	14	7	1	7	51	7	41	7	5	7	2	7	25	7	57	7	26	7	13	7	38
8	23	8	16	8	1	8	6	8	18	8	18	8	27	8	23	8	32	8	15	8	1
9	9	9	19	9	1	9	3	9	24	9	15	9	51	9	11	9	4	9	7	9	16
10	8	10	15	10	43	10	1	10	26	10	2	10	1	10	8	10	19	10	26	10	25
11	23	11	15	11	1	11	16	11	15	11	1	11	5	11	1	11	16	11	24	11	14
12	63	12	9	12	30	12	27	12	1	12	26	12	13	12	9	12	4	12	1	12	1
13	1	13	1	13	1	13	18	13	1	13	38	13	16	13	2	13	18	13	12	13	6
14	1	14	4	14	8	14	1	14	17	14	56	14	24	14	26	14	3	14	13	14	7
15	8	15	37	15	17	15	36	15	16	15	2	15	1	15	19	15	21	15	15	15	8
16	9	16	13	16	16	16	24	16	27	16	24	16	3	16	19	16	22	16	12	16	1
17	17	17	23	17	19	17	1	17	29	17	3	17	8	17	22	17	17	17	1	17	15
18	14	18	1	18	5	18	6	18	1	18	13	18	6	18	24	18	16	18	26	18	18
19	23	19	30	19	1	19	7	19	1	19	1	19	28	19	2	19	1	19	1	19	12
20	5	20	5	20	1	20	8	20	13	20	42	20	41	20	3	20	1	20	1	20	19
21	1	21	1	21	7	21	1	21	15	21	1	21	1	21	3	21	2	21	13	21	18
22	12	22	1	22	8	22	23	22	1	22	16	22	6	22	1	22	16	22	9	22	1
23	11	23	12	23	1	23	1	23	18	23	1	23	8	23	12	23	1	23	4	23	23
24	9	24	1	24	1	24	3	24	1	24	1	24	1	24	1	24	15	24	12	24	12
25	1	25	1	25	1	25	5	25	1	25	1	25	1	25	1	25	2	25	1	25	1
Total	343	Total	298	Total	315	Total	300	Total	305	Total	332	Total	330	Total	317	Total	314	Total	307	Total	300
Avarage	13,72	Avarage	11,92	Avarage	12,6	Avarage	12	Avarage	12,2	Avarage	13,28	Avarage	13,2	Avarage	12,68	Avarage	12,56	Avarage	12,28	Avarage	12

12)		12)		12)		12)		12)		12)		12)		12)		12)		12)		12)	
12.12)	Spots	12.13)	Spots	12.14)	Spots	12.15)	Spots	12.16)	Spots	12.17)	Spots	12.18)	Spots	12.19)	Spots	12.20)	Spots	12.21)	Spots	12.22)	Spots
1	22	1	1	1	15	1	1	1	12	1	15	1	35	1	2	1	47	1	20	1	13
2	12	2	8	2	15	2	22	2	16	2	16	2	10	2	7	2	12	2	21	2	3
3	40	3	19	3	13	3	16	3	13	3	12	3	19	3	6	3	26	3	1	3	54
4	18	4	1	4	11	4	18	4	1	4	1	4	4	4	10	4	18	4	16	4	1
5	8	5	26	5	1	5	1	5	22	5	3	5	1	5	16	5	1	5	14	5	14
6	11	6	43	6	23	6	16	6	19	6	18	6	5	6	41	6	1	6	1	6	60
7	5	7	1	7	5	7	18	7	24	7	28	7	12	7	15	7	12	7	5	7	4
8	8	8	1	8	18	8	1	8	1	8	34	8	31	8	1	8	13	8	7	8	28
9	2	9	6	9	33	9	33	9	1	9	1	9	1	9	26	9	18	9	18	9	27
10	4	10	7	10	14	10	1	10	29	10	1	10	16	10	21	10	1	10	19	10	3
11	13	11	9	11	8	11	26	11	1	11	8	11	12	11	1	11	6	11	27	11	7
12	4	12	13	12	7	12	15	12	30	12	17	12	16	12	1	12	9	12	14	12	2
13	8	13	18	13	1	13	7	13	5	13	19	13	1	13	3	13	1	13	16	13	7
14	36	14	12	14	14	14	14	14	16	14	1	14	40	14	4	14	45	14	1	14	1
15	23	15	22	15	16	15	23	15	47	15	23	15	1	15	18	15	9	15	2	15	12
16	12	16	1	16	30	16	12	16	1	16	24	16	13	16	16	16	12	16	13	16	18
17	15	17	9	17	12	17	2	17	6	17	1	17	1	17	17	17	31	17	26	17	15
18	2	18	4	18	26	18	3	18	1	18	6	18	18	18	15	18	2	18	1	18	1
19	16	19	33	19	1	19	15	19	15	19	7	19	3	19	23	19	6	19	7	19	23
20	22	20	26	20	12	20	46	20	18	20	8	20	9	20	15	20	7	20	6	20	5
21	25	21	5	21	5	21	1	21	12	21	36	21	25	21	1	21	1	21	4	21	9
22	2	22	1	22	1	22	12	22	1	22	1	22	22	22	1	22	19	22	5	22	15
23	1	23	10	23	18	23	1	23	3	23	1	23	12	23	5	23	3	23	2	23	1
24	1	24	5	24	1	24	3	24	1	24	1	24	1	24	8	24	1	24	23	24	6
25	1	25	1	25	44	25	5	25	1	25	2	25	1	25	1	25	1	25	5	25	5
Total	311	Total	282	Total	344	Total	312	Total	296	Total	284	Total	309	Total	274	Total	302	Total	274	Total	334
Avarage	12,44	Avarage	11,28	Avarage	13,76	Avarage	12,48	Avarage	11,84	Avarage	11,36	Avarage	12,36	Avarage	10,96	Avarage	12,08	Avarage	10,96	Avarage	13,36

Total counted plants 5	550	Total counted plants 6	550
Total counted GUS spots 5	1771	Total counted GUS spots 6	6630
Total avarage 5	3,22	Total avarage 6	12,05454545
Total counted plants7	550	Total counted plants 12	550
Total counted GUS spots 7	1711	Total counted GUS spots 12	6783
Total avarage 7	3,110909091	Total avarage 12	12,33272727
STD	0,0778	STD	0,198