Transforming diploid potato: an efficiency issue

Douwe Rietveld, Tom Schermer, Marcel Proveniers, 2022

Abstract

Improving the quality of potato cultivars remains a costly and time-inefficient process because most potato cultivars are tetraploid. Diploid lines already exist and allow for easier and more precise genetic engineering via transformation and breeding. To this day, an efficient protocol for diploid potato transformation remains elusive. In this research, we aim to improve the diploid transformation protocol and use it to generate a new compact diploid *Solanum tuberosum ERECTA* (*Ster*)-knockout plant which is suitable for seed production in vertical farms, as *ER*-knockouts in both *Arabidopsis thaliana* and the closely related tomato show compact shoots. Given current difficulties in transforming- and in vitro growing of diploid True Potato Seed (TPS) plants, multiple TPS genotypes and tissues were grown in vitro, and additional sample preparation was added from a cucurbit transformation protocol for more efficient *Agrobacterium*-mediated transformation. Finally, a *Ster*-knockout-assembly was designed for genome editing via CRISPR/Cas-9. New features of this assembly are the more efficient intronized Cas-9 nuclease coding sequence and *RUBY* as a reporter gene. Using the unconventional starting material of nodes and petioles, growth of callus tissue was observed. However, most transformation- and in vitro growth experiments were disrupted by fungal infections. Therefore, it remains unclear whether additional sample preparation techniques for *A. tumefaciens* infiltration are beneficial due to infections resulting in small sample sizes, which emphasizes the need for an effective sample sterilization method.

Layman's summary

Potato is one of the most important crops worldwide for our food supply. Because the global food demand is expected to grow in the future, crops like potato need to grow more efficiently. Unfortunately the high genomic complexity of potato makes it harder to do research and breed better crops than in most other crops like tomato or rice. Due to this, most protocols for genetic alteration of diploid potato are less efficient. Unlike most other crops which are grown via seed, potato is propagated by tubers. To sell potatoes commercially, large quantities have to be generated first. This is a time-inefficient and costly process that uses a substantial part of the global potato yield each year. Growing potato via seed may revolutionize potato cultivation. Potato plants could be used for growing seeds in vertical farms (highly controlled plant growing facilities). In vertical farms, space is limited, so plants need to be as short as possible. In this research, we aim to create a shorter potato plant variety that is suitable for growing seeds in vertical farms while improving the protocol for genetic alteration. We monitored growth of potato plants in sterile conditions, experimented with methods to increase likelihood of genetic alteration and designed a genetic construct to genetically alter potato plants (to make them shorter). Our results show the need for a more efficient protocol and (possibly) generated a potato variety with a shorter shoot.

Introduction

Innovating potato as a crop

Potato (*Solanum tuberosum*) is one of the major food crops in the world, as it makes up an important part of global human nutrition (Stokstad, 2019). Where other crops such as rice and wheat see an increase in yield of 1% per year thanks to effective breeding, potato lags behind and is increasingly affected by pests and abiotic stresses (Khan et al., 2015, Bethke *et al.,* 2014). This is due to the high heterozygosity and tetraploidity of the potato genome, which makes efficient breeding difficult. Having four copies of every chromosome means that more recombination takes place during breeding, increasing the chances of detrimental genes expressing in the next generation of potato plants. Therefore, the current widespread method of propagation in potato is clonally achieved via tubers, which yields no genetic variation over generations, has a low multiplication rate and a high carbon footprint (Zhang *et al*., 2021), as part of the harvest is used for propagation of new plants rather than commercial yield. In search of new varieties, large amounts of potato plants grown from tetraploid 'True Potato Seeds' (TPS) are screened for favorable agricultural characteristics. This method requires extensive screening protocols, where thousands of potato seedlings are screened for acceptable characteristics (Eggers *et al.,* 2021). As there are around 50 characteristics to a potato plant that influence commercial value of a potato variety (Eggers *et al.,* 2021), it is near impossible to breed all of these traits into a single variety using this method. Therefore, innovations on potato breeding stay relevant.

Using aimed genetic transformation methods could yield better potato plants in shorter timespans, which is becoming a necessity in order to fulfill the increasing food demands (van Dijk *et al.,* 2021). Next to large phenotypic screening of potato, research groups have therefore also transformed tetraploid potato for nearly forty years in search for methods to create better potato varieties (Sree Ramulu *et al.*, 1983). Transformation would offer a more accurate method of generating commercial varieties, as gene editing with the CRISPR/Cas-9 system allows for more precise modification of beneficial traits on a genetic level, which was performed for the first time in 2015 (Butler *et al.,* 2015). However, tetraploidity of the potato genome still poses problems, as effective transformation requires four successful transformation events instead of two (in diploid genomes). During subsequent breeding after transformation, the high allelic heterozygosity of the potato genome also increases the chances of deleterious alleles expressing after breeding (Zhang *et al.,* 2021). In the past, different transformation methods have been tested in diploid potato like electroporation and *Agrobacterium tumefaciens* (*A. tumefaciens,* a bacterium that is often used to transform plants by inserting DNA in plant tissue)-mediated gene transfer (De Vries-Uijtewaal *et al.,* (1989), Masson *et al.,* (1989), Dönmez *et al.,* (2019), Brown *et al.,* (1991) and Nadolska-Orczyk *et al.,* (2007), and Visser (1991)), but none of these used the CRISPR/Cas-9 genome editing system. However, successful application of CRISPR/Cas-9 diploid potato varieties already exists (Enciso Rodriguez *et al.,* 2019, Zhang *et al.,* 2021, Eggers *et al.,* 2021), allowing for more precise breeding, fewer required genetic alterations, eventually propagation via seed and accumulative breeding.

If potato can be genetically modified to invest less energy in non-edible parts without ultimately decreasing crop fitness, this could increase crop yield on a large scale, as has happened in crops like wheat (Pearce, 2021). Strategies to reach this are for instance creating less carbon sinks in stolons, or by growing crops with more compact shoots. In *Arabidopsis thaliana (Arabidopsis)*, the Landsberg Erecta ecotype is well known for its shorter, more compact shoot architecture thanks to the lack of ERECTA (ER) function, which influences the length of many shoot organs (Torii *et al.,* 1996). In tomato (*Solanum lycopersicum*), plants with shorter internodes and more compact inflorescences can be generated as shown by Villagarcia *et al.* (2012) and by Kwon *et al*. (2020), of which the latter used CRISPR/Cas-9 genome editing to knock out *SlER*. As potato is genetically closely related to tomato, this approach could show similar desired architectural changes in potato as well. Next to *SlER*, other gene mutations and/or GMO-phenotypes in *Arabidopsis* and other plants like tobacco yield shorter shoots, such as overexpression of *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*) (Proveniers *et al.,* 2007, Gomez-Mena and Sablowski, 2008, Proveniers, unpublished data). *ATH1* is a TALE-domain

homeobox transcription factor that expresses during the vegetative phase of *Arabidopsis* development and is associated with rosette habit (Quaedvlieg *et al.,* 1995, Proveniers *et al.,* 2007). If knocking out *ER* or overexpressing *ATH1* in potato would also lead to a compact plant phenotype, this could improve seed yield and introduce potato to agricultural settings where space is an even more limiting factor, like in vertical farms. The potato breeding company Solynta aims to generate compact potato plants for higher production of diploid, (largely) homozygous True Potato Seeds. For high seed production, vertical farm settings with compact potato plants that operate year-round would generate the highest yields, which is an incentive for creating compact potato plants in this research.

Improving transformation efficiency in diploid potato

Because of low efficiency of earlier diploid potato transformation experiments (a single homozygous transformant out of 149 recipient samples, Eggers *et al.*, 2021), several steps in the transformation process could be improved for higher efficiency. A recently published article regarding diploid potato transformation still uses a (slightly altered) version of a protocol from over thirty years ago (Visser, 1991; Eggers *et al.*, 2021). Visser (1991) describes a protocol to transform potato with *A. tumefaciens.* Because transformation (with *A. tumefaciens*) requires sterile conditions, potato plants are grown in sterile pots with agar where plant material is immersed in diluted sodium hypochlorite solution beforehand for tissue sterilization. The ability of *A. tumefaciens* to insert DNA into the tissue determines a large part of transformation efficiency, meaning that this process should be as optimal as possible. Factors such as the efficiency of the CRISPR/Cas-9 construct, the Cas-9 nuclease and guide RNA's are also important when generating transformed (potato) plants (Belhaj *et al.,* 2015). Grützner *et al.* (2020) already showed that in a construct used to transform Arabidopsis, *Catharanthus roseus* and tobacco, the use of 13 introns in the coding sequence of the Cas-9 nuclease greatly increased editing efficiency in these different species, up to 70% (Grützner et al., 2020). Another common transformation feature is the use of Green Fluorescent Protein (GFP) as reporter gene in callus tissue (Murray *et al.,* 2004). While GFP reports successful transformation, detection of the signal requires a fluorescence microscope. '*RUBY*' is an open reading frame that can be used to report transformed tissues, with the benefit of turning all transformed tissues bright red, visible without fluorescence detection (He *et al.,* 2020). Other factors also influence transformation- and regeneration efficiency, such as the infiltration of *A. tumefaciens* in the plant tissue and dealing with internal microbes carried by potato plants (Buchholz *et al.,* 2019). Xin *et al.,* (2022) describe a protocol for *A. tumefaciens* mediated transformation of cucurbit plants like cucumber and watermelon, with extra preparation of the plant samples. By scratching, sonicating and vacuum-infiltration methods, transformation efficiency of internode tissues increased from 0% to 3,17% (Xin *et al.,* 2022), thanks to the wounding of samples for better infiltration of *A. tumefaciens.* Therefore, the use of sample preparation, zCas9i and *RUBY* make good candidate techniques for improved efficiency of transforming diploid TPS-potato.

Aims of the project

The goal of this project is to generate a new diploid *ERECTA* (*ER*)-knockout potato plant that is suitable for seed production in vertical farms, like the more compact tomato plants shown by Kwon *et al* (2020). To reach this we aim to transform potato plants using *A. tumefaciens* combined with CRIPSPR-Cas9 genome editing. As earlier protocols have shown low efficiency in diploid potato transformation experiments (one homozygous out of 149 samples, Eggers *et al.*, 2021), we aim to increase this efficiency. Therefore, our research question is: "How can we improve transformation efficiency in diploid TPS-grown potato?" We search for improvements in transformation efficiency using the protocol by Visser (1991, also used by Eggers *et al.,* 2021), by experimenting with sample preparations (Xin *et al.,* 2022) using an *A. tumefaciens*–strain containing an 35S::ATH1-GFP construct. This construct overexpresses AtATH1(-GFP), which enhances rosette habit and decreases shoot length (Proveniers *et al.,* 2007). By investigating GFP-signal using fluorescence microscopy we confirm eventual transformation events in sample preparation experiments. For the *StER* knockout

construct, we design four guide-RNA sequences for CRISPR-Cas9 with Benchling, apply *RUBY* (He *et al.,* 2020) as reporter gene (no fluorescence microscopy needed) and the intronized Cas-9 sequence (Grützner *et al.,* 2021) to increase genome editing efficiency.

Materials & methods

Growth conditions

All plant material were grown in long-day conditions (16 hours light, 8 hours dark) at a temperature of 22°C. Light intensity: 140 µmol·m-2·s-1, humidity: 70%. Plants in greenhouses at the Botanical gardens were grown with fluctuating light intensities, humidity and (sometimes very high) temperature. After 2 weeks, plants received a 1 ‰ mix of nutrients (20/20/20) 2 times each week. In vitro plants were grown from axillary buds and apical meristem cuttings on MS20 (1x Murashige and Skoog medium, 8 g/L agar and 20g/L sucrose).

Plant material

For transformation of potato plants, petiole-, node- and internode tissue was harvested from potato plants with a length of around 1 cm (unless indicated otherwise). Nodes were cut clearly around the axillary meristem zone to avoid later interference of the meristematic cells with callus tissue. A total of 49 TPS-grown lines of the breeding company Solynta (Research Panel 001-056) as well as one inbred plant line were received. For in vitro growth, both Shoot Apical Meristems and axillary meristems were used.

Sterilization of plant material

Bleaching solution was created using Chlorix (4,5% sodium hypochlorite), Milli-Q (MQ) and 0,1% Sodium dodecyl sulfate (SDS). Sodium hypochlorite concentrations of 0,09%, 1% and 2% were used. For sterilization, plant material was immersed in bleaching solution for five minutes (unless stated otherwise), after which 3 washes in MQ were performed by holding the material with sterile foreceps and flushing with a pipet. After washing plant material was placed on growth medium.

Growth media

Growth media used were as described by Visser (1991), with the alteration of all plates containingMS20 (1x Murashige and Skoog medium, 8 g/L agar and 20g/L sucrose). Furthermore, selection media also contained vancomycin (200 µg /mL) from the start of July on. In PACM medium (M100 medium) and M13 medium, we used 2,5mg/L BA instead of kinetin because of higher bioactivity.

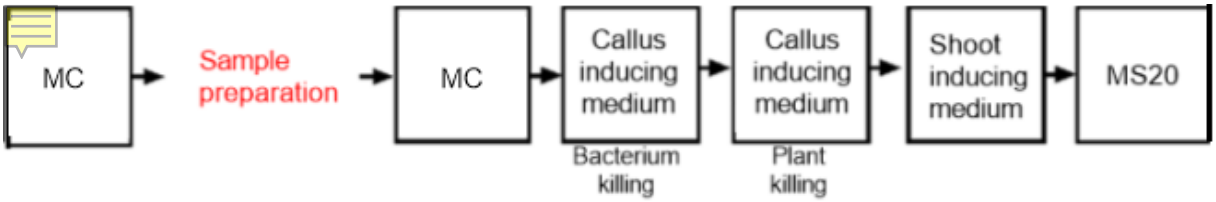

Figure 1: Schematic workflow of transformation- and regeneration media for potato transformation derived from Visser (1991).

Callus induction medium (M400) also contains 200 µg /mL vancomycin when stated in experiments.

Transformation protocols

An *A. tumefaciens*-stock from the C58C1-strain was grown in LB medium with spectinomycin (20 µg /mL) and rifampicin (80 µg /mL). Two methods of diluting *A. tumefaciens* stocks were used for experiments: 1) when the stock solution has reached a high cellular optical density (OD) of OD600 > 1, a new solution was diluted 2:5 directly from the stock with liquid LB (50ml with 75mL, Eggers *et al.,* 2021), which was used for incubation. 2) diluting *A. tumefaciens* stock with liquid LB to an OD600 of

+- 0,5, measured with the Spectronic Genesys 5. For sample preparations, scalpel scratching and sonication were used to create more wound-surface on explants for *A. tumefaciens* to infiltrate during incubation. Scratching creates wounds at the surface of explants, while sonication uses high-pitched sound to damage tissues everywhere. Sonication was performed using the Diagenode BioRuptor Standard (power setting and treatment duration differ per experiment). Metal probes for sonication that enter the sterile tubes with plant samples are washed in 70% ethanol and sterilized in fire beforehand. Vacuum infiltration was performed by immersing plant material in *A. tumefaciens* suspension in a plastic vacuum chamber (interior cleaned with 70% ethanol) with low air pressure. After pressure was low enough (about 50% atmosphere), the vacuum pump was detached and sterile air via the flow cabinet was slowly let back in. Immersed internodes were shortly blotted dry on sterile filter paper to drain off excess fluid and placed back on the MC plates in growth chambers. After 2 days of incubation, internodes were initially placed on M400 plates as described by Visser, which contain cefotaxime (200 µg /mL) to kill *A. tumefaciens* (from July on, M400 plates also contained 200 µg /mL vancomycin). After 3-5 days, internodes were placed on M400 medium containing both cefotaxime and kanamycin (50 µg /mL) to select for internodes that received kanamycin resistance from *A. tumefaciens.* Later on in the project, vancomycin (200 µg /mL) was also added to M400 and M13 plates. M400 plates were refreshed every week. After 3 weeks, formed calli were removed and placed on M13 shoot inducing medium by Visser (1991).

Plasmids and oligonucleotides

Table 1: Plasmids and oligonucleotide sequences used for CRISPR/Cas-9 molecular cloning of the *ER* knockout construct.

For oligonucleotides, the 4-bp sequence in front of the period (.) is the overhang sequence. ''gRNA 1-4'' are guide RNA sequences used by the CRISPR-Cas9 system to locate different sequences in the genome: gRNA 1 and 2 guide the Cas-9 nuclease to *StER* exon 1 and -3 respectively, gRNA 3 and 4 to *StER* exon 27, the last exon.

PLASMIDS USED FOR CRISPR/CAS9:	
Carbenicillin resistance:	Streptomycin/spectinomycin resistance:
pICH47732 RED POS1	pICH41822 end linker POS7
pICH47742 RED POS2	pICH50866 end linker level M
pICH47751 RED POS3	
pICH47761 RED POS4	Kanamycin resistance:
pICH47781 RED POS6	pICSL4723
pICH47791 RED POSB1	

OLIGONUCLEOTIDES:		
	Forward	Reverse
gRNA1	ATTG.GGTTCTGTGGTGTCTGATGA	AAAC.TCATCAGACACCACAGAACC
gRNA2	ATTG.GAAAATAGGTTCTGCATTGT	AAAC.ACAATGCAGAACCTATTTTC
gRNA3	ATTG.GAGGTCATATCCCAGAATAG	AAAC.CTATTCTGGGATATGACCTC
gRNA4	ATTG.GGCAGCAAACAATGCTGTAA	AAAC.TTACAGCATTGTTTGCTGCC

Cloning protocols

Ordered oligonucleotides were diluted in MQ to a concentration of 10 µM and heated to 98°C for 5 minutes for hybridization, and then cooled down to room temperature. For cloning of the gRNA spacers into the dsRED-plasmids the Esp3I-restriction enzyme was used (for BsmBI sites). Components for the Level 1 digestion-ligation-reaction and cycling temperatures and durations are shown in Table 2.

Table 2: Protocol for Level 1 Golden Gate cloning of oligonucleotide sequences into dsRED plasmids.

Reaction was prepared on ice before starting temperature cycling. Restriction enzyme used for BsmbI

sites is Esp3I. To experiment with efficiency of the level 1 reaction, we also tested concentrations of 500fmol hybridized oligonucleotides.

Level 1 molecular cloning reaction		Cycling	20-30 cycles
20fmol	dsRED	37°C	3 minutes
50fmol = 1µL	Hybridized oligonucleotides	16°C	5 minutes
1µL	10x G buffer	50°C	10 minutes
1µL	10mM ATP	80°C	10 minutes
0,5µL	Esp3I (BsmBI)	12°C	
0,5µL	T4 DNA Ligase		
	H20		
10µL	Total		

After the Level 1 molecular cloning reaction, the dsRED plasmids were transformed into competent *E. coli* cells using heat shock. 5 µL of digestion/ligation reaction was added to competent *E. coli* cells and incubated for 15 minutes on ice. The heat shock was performed by putting the *E. coli* cells 30 seconds in a heat block of 42°C and immediately back on ice for one minute. Liquid LB was added to the cells to a volume of 1mL after which they are placed in a 37°C incubator for one hour. After incubation, 100 µL of *E. coli* culture was plated on an LB carbenicillin (100 µg /mL) plate. The remaining *E. coli* culture was centrifuged for 10 minutes at 2000 rpm, resuspended and plated on another LB carb-plate. Plates were stored overnight in the 37°C incubator. The overnight formed bacterial colonies were either white or purple, depending on successful insertion of the fragment into the acceptor plasmid. White colonies were selected and used for further experiments. Non-genomic plasmid DNA from *E. coli* cells were isolated using the E.Z.N.A. Plasmid DNA Mini Kit I (Omega-Biotek). After isolation, glycerol stocks of level 1 plasmids were created from *E. coli*. Stocks were stored at -80°C. Isolated DNA was used in the next molecular cloning step, the Level 2 digestion-ligation reaction with the BpiI restriction enzyme. The Level 2 reaction was similarly performed as the described Level 1 reaction, except for differences in reaction components being a different acceptor pICSL4723 backbone vector and the Level 1 inserts containing the gRNA oligonucleotide sequences (Table 1).

Bacterial glycerol stocks

Glycerol stocks of *E.coli* DH5α bacteria were made by adding 600 µL bacterial culture in LB (with appropriate antibiotics) to 400uL 60% sterile glycerol solution, creating 25% glycerol stocks. Stocks are stored in -80°C.

Plasmid gel analysis

For gel analysis of DNA samples, 1% agarose gel with 25 µL ethidium bromide per liter Tris-borate-EDTA (TBE) buffer was used (2,5 µL in a normal 100mL gel, 1,5 µL in a 60mL (small) gel). Samples were prepared by incubating one hour in 37°C. DNA from the pICH47781 RED POS6 stock was chosen because of its relatively high and convenient concentration (200 ng/µL). In every 30 µL DNA sample 6 µL of loading dye was added. The ladder used is 1kb Plus Ladder (Thermo Scientific). The gel was immersed in TBE buffer and run at 120V.

Assembling plasmids

Benchling [Biology Software], (2022) was used to create the genetic assembly with guide RNA's used for this work (assembly wizard), as well as creating alignment with sequencing samples from gRNA's. Retrieved from https://benchling.com.

Results

In vitro growth of diploid potato

A total of 49 genotypically different TPS-grown hybrid potato plants were available from Solynta, called "Research Panel"- plants (RP), and a single inbred line. With these different plants, the aim was

to monitor differences in in vitro growth- and transformation efficiencies. The RP048 plant did not germinate and a few genotypes did not survive transplanting. At the moment the thrips pest was discovered, some genotypes were not yet inside sterile agar pots and had to be discarded. The genotypes that were discarded and the ones that died shortly after transplanting could not be taken into account for the in vitro growth experiment. Surface-sterilization of Shoot Apical Meristems (SAM) or axillary meristems was performed using both a 2%- and 1% sodium-hypochlorite solution with 0,1% SDS for five minutes, as described by Visser (1991). After sterilization SAMs were placed in the sterile agar MS20 pots. Visser describes that SAMs should be transplanted to new pots after every four weeks of growth. We monitored survival of the different RP-genotypes during in vitro growing, with the aim to yield as many sterile growing genotypes as possible (Figure 1). The number of surviving genotypes is higher for the 1% sodium-hypochlorite solution, and stabilizes at four genotypes during further observation of in vitro growth. Often, in vitro growing plants had to be transplanted more frequently than once in every four weeks (as described by Visser, 1991) to evade (endogenous) infection. Over time, infections emerged numerous times to all genotypes, with the most common being fungal infections (Figure 1B). When pots had small infections, meristems or plants that seem clearly out of reach of the contamination were transplanted to new sterile pots. However, many genotypes were lost due to contaminations directly surrounding the meristems, with no chance of successful (sterile) transplantation and no back-up material due to the thrips pest. SAM's of plants that were tall enough to be out of reach of the fungal infections were transplanted to new pots. The detailed course of the survival of the RP-plants can be found in Supplementary Table 1.

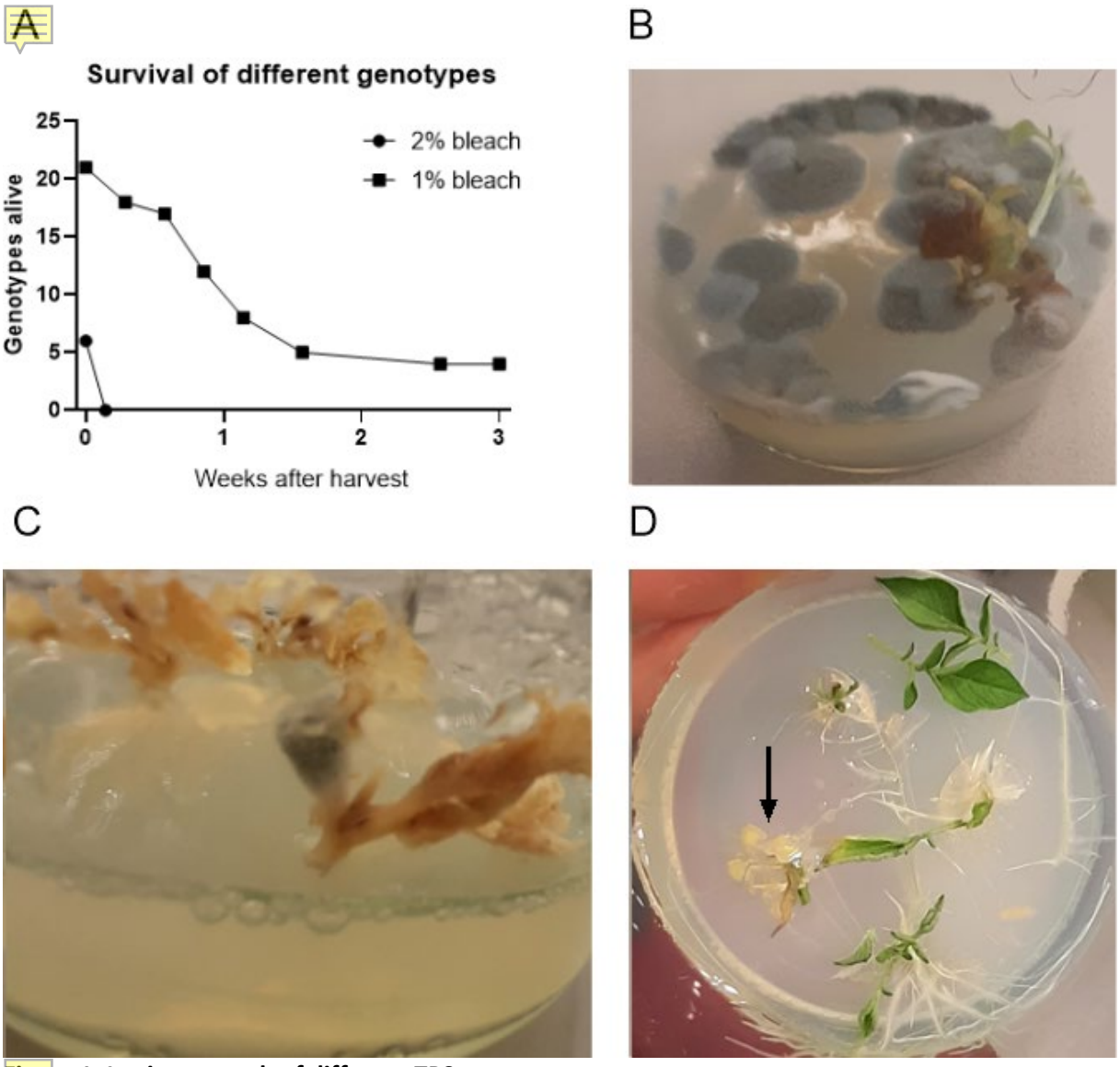

Figure 1: In vitro growth of different TPS-grown-potato genotypes.
A: timeline of sterile genotypes (or infected pots with a chance of surviving) after 2%- or 1% bleach treatment of potato SAM's in vitro.
B: fungal infection in MS20 pot (RP007), 14 days after bleaching.
C: SAMs (RP021) treated with 2% bleach solution. Plant material is killed by bleach, while fungal infections still emerged after 15 days.
D: Bacterial infections emerging 23 days after bleaching from in vitro potato plant roots (RP001) in MS20 pot (arrow); less harmful than fungal infections for the survival of plants.

Out of 49 total genotypes, 27 different genotypes were transplanted into MS20 agar. The 6 different genotypes that were sterilized with the 2% bleach solution as described by Visser (1991) all died (Figure 1A, Figure 1C, Supplementary table 1). Although the 2% bleach treatment killed all plant material, in some cases fungal spores were still found (Figure 1C). This shows ineffectiveness of the bleaching treatment regarding fungal infection. Subsequent transplanting therefore lowered the bleach percentage to 1% sodium-hypochlorite, which was less harmful to plant material. Sometimes, yellow-colored bacteria emerged from newly formed plant roots inside sterile pots after weeks of seemingly sterile growth, suggesting infection of medium through TPS endogenous microbes (Figure 1D). In the end, the sterile appearing genotypes that survived longer than three weeks after 1% bleach treatment were RP001, RP002, RP005 and RP011 (4 out of 21, Supplementary figure 1). Plant material from these genotypes was used for transformation experiments.

Skipping in vitro growth: adult TPS plant material remained sterile at very low bleach concentrations
Next to issues regarding in vitro growth, Eggers *et al.* have reported low transformation efficiency in potato. Tactics to improve the speed up until transformation and skipping the in vitro growth procedure were therefore researched. To speed up transformation procedures it was tested whether adult material from soil-grown potato plants could also be used for transformation with *A. tumefaciens*. In this way, the aim is to completely skip the extensive in vitro growing procedure. Internode- and node material from RP001 and RP035-plants, which were adult plants growing for 16-20 weeks, was chosen because the phenotypic characteristics (e.g. shoot- or stolon length) of these genotypes were average in comparison to the rest of lines (Schermer, unpublished data). This is for better phenotypic comparison between eventual transformants and other lines in follow up experiments . However, in the pilot experiment a 0,09% sodium-hypochlorite concentration was used and (inter)node ends were not cut off. Regardless, RP001 internodes on MS20-plates remained sterile for at least two weeks after different durations of bleaching treatments: 5-, 10-, 15- and 20 minutes (Figure 2).

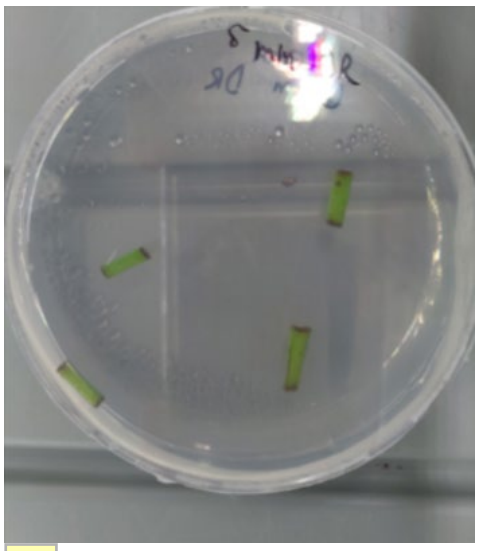

Figure 2: Bleach experimenting with RP001 internodes
Close-up of MS20 plate with internodes after 5 minutes of bleaching.

Visser (1991) describes that internodes must be of 1 cm in length. We confirmed this by comparing survival rates of (inter)nodes of +-0,5 cm to (inter)nodes of +-1 cm after bleaching with 1% sodium-hypochlorite solution (Supplementary Figures 2A and 3).

Testing sample preparation methods to increase transformation efficiency
Methods such as surface scratching and sonication create more wound surface on plant tissues to be infiltrated by *A. tumefaciens,* which enhances the ability of *A. tumefaciens* to transform tissues of cucurbit plants (Xin *et al.*, 2022). Vacuum infiltration also helps *A. tumefaciens* to infiltrate plant tissues better (Xin *et al.*, 2022). Since a higher percentage of successful transformation events in cucurbit plants were previously achieved, this raises the question whether these methods could also improve transformation efficiency in the sturdy TPS-potato shoot material. After pre-incubation, we applied microbrush-scratching, sonication and vacuum infiltration as performed by Xin *et al.* to the samples. The application of transformation-improving methods was thus tested on sterile (inter)node material.

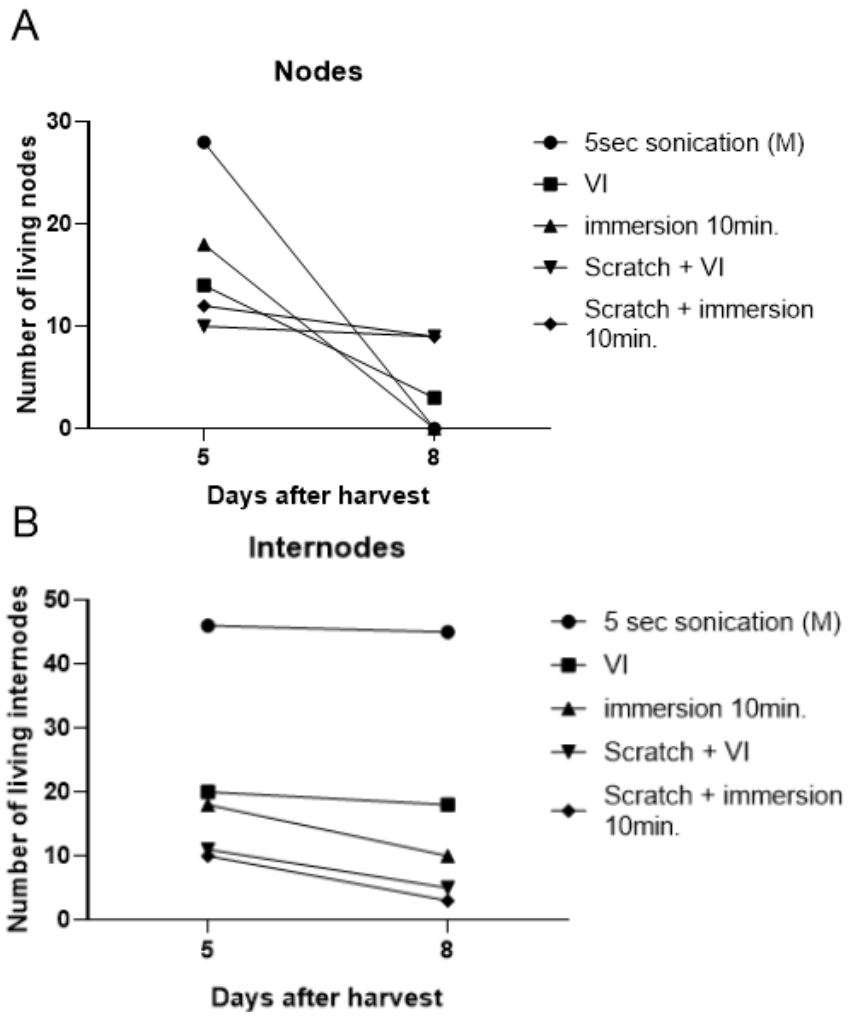

Figure 3: Applying sample preparation methods by Xin *et al.* (2022) on nodes and internodes. A: Different treatments performed on node tissues of an adult RP035 plant, 5 days after harvest. B: Different treatments performed on internode tissues of an adult RP035 plant, 5 days after harvest. Treatments included: surface scratching of plant material with a scalpel, vacuum infiltration (VI) or immersion, and/or sonication at medium power (M) for five seconds, the same for both nodes- and internodes. Samples that are clearly green (or growing buds or calli) are defined as 'living', samples that are brown, bleached or infected seen as 'dead'.

The optimal combination of treatments for the cucurbit plants melon and cucumber was reported to be the use of scratching, 10 seconds of sonication at 100W and vacuum infiltration (Xin *et al.,* 2022). Here, 5 seconds of sonication at medium power are chosen instead of 10 seconds because of the higher expected sturdiness of cucurbit plants. A series of different sample preparations were used on node- and internode tissues of the RP035 TPS-grown potato plant (Figure 3). Unfortunately, due to fungal infections the nodes treated with 10 minutes of immersion died while the uninfected scratch +10 minutes immersion did not, as well as scratching and immersion treatments combined (Figure 3A).

Incubation of plant samples and *A. tumefaciens* resulted in infections

After sample preparation, the next step in transformation is to expose the plant material to *A. tumefaciens* for transfer of genetic material. Because the aim of this project is to generate potato plants with shorter shoots and our *StER* construct was not yet ready for use, we used the C58C1- *A. tumefaciens*-35S::ATH1-GFP-line with kanamycin resistance to perform transformation experiments

(Rutjens *et al.,* 2009). This construct encodes for overexpression of AtATH1-GFP and has already effectively created mutant phenotypic lines in *Arabidopsis thaliana* by transformation (Proveniers, unpublished data). Upon successful transformation, GFP-signal is visible in calli when using fluorescence microscopy. From transformed calli, new transgenic shoots can be generated which contain the inserted DNA. We used both nodes and internodes for transformation and regeneration experiments with the 35S::ATH1-GFP line. After incubation, samples were transferred to M400 plates containing cefotaxime to kill *A. tumefaciens.*

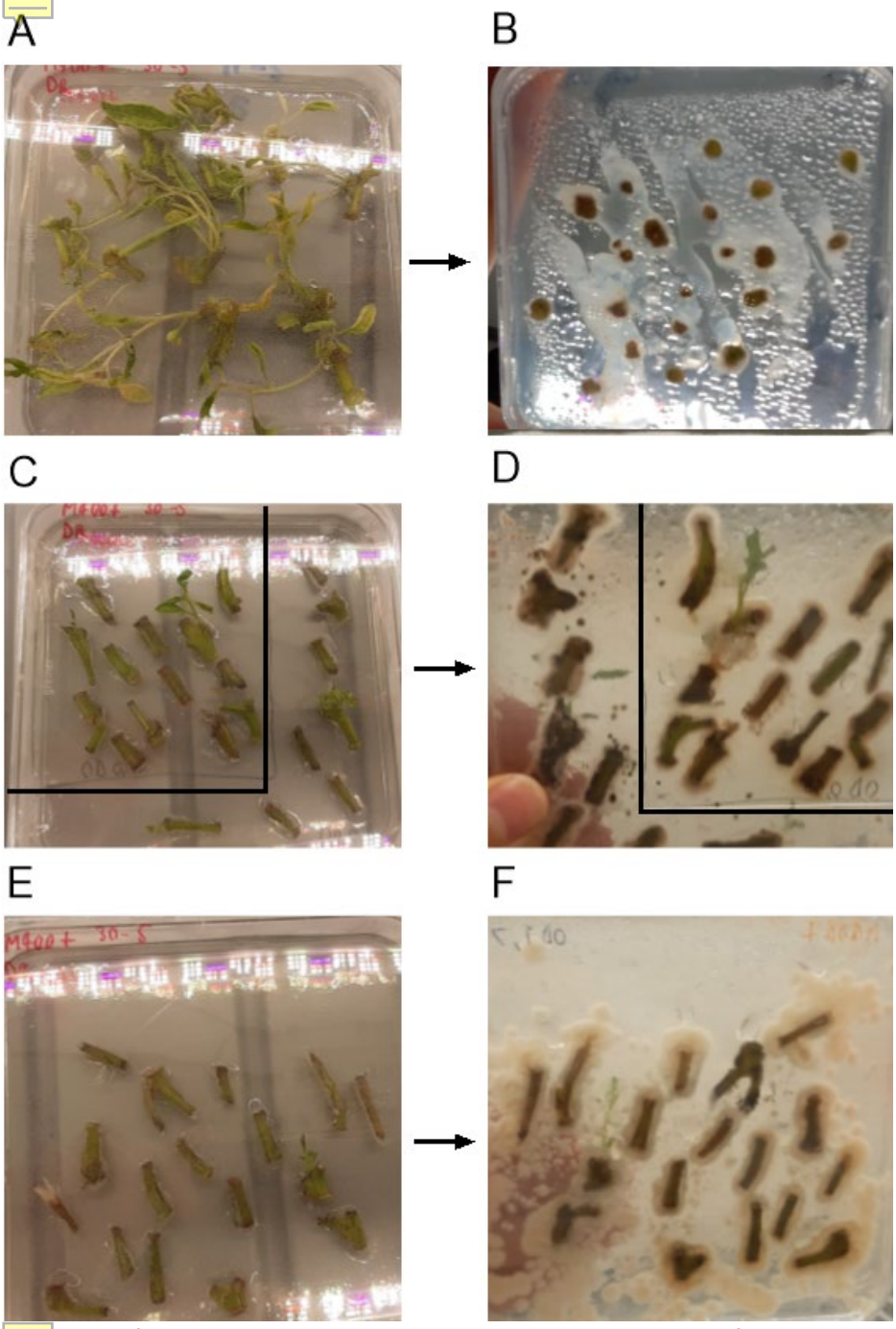

Figure 4: Infections on M13 and M400 plates, possibly *A. tumefaciens*.
A and B are the same experiment, C, D, E and F are another experiment. Pictures from A, C and E are taken from above, B D and F are from below for a better view.
A: Callus formation on RP001 nodes after 19 days (M400 plate with kanamycin).
B: Picture from calli from (A) two days after transplantation to M13 shoot inducing medium, probably with *A. tumefaciens* infections, OD600 = above 1,0.

C and D: M400 plates with dividing line with nodes and internodes immersed in *A. tumefaciens* cultures with different OD600's: 0,5 inside the square area; 0,9 outside the square area. Picture from D was taken four days after treatment.
E: M400 plate with nodes and internodes, treated with *A. tumefaciens* immersion (OD600 = well above 1,0).
F: Infections on plate in E four days after transfer.

Although a control experiment shows that cefotaxime kills *A. tumefaciens* even with high optical densities (Supplementary Figure 4), calli were still killed due to an infection (Figure 4A and 4B). There was a probability that this infection was caused by overproliferation of *A. tumefaciens* given its phenotypical similarity to *A. tumefaciens* colonies observed earlier (Figure 4B, -D and -F, Supplementary Figure 4). To make sure no *A. tumefaciens* infections would persist after incubation, vancomycin as additionally added as an extra antibiotic to M400- and M13 plates. Next to that, we also used an *A. tumefaciens* optical density (OD600) that was lower than the 2:5 stock dilution (OD600 above 1,0) to decrease chances of overproliferation.

RP005 nodes and petioles show growth of callus tissues after application of sample preparation

The protocol of Visser describes that internodes should be used for transformation and regeneration procedures, because of vascular tissues inside internodes that allow for better infiltration of *A. tumefaciens* (Visser, 1991). However, vascular tissues are found in many tissues in (potato) plants, meaning that more (shoot) tissues could be used for transformation- and callus regeneration experiments. Calli grow on cut-sites of plant samples, meaning that petiole- and node tissue can also be used, if the cut-site is clearly devoid of axillary meristem tissue to avoid chimeric tissue growth, as seen in Figure 4A. Another transformation experiment was performed with the in vitro growing RP005- and RP001 plants. Effects of vacuum infiltration and high power-sonication for 15- and 30 seconds were tested to explore the upper limits of effectiveness of sonication. Calli formed on node- and petiole tissues (Figure 5A and B). Within 38 days after the first formed callus, more calli appeared on both nodes and petioles. Unfortunately, internodes that survived sonication treatments died from infections (data not shown). Duration of sonication influences the browning of the tissue material as internodes (that did not die from infections yet) that were subjected to 30 seconds of sonication are darker brown than in samples subjected to 15 seconds of sonication (data not shown). The difference in color is only small because both pictures are taken of M400 selection plates with kanamycin, which browns and kills non-transformed plant tissue.

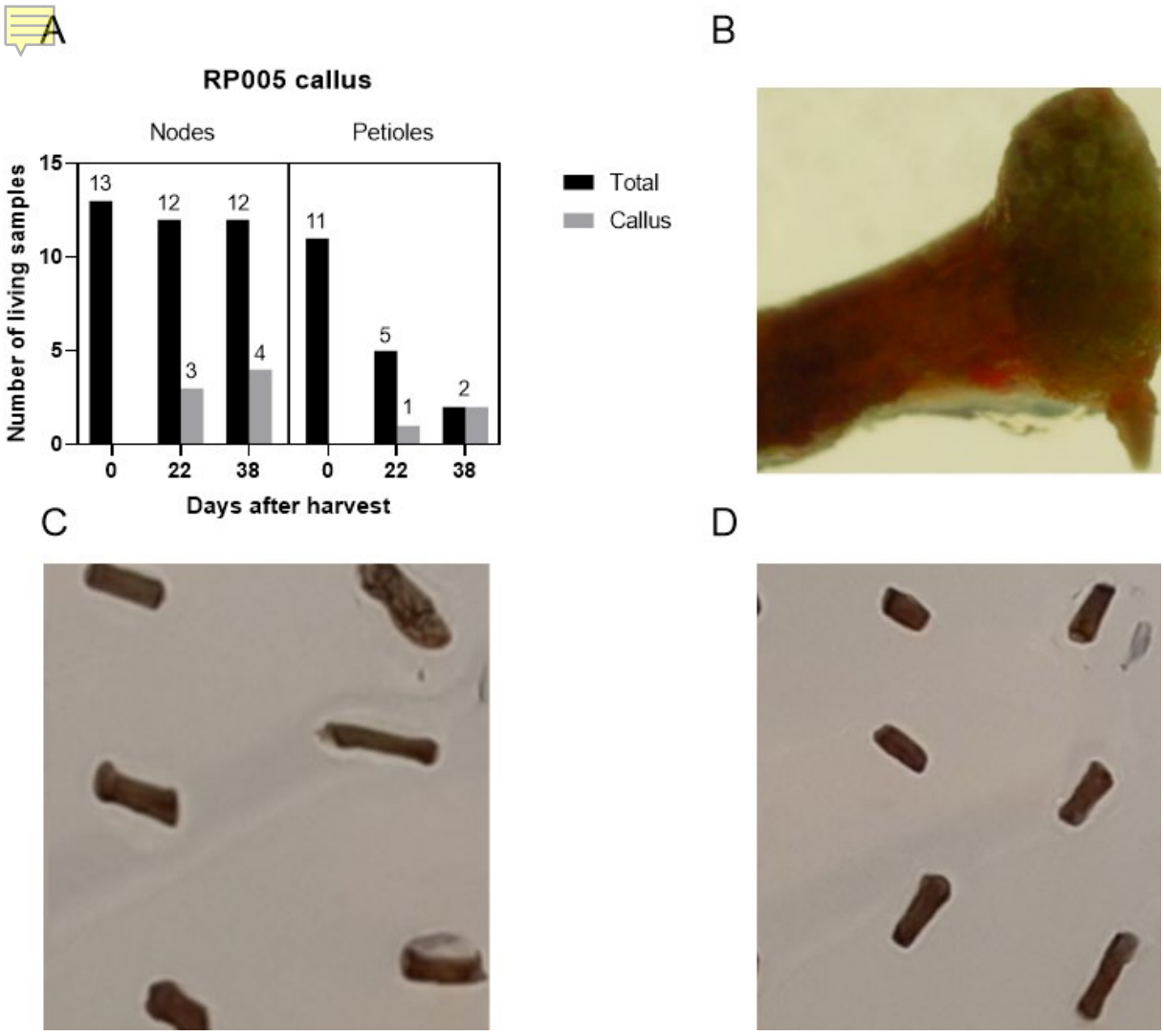

Figure 5: Callus formation on RP005 nodes and petioles, and effects of sonication on internodes.
A: Number of nodes and petioles that survived for 22 and 38 days after harvest. After 22 days, both nodes and petioles had started to form calli. After 38 days, 4 out of 12 node samples had formed callus tissue and all living petioles (2).
B: Close-up image of node with callus on right end (modified image brightness with Paint 3D).
C: Internode browning after 15 seconds of sonication.
D: internode browning after 30 seconds of sonication.

Tissues that formed callus still portrayed browning in the rest of the (inter)node/petiole (Figure 5B). However, the callus tissue is still green (Supplementary figure 5A), meaning that it is probably still alive. This could indicate that it received kanamycin resistance from *A. tumefaciens,* meaning that transformation occurred.

<u>Assembling the *StER* construct with Golden Gate cloning for higher genome editing efficiency and RUBY reporter gene</u>

The transformation experiments thus far only focused on improving the ability of *A. tumefaciens* to infiltrate and infect plant tissues. However, for successful genetic transformation, incorporation of the inserted DNA into the plant genome is also necessary. As Eggers *et al.* (2021) described, their transformed diploid potato plants only incorporated the DNA 6 times per 149 transformants with only a single successful homozygous mutation event. To improve genome editing-efficiency we aimed to assemble a genetic construct with Golden Gate cloning encoding for the CRISPR/Cas-9-

mediated knockout of *ER* that is easy to use (Figure 6). This method uses the CRISPR-associated-protein 9 (zCas9i) that uses four guide RNA (gRNA) sequences that target for regions in the potato genome where the zCas9i cleaves. The first two gRNA sequences are used to guide the zCas9i nuclease to sites in the first and third exon, and the third and fourth gRNA's are designed for sites in the 27th exon of *ER* (Table 1). In this way, the nuclease cleaves around the start and end of the coding *ER* sequence, which enhances chances of an *ER*-gene knockout. To make the process of transformation easier and faster, we use the '*RUBY*' open reading frame as reporter genes (He *et al.,* 2020). *RUBY* is an artificial DNA open reading frame that encodes for all necessary enzymes to convert the amino acid tyrosine into betalain, a natural plant product that appears bright red (Strack *et al.,* 2003). Tyrosine is present in all cells, which gives RUBY high applicability and reliability as reporter over most anthocyanins, which differ more and require more enzymes (Misyura *et al.,* 2013, Li *et al.,* 2016). Together with the PcUbi (Parsley Ubiquitin, Kishi-Kaboshi *et al.,* 2019) promoter, *RUBY* offers a potentially easier and faster method to detect transformation events. Upon transformation, the red color will already be visible in calli with *RUBY* instead of in regenerated shoot tissues when using GFP (He *et al.,* 2020). Whereas GFP or GUS-staining as reporter genes require fluorescence detection methods or expensive chemicals, *RUBY* does not and is non-invasive.

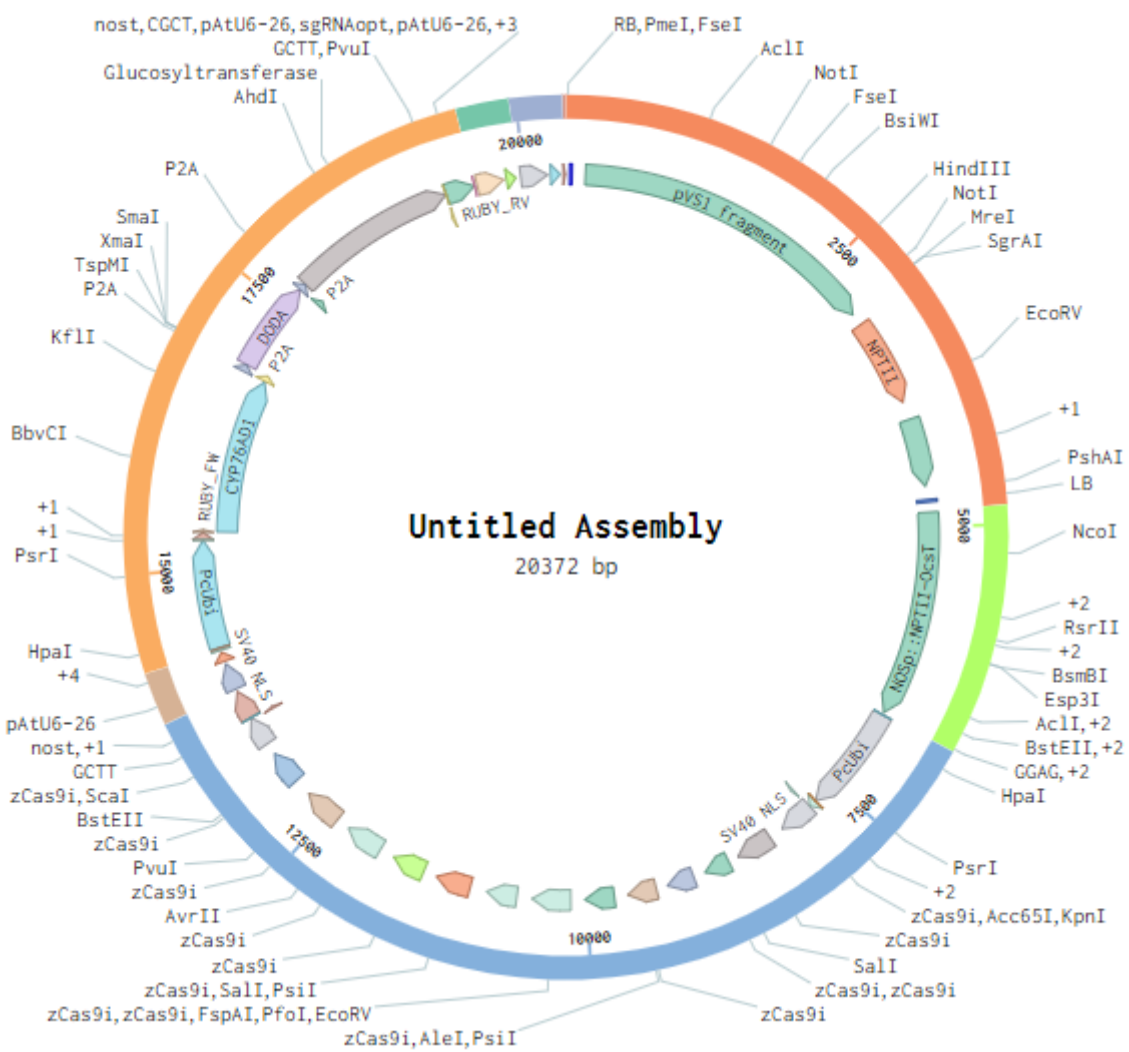

Figure 6: Genetic assembly with CRISPR/Cas-9, gRNA's and *RUBY* for diploid potato transformation. *StER* knockout level 2 assembly (to be) used to knock out *ER* expression in diploid potato. The plasmid contains kanamycin resistance (NPTII with NOS promoter), the *RUBY* open reading frame,

the intronized Cas-9 coding sequence (both with the PcUbi promoter) as described by Grützner *et al*. (2021) and four guide RNA sequences.

The intronized Cas-9-coding sequence also uses the PcUbi promoter. NPTII with nopaline synthase (NOS)-promoter is for kanamycin selection. Taken together, this genetic assembly is designed to knock-out expression of native *ER* in potato in combination with a fast reporter system.
To assemble this construct, Esp3I was used as restriction enzyme (for BsmBI sites) for the first molecular cloning reaction, where diluted hybridized oligonucleotide gRNA spacers are cloned in their respective level 1 plasmid. After cloning and heat shock treatments with *E. coli,* transformed bacteria were plated on selective medium (LB agar with carbenicillin (100µg/mL), Figure 7A). When the digestion reaction with the Esp3I enzyme had taken place correctly, colonies should be white. If not, colonies are purple because in the case that the level 1 molecular cloning reaction failed to incorporate the desired gRNA spacer, the dsRED cassette is still present (causing the colony to appear red).

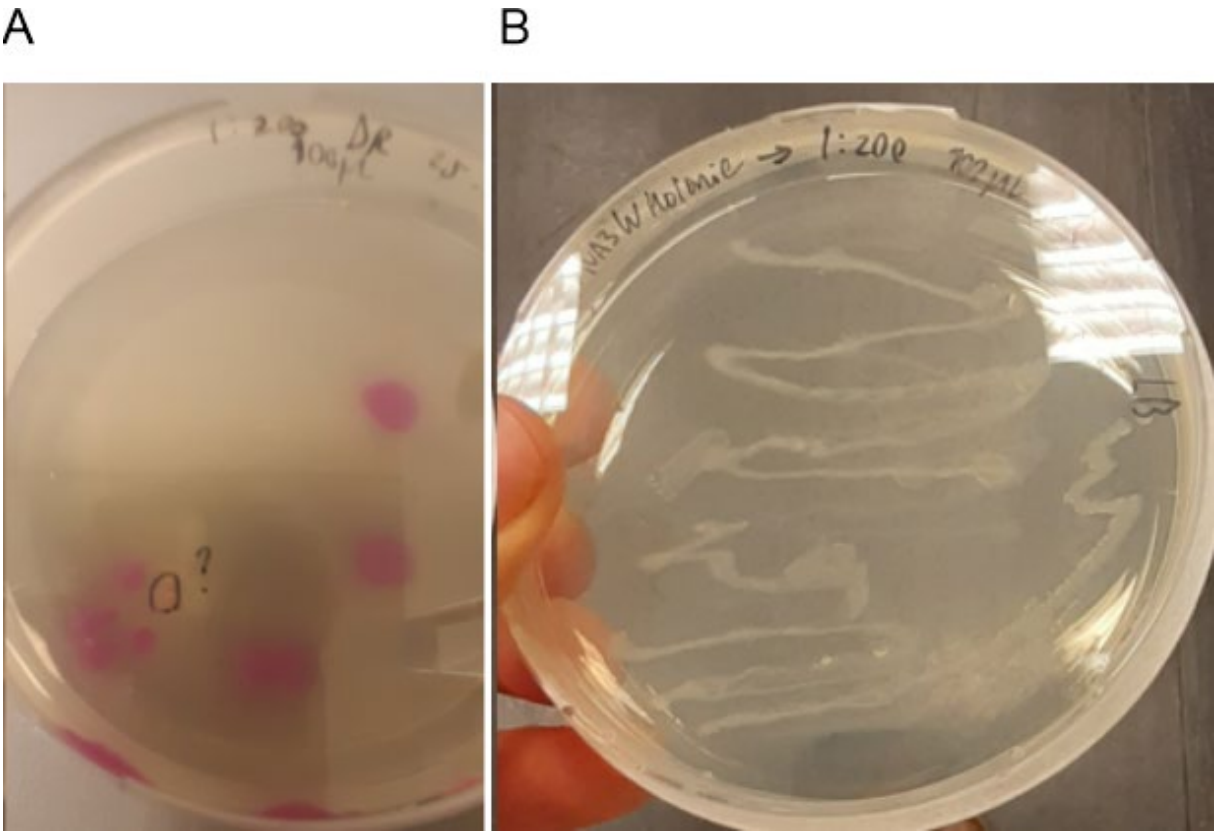

Figure 7: *E. coli* growth on LB selection plates
A: LB carbenicillin (100 µg /mL) plate with one white and multiple purple colonies. 1:200 means an oligonucleotide concentration of 50fmol.
B: White colony from left picture inoculated on another LB carbenicillin plate as backup.

The *E. coli* cells took up the dsRED plasmids because colonies were able to grow on carbenicillin medium (Figure 7A, control experiment: Supplementary figure 7). However, the percentage of white colonies among purple ones was very low (data not shown). The few white colonies were plated to new LB carbenicillin plates for confirmation via sequencing and storage. Due to several replicate experiments resulting in no white colonies, the efficiency of the restriction enzyme Esp3I used in the reactions was tested using Gel analysis. ThermoFisher Scientific recommends the use of Buffer Tango and Dithiothreitol (DTT) for molecular cloning reactions with Esp3I, thus effects of DTT were also tested (Figure 8B). Combinations of the used Esp3I-stock (Figure 8A) and new Esp3I-stock (Figure 8B) and BpiI-restriction enzymes were tested (BpiI as control reaction for the Esp3I enzyme reaction), as

well as Tango- or G-buffers and addition of DTT. DNA samples from the dsRED-plasmid stocks were used.

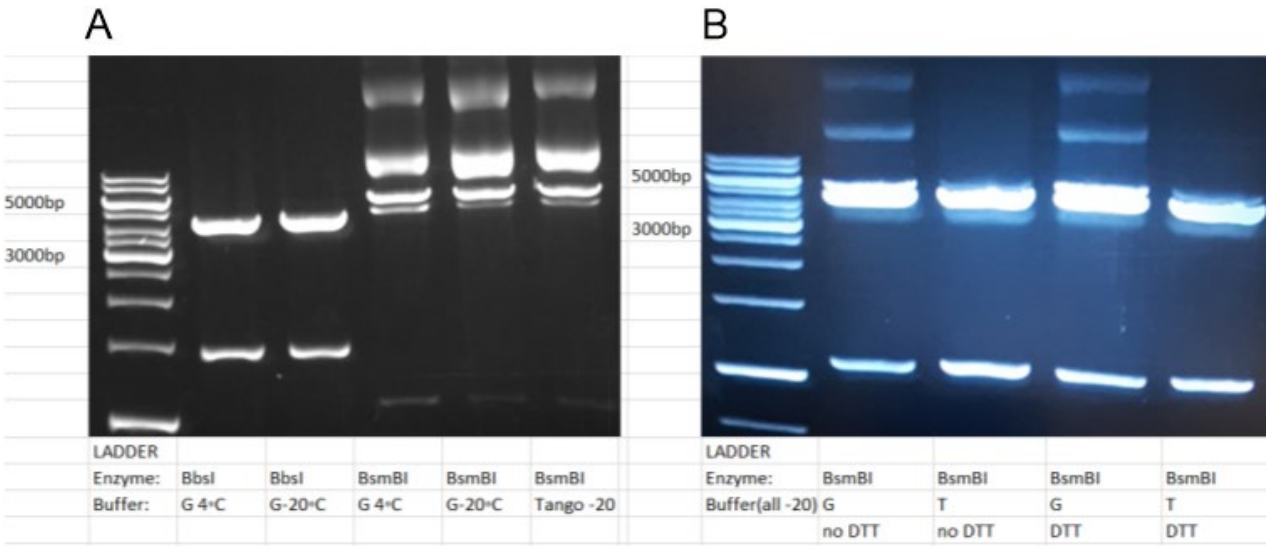

Figure 8: Gel analysis to compare activity of Esp3I- and BpiI aliquots.
A: Fragment of Gel analysis. Restriction enzyme Esp3I (initial aliquot). Stock samples were used for digestion by Esp3I. Plasmid vector used is: 'gRNA3': dsRED 47781.
B: Gel analysis with Esp3I restriction enzyme. Abbreviations: 'G' = buffer G added, 'T' = buffer Tango added, 'DTT' = dithiothreitol added.

Using the dsRED plasmids, a fragment of just under 6000bp is expected to be visible without active restriction enzyme. With active restriction enzymes, Esp3I splits the vector into two fragments of around 1200- and 4600 bp, whereas BpiI yields two fragments of around 1500- and 4300bp. Band size and intensity of the Esp3I enzyme in the first Gel analysis shows lower enzymatic activity than in the second blot (Figure 8B), because almost no bands are visible with a size of around 1200kb in Figure 8A (right three lanes, BsmbI). Also, more plasmid is digested when Buffer Tango is added than with Buffer G (Figure 8B), as is seen when looking at the dimmer bands in the lanes with Buffer Tango around 5000Bp, just above the brightest bands. Addition of 20mM DDT shows no clear effect in this gel analysis.
To verify if the reaction had taken place correctly, samples from every guide RNA were sent *in triplo* to Macrogen for sequencing to increase chances of finding the right alignments. For this sequencing the primer sequence used was GAACCCTGTGGTTGGCATGCACATAC (0229F). Alignments of sequencing results and the designed plasmids were created in order to check if the samples were cloned correctly (Supplementary figure 8). Samples 1-2 (for gRNA 1), 2-3 (for gRNA 2) and all three samples for gRNA 4 show no mismatches regarding the cloning of the gRNA sequences, with sample 2-3 showing some mismatches in the gRNA sequence (Supplementary figure 8B). For dsRED pICH47781 (for guide RNA 3) we did not obtain successfully cloned plasmids and thus no alignment results could be made.

Conclusion and discussion

To improve the current process of potato cultivation, several obstacles have yet to be overcome. Diploid inbred potato lines are promising in terms of breeding. Transforming diploid potato to generate lines that are suitable for high seed production would help alleviate the costs and footprint of current propagation methods and revolutionize the potato industry. However, transformation procedures for diploid TPS-grown potato are prone to low efficiencies, as is shown in this research. In this work we aimed to create a more compact potato line using an optimized protocol for transformation and in vitro growth. This work emphasizes the need for an effective sterilization

protocol, as infections interfered frequently with in vitro growth- and transformation experiments. Effects of sample preparations were hard to attribute to treatments due to these infections, but we nonetheless grew calli on potato shoot tissues with a chance of transformation with 35S::ATH1-GFP. To further optimize transformation efficiency we aimed to create our *StER*-assembly using Golden Gate cloning with increased CRISPR/Cas-9 genome editing efficiency in combination with the RUBY reporter gene(s) but ran into problems with low efficiency of molecular cloning reactions.

We showed that bleaching is lethal in too high dosage (2% sodium hypochlorite, Visser (1991)) and ineffective in killing all fungal spores, as some of them may possibly be endophytic fungi out of reach of the bleaching treatment (Zhang *et al.,* 2022, Götz *et al.,* 2006). The bleaching has a chance of effectively surface-sterilizing plant material, depending on the species of fungi living on the tissue (Visconti *et al.,* 2021). However, it must not reach everywhere inside it if the material is to survive. Internodes of 0,5 cm died from 1% bleaching treatment because the chlorine compounds reached inside all of the tissue, while inner parts of 1 cm internodes probably remained intact because they retained their green color. Bleaching experiments did not point out clearly whether fungal spores are endogenous or not to potato shoots, because short internode samples where all of the sample material was bleached still got infected later on. The seemingly sterile bleaching experiment with 0,09% bleach could be attributed to the species of fungi (Visconti *et al.,* 2021) or the fact that we did not treat it with *A. tumefaciens,* since we never managed to keep material sterile that long again with such low a bleach concentration. Our most important finding about bleaching protocols is that a more effective sterilization method for diploid TPS potato is needed. Infections interfered many times with our in vitro- and transformation experiments, what made obtaining reliable results of sonication- or vacuum infiltration hard.

Despite problems with sterilization of plant material, a few genotypes have shown to be able to grow over longer periods of time (over 3 weeks) in vitro. However, there is no indication that this can be attributed to the genotypes of these plants, and transplantations were sometimes needed to keep plants sterile when fungal infections arose from other plants in the same in vitro-pot. The yellow bacterial colonies growing from newly emerged roots in in vitro growing plants are also an indication for endophytic microbes. According to Sturz (1995), healthy potato plant tissue carries internal bacterial cultures. Whether the observed yellow bacterial colonies were harmful for plant health could be investigated with disease assays or genetic experiments like qPCR with genetic material from the bacteria for identification. If bacterial cultures in potato interfere with *A. tumefaciens* experiments, low antibiotic dosage in in vitro growth medium may be of use, since *A. tumefaciens* is grown with antibiotic resistance (rifampicin and spectinomycin). An assay with isolated bacteria growing on medium with antibiotics may point out effective strategies for in vitro growing with antibiotics.

Cefotaxime, vancomycin and kanamycin are bactericides (kanamycin also represses plant growth, Duan *et al.,* 2009), which means that fungi may survive in their presence. Alternatives like fungicide or biocide can offer possible solutions to fungus-related problems with diploid potato. Although harmful to plants, fungi- or biocides may still be more effective in fighting fungal infections than bleaching. Another way to grow sterile TPS-plants is to germinate seeds in vitro using GA, although other propagation techniques such as microtuber growth are favored over TPS germination because of unreliable TPS-germination rates and probably high in vitro humidity (Pallais, 1987, Buckseth *et al.*, 2022, Marcel Proveniers, personal communication, 2022). For this research TPS was germinated in soil. However, experiments with TPS germinating in vitro and for instance chlorine gas sterilization like in Arabidopsis may offer a way out of bleaching protocols (Lindsey *et al.*, 2017).

Control experiments with *A. tumefaciens* survival showed that cefotaxime was effective, although *A. tumefaciens* apparently had better survival chances in the presence of plant material when incubating on cefotaxime plates, as is seen in Figures 4C-F. Another possibility is that the *A. tumefaciens* optical density so high that the 2:5 dilution described by Eggers (2021) resulted in too high concentrations for effective transformation. Therefore, we used vancomycin as extra antibiotic to kill *A. tumefaciens.* However, we have no results that indicate whether vancomycin was effective in killing *A. tumefaciens* or not in the presence of plant material, which would require genetic

analysis of isolated bacterial DNA.
The protocol of Visser described that internodes should be used for transformation and regeneration. However, our results suggested that nodes and petioles can be used for transformation- and subsequent regeneration processes as well. Visser (1991) evaded the use of nodal tissue because the axillary meristem may cause regenerating shoots to be chimeric. Our results suggest that nodes and petioles may be effective as well in forming callus and surviving regeneration procedures (Figure 5A), as calli formed over time on petioles and nodes. However, confirmation of transformation by observation of GFP signal is yet to be performed. This also applies to petiole tissues in this experiment (Supplementary figure 5B). We applied sample preparation as by Xin *et al.* on these samples, which shows that calli can still grow after these (relatively) harsh treatments. Longer sonication treatment (30 seconds high power) turned plant samples darker brown than shorter treatments (15 seconds high power), showing that sonication decreases sample survival (Xin *et al.,* 2022). Due to infections and therefore reduced sample sizes we did not find a significant increase in survival- or callus formation rates between the different tissues and treatments (Scratching, sonication and vacuum infiltration, Figure 3).
Also, the protocol of Visser prescribes that kanamycin be used as plant selection agent. Our results showed that non-transformed plant tissues turned brown and died on kanamycin medium, but not the RP001 nodes from Figure 4A. Possible causes for this could have been improper preparation of the medium, kanamycin only killing plant samples that are more vulnerable because of earlier harsh treatments, or differences between the RP-genotypes in kanamycin resistance. If the combination of sample preparations by Xin *et al.* and selection on kanamycin was too harsh for plant material, more incubation time prior to selection with kanamycin may help plant samples survive better. However, none of the samples in Figure 4A showed browning like the nodes and petioles in Figure 5C-D (medium with kanamycin), so the medium in Figure 4A may have lacked an effective kanamycin dose. This suggests that in this experiment selection for transformed tissues may not have taken place. It is unclear whether the infections in Figure 4B were *A. tumefaciens* or not because the plate in Figure 4A remained sterile for at least three weeks after immersion treatment.
In the Golden Gate cloning steps for the creation of the *StER* knockout we encountered numerous obstacles. Gel analysis showed the initially used restriction enzyme Esp3I aliquot to be less active than another Esp3I aliquot. Although these were different gels and experiments, we can compare both gels to the ladder, which is the same for both gels. Figure 8B shows that the Esp3I enzyme digested the dsRED plasmids, and *E.coli* colonies grew on LB carbenicillin plates, indicating a successful heat shock (Figure 7A and Supplementary figure 7). Considering these parts of the experiment were successful, the most probable explanations for the low number of white *E. coli* colonies involve the creation of recombinant DNA since the reaction mixture and heating steps were performed according to the Golden Gate protocol (Table 2, Materials and Methods). Examples are DNA methylation by *E. coli* or incorrect DNA ligase activity. If DNA methylation inhibits the level 1 cloning reaction, addition of demethylation enzymes such as methylguanine methyltransferase (MGMT) to the reaction mix may increase the occurrence of correct level 1 cloning reactions and thus white *E. coli* colonies. To investigate effectivity of T4 DNA ligase, a gel analysis can be performed with plasmid samples digested by a restriction enzyme. After digestion, T4 DNA ligase can be used for ligation. Enzymatic activity can be tested by comparing positions of DNA (plasmid) fragments equal in mass, since ligated circular DNA travels slower through agarose than non-ligated linear DNA fragments.
To conclude, this work emphasized the need for a new sterilization protocol for research in TPS-diploid potato. We showed that we were able to grow calli on node- and petiole samples treated with methods described by Xin *et al.* (2022) within a month. Still, more research is needed to confirm findings of this work; experiments had mostly small sample sizes and were often disturbed by infections.

References

Belhaj, Khaoula, Angela Chaparro-Garcia, Sophien Kamoun, Nicola J Patron, en Vladimir Nekrasov. 'Editing Plant Genomes with CRISPR/Cas9'. *Current Opinion in Biotechnology*, Food Biotechnology • Plant Biotechnology, 32 (1 april 2015): 76–84. https://doi.org/10.1016/j.copbio.2014.11.007.

Bethke, Paul C., Atef M. K. Nassar, Stan Kubow, Yves N. Leclerc, Xiu-Qing Li, Muhammad Haroon, Teresa Molen, John Bamberg, Max Martin, en Danielle J. Donnelly. 'History and Origin of Russet Burbank (Netted Gem) a Sport of Burbank'. *American Journal of Potato Research* 91, nr. 6 (1 december 2014): 594–609. https://doi.org/10.1007/s12230-014-9397-5.

Brown, C. R., C. -P. Yang, S. Kwiatkowski, en K. D. Adiwilaga. 'Insert Copy Number, Chromosome Number, Pollen Stainability, and Crossability OfAgrobacterium-Transformed Diploid Potato'. *American Potato Journal* 68, nr. 5 (1 mei 1991): 317–30. https://doi.org/10.1007/BF02853669.

Buchholz, Franziska, Livio Antonielli, Tanja Kostić, Angela Sessitsch, en Birgit Mitter. 'The bacterial community in potato is recruited from soil and partly inherited across generations'. *PLoS ONE* 14, nr. 11 (8 november 2019): e0223691. https://doi.org/10.1371/journal.pone.0223691.

Buckseth, Tanuja, Jagesh K. Tiwari, Rajesh K. Singh, Vinod Kumar, Ashwani K. Sharma, Dalamu Dalamu, Vinay Bhardwaj, e.a. 'Advances in innovative seed potato production systems in India'. *Frontiers in Agronomy* 4 (2022). https://www.frontiersin.org/articles/10.3389/fagro.2022.956667

Butler, Nathaniel M., Paul A. Atkins, Daniel F. Voytas, en David S. Douches. 'Generation and Inheritance of Targeted Mutations in Potato (Solanum Tuberosum L.) Using the CRISPR/Cas System'. *PLOS ONE* 10, nr. 12 (14 december 2015): e0144591. https://doi.org/10.1371/journal.pone.0144591.

Dönmez, Betül Ayça, Sarbesh Das Dangol, en Allah Bakhsh. 'Transformation Efficiency of Five Agrobacterium Strains in Diploid and Tetraploid Potatoes'. *Sarhad Journal of Agriculture 35*, nr. 4 (december 2019): 1344-1350. https://doi.org/10.17582/journal.sja/2019/35.4.1344.1350.

Duan, Hongying, Xiaosheng Ding, Jianying Song, Duan Zhikun, Yanqing Zhou, en Chune Zhou. 'Effects of kanamycin on growth and development of arabidopsis thaliana seedling, cotyledon and leaf'. *Pakistan Journal of Botany* 41 (1 augustus 2009).

Eggers, Ernst-Jan, Ate van der Burgt, Sjaak A. W. van Heusden, Michiel E. de Vries, Richard G. F. Visser, Christian W. B. Bachem, en Pim Lindhout. 'Neofunctionalisation of the Sli Gene Leads to Self-Compatibility and Facilitates Precision Breeding in Potato'. *Nature Communications* 12, nr. 1 (6 juli 2021): 4141. https://doi.org/10.1038/s41467-021-24267-6.

Enciso-Rodriguez, Felix, Norma C. Manrique-Carpintero, Satya Swathi Nadakuduti, C. Robin Buell, Daniel Zarka, en David Douches. 'Overcoming Self-Incompatibility in Diploid Potato Using CRISPR-Cas9'. *Frontiers in Plant Science* 10 (2019). https://www.frontiersin.org/articles/10.3389/fpls.2019.00376.

Gómez-Mena, Concepción, en Robert Sablowski. 'ARABIDOPSIS THALIANA HOMEOBOX GENE1 Establishes the Basal Boundaries of Shoot Organs and Controls Stem Growth'. *The Plant Cell* 20, nr. 8 (augustus 2008): 2059–72. https://doi.org/10.1105/tpc.108.059188

Götz, Monika, Helgard Nirenberg, Sibylle Krause, Heike Wolters, Siegfried Draeger, Arno Buchner, Jana Lottmann, Gabriele Berg, en Kornelia Smalla. 'Fungal endophytes in potato roots studied by traditional isolation and cultivation-independent DNA-based methods'. *FEMS Microbiology Ecology* 58, nr. 3 (1 december 2006): 404–13. https://doi.org/10.1111/j.1574-6941.2006.00169.x.

Grützner, Ramona, Patrick Martin, Claudia Horn, Samuel Mortensen, Erin J. Cram, Carolyn W. T. Lee-Parsons, Johannes Stuttmann, en Sylvestre Marillonnet. 'High-Efficiency Genome Editing in Plants Mediated by a Cas-9 Gene Containing Multiple Introns'. *Plant Communications*, Technology and Applications in Plants, 2, nr. 2 (8 maart 2021): 100135. https://doi.org/10.1016/j.xplc.2020.100135.

He, Yubing, Tao Zhang, Hui Sun, Huadong Zhan, en Yunde Zhao. 'A Reporter for Noninvasively Monitoring Gene Expression and Plant Transformation'. *Horticulture Research* 7, nr. 1 (19 september 2020): 1–6. https://doi.org/10.1038/s41438-020-00390-1.

Khan, Mudasir Hafiz, Zahoor Ahmad Dar, en Sher Ahmad Dar. 'Breeding Strategies for Improving Rice Yield—A Review'. *Agricultural Sciences* 06, nr. 05 (2015): 467.https://doi.org/10.4236/as.2015.65046.

Kishi-Kaboshi, Mitsuko, Ryutaro Aida, en Katsutomo Sasaki. 'Parsley ubiquitin promoter displays higher activity than the CaMV 35S promoter and the chrysanthemum actin 2 promoter for productive, constitutive, and durable expression of a transgene in Chrysanthemum morifolium'. *Breeding Science* 69, nr. 3 (september 2019): 536–44. https://doi.org/10.1270/jsbbs.19036.

Kwon, Choon-Tak, Jung Heo, Zachary H. Lemmon, Yossi Capua, Samuel F. Hutton, Joyce Van Eck, Soon Ju Park, en Zachary B. Lippman. 'Rapid Customization of Solanaceae Fruit Crops for Urban Agriculture'. *Nature Biotechnology* 38, nr. 2 (februari 2020): 182–88. https://doi.org/10.1038/s41587-019-0361-2.

Li, Penghui, Beibei Chen, Gaoyang Zhang, Longxiang Chen, Qiang Dong, Jiangqi Wen, Kirankumar S. Mysore, en Jian Zhao. 'Regulation of Anthocyanin and Proanthocyanidin Biosynthesis by Medicago Truncatula BHLH Transcription Factor MtTT8'. *New Phytologist* 210, nr. 3 (2016): 905–21. https://doi.org/10.1111/nph.13816.

Lindsey, Benson E., Luz Rivero, Chistopher S. Calhoun, Erich Grotewold, en Jelena Brkljacic. 'Standardized Method for High-Throughput Sterilization of Arabidopsis Seeds'. *Journal of Visualized Experiments: JoVE*, nr. 128 (17 oktober 2017): 56587. https://doi.org/10.3791/56587.

Masson, J., D. Lancelin, C. Bellini, M. Lecerf, P. Guerche, en G. Pelletier. 'Selection of Somatic Hybrids between Diploid Clones of Potato (Solanum Tuberosum L.) Transformed by Direct Gene Transfer'. *Theoretical and Applied Genetics* 78, nr. 2 (1 augustus 1989): 153–59. https://doi.org/10.1007/BF00288791.

Misura, Maksym, Joseph Colasanti, en Steven J. Rothstein. 'Physiological and Genetic Analysis of Arabidopsis Anthocyanin Biosynthesis Mutants under Chronic Adverse Environmental Conditions'. *Journal of Experimental Botany* 64, nr. 1 (januari 2013): 229–40. https://doi.org/10.1093/jxb/ers328.

Murray, F., R. Brettell, P. Matthews, D. Bishop, en J. Jacobsen. 'Comparison of Agrobacterium-Mediated Transformation of Four Barley Cultivars Using the GFP and GUS Reporter Genes'. *Plant Cell Reports* 22, nr. 6 (januari 2004): 397–402. https://doi.org/10.1007/s00299-003-0704-8.

Nadolska-Orczyk, Anna, Aleksandra Pietrusinska, Agnieszka Binka-Wyrwa, Dominik Kuc, en Wacław Orczyk. 'Diploid Potato (Solanum Tuberosum L.) as a Model Crop to Study Transgene Expression'. *Cellular & Molecular Biology Letters* 12, nr. 2 (1 april 2007): 206–19. https://doi.org/10.2478/s11658-006-0064-9.

Pallais, Noel. 'True potato seed quality'. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik* 73 (1 april 1987): 784–92. https://doi.org/10.1007/BF00289380.

Pearce, Stephen. 'Towards the replacement of wheat "Green Revolution" genes'. *Journal of Experimental Botany* 72, nr. 2 (2 februari 2021): 157–60. https://doi.org/10.1093/jxb/eraa494.

Proveniers, Marcel, Bas Rutjens, Marco Brand, en Sjef Smeekens. 'The Arabidopsis TALE Homeobox Gene ATH1 Controls Floral Competency through Positive Regulation of FLC'. *The Plant Journal* 52, nr. 5 (2007): 899–913. https://doi.org/10.1111/j.1365-313X.2007.03285.x.

Quaedvlieg, N, J Dockx, F Rook, P Weisbeek, en S Smeekens. 'The homeobox gene ATH1 of Arabidopsis is derepressed in the photomorphogenic mutants cop1 and det1.' *The Plant Cell* 7, nr. 1 (januari 1995): 117–29. https://doi.org/10.1105/tpc.7.1.117.

Rutjens, Bas, Dongping Bao, Evelien Van Eck-Stouten, Marco Brand, Sjef Smeekens, en Marcel Proveniers. 'Shoot Apical Meristem Function in Arabidopsis Requires the Combined Activities of Three BEL1-like Homeodomain Proteins'. *The Plant Journal* 58, nr. 4 (2009): 641–54. https://doi.org/10.1111/j.1365-313X.2009.03809.x.

Stokstad, E. 'The New Potato. Breeders Seek a Breakthrough to Help Farmers Facing an Uncertain Future.' *Science*, februari 2019, pp 574-577. https://doi.org/10.1126/science.363.6427.574.

Strack, Dieter, Thomas Vogt, en Willibald Schliemann. 'Recent Advances in Betalain Research'. *Phytochemistry* 62, nr. 3 (februari 2003): 247–69. https://doi.org/10.1016/s0031-9422(02)00564-2.

Sturz, A. V. 'The Role of Endophytic Bacteria during Seed Piece Decay and Potato Tuberization'. *Plant and Soil* 175, nr. 2 (1 augustus 1995): 257–63. https://doi.org/10.1007/BF00011362.

Torii, K. U., N. Mitsukawa, T. Oosumi, Y. Matsuura, R. Yokoyama, R. F. Whittier, en Y. Komeda. 'The Arabidopsis ERECTA Gene Encodes a Putative Receptor Protein Kinase with Extracellular Leucine-Rich Repeats'. *The Plant Cell* 8, nr. 4 (april 1996): 735–46. https://doi.org/10.1105/tpc.8.4.735.

Villagarcia, Hector, Anne-Claire Morin, Elena D. Shpak, en Mariya V. Khodakovskaya. 'Modification of tomato growth by expression of truncated ERECTA protein from Arabidopsis thaliana'. *Journal of Experimental Botany* 63, nr. 18 (1 november 2012): 6493–6504. https://doi.org/10.1093/jxb/ers305.

Visconti, Vincent, Karim Rigalma, Emmanuel Coton, en Philippe Dantigny. 'Impact of the Physiological State of Fungal Spores on Their Inactivation by Active Chlorine and Hydrogen Peroxide'. *Food Microbiology* 100 (december 2021): 103850. https://doi.org/10.1016/j.fm.2021.103850.

Visser, Richard G. F. 'Regeneration and Transformation of Potato by Agrobacterium Tumefaciens'. In *Plant Tissue Culture Manual: Supplement 7*, onder redactie van K. Lindsey, 301–9. Dordrecht: Springer Netherlands, 1997. https://doi.org/10.1007/978-94-009-0103-2_16.

Vries-Uijtewaal, E. de, L. J. W. Gilissen, E. Flipse, K. Sree Ramulu, W. J. Stiekema, en B. de Groot. 'Fate of Introduced Genetic Markers in Transformed Root Clones and Regenerated Plants of Monohaploid and Diploid Potato Genotypes'. *Theoretical and Applied Genetics* 78, nr. 2 (1 augustus 1989): 185–93. https://doi.org/10.1007/BF00288798.

Wang, Yonghong, en Jiayang Li. 'Genes Controlling Plant Architecture'. *Current Opinion in Biotechnology*, Plant biotechnology/Food biotechnology, 17, nr. 2 (1 april 2006): 123–29. https://doi.org/10.1016/j.copbio.2006.02.004.

Xin, Tongxu, Haojie Tian, Yalin Ma, Shenhao Wang, Li Yang, Xutong Li, Mengzhuo Zhang, e.a. 'Targeted Creating New Mutants with Compact Plant Architecture Using CRISPR/Cas-9 Genome Editing by an Optimized Genetic Transformation Procedure in Cucurbit Plants'. *Horticulture Research*, 20 januari 2022, uhab086. https://doi.org/10.1093/hr/uhab086.

Yokoyama, R., T. Takahashi, A. Kato, K. U. Torii, en Y. Komeda. 'The Arabidopsis ERECTA Gene Is Expressed in the Shoot Apical Meristem and Organ Primordia'. *The Plant Journal: For Cell and Molecular Biology* 15, nr. 3 (augustus 1998): 301–10. https://doi.org/10.1046/j.1365-313x.1998.00203.x.

Zhang, Chunzhi, Zhongmin Yang, Dié Tang, Yanhui Zhu, Pei Wang, Dawei Li, Guangtao Zhu, e.a. 'Genome Design of Hybrid Potato'. *Cell* 184, nr. 15 (22 juli 2021): 3873-3883.e12. https://doi.org/10.1016/j.cell.2021.06.006.

Zhang, Jiaxin, Md Samiul Islam, Jieyu Wang, Yang Zhao, en Wubei Dong. 'Isolation of Potato Endophytes and Screening of Chaetomium Globosum Antimicrobial Genes'. *International Journal of Molecular Sciences* 23, nr. 9 (januari 2022): 4611. https://doi.org/10.3390/ijms23094611.