

Developing novel methods for gene editing in trees

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THE FACULTY OF SCIENCES MASTER IN BIOTECHNOLOGY

**Developing novel methods for gene editing in
trees**

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Under the supervision of
Dr. Amir Raz
Prof. Martin Goldway

**Thesis submitted in partial fulfillment of the requirements for the master of
science degree in biotechnology**

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Contents

1 Abstract	7
2 Introduction	7
2.1 Trees and their vital role as source for food security	7
2.2 Classical plant improvement methods for trees on a human time scale	7
2.3 Genetic modification as a viable solution for trees improvement	8
2.4 Exogenous DNA delivery methods	8
2.4.1 Biolistic particle delivery system or Gene gun	8
2.4.2 Protoplast transformation	8
2.4.3 Agrobacterium-mediated transformation	9
2.5 Site-specific endonucleases	9
2.5.1 Zinc Finger Nucleases (ZFNs)	9
2.5.2 Transcription Activator-like Effector Nucleases (TALENs)	10
2.5.3 Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)	10
2.6 Genetic Editing bottleneck	10
2.7 Advance genetic Editing technologies	11
2.8 Green fluorescent protein (GFP) as a reporter gene for transformation	12
2.9 Poplar as a model organism for trees	12
2.10 Apple tree as a commertial crop	12
2.11 Hypothesis	13
2.12 Milstones	13
2.13 Goal	13
3 Materials and methods	13
3.1 Plant matirial	13
3.2 Matirials	14
3.2.1 antibiotics	14
3.2.2 Kits	14
3.2.3 Instruments	14
3.3 Protocols	14
4 Experiments scheme	15
4.1 Implementing De-novo maristem induction and transformation.	15
4.1.1 Construction of plasmids containing DRs, Cas9 and sgRNAs	15
4.1.2 Infection methods experiment	17
4.1.3 DRs experiment	18

4.1.4	Scale-up experiment	19
4.1.5	High humidity experiment	19
4.1.6	Poplar infections	19
4.1.7	Identification of new transcription factors that are development regulators in poplar	19
4.1.8	Validation of the RNA-seq analysis with real-time quantitative PCR (RT-qPCR)	19
5	Results	20
5.1	Implementing De-novo maristem induction and transformation.	20
5.1.1	Construction of plasmids containing DRs, Cas9 and sgRNAs	20
5.1.2	Infection methods experiment	20
5.1.3	DRs experiment	21
5.1.4	Scale-up experiment	22
5.1.5	High humidity experiment	22
5.1.6	Poplar infections	22
5.1.7	Identification of new transcription factors that are development regulators in poplar	22
5.1.8	Validation of the RNA-seq analysis with real-time quantitative PCR (RT-qPCR)	23
5.1.9	<i>WOX1</i> gene isolation from PopAT shoot tip	24
6	Conclusions	25
7	Acknowledgements	27
8	Supplementary information	27
8.1	Protocols	29
8.2	Gel agarose for DNA electrophoresis	29
8.3	E.coli Heat-shock competent preparation (Chang et al. 2017)	29
8.4	E.coli heat-shock transformation (Chang et al. 2017)	30
8.5	CTAB protocol (Porebski et al. 1997)	30
References		31

List of Figures

1	Agricultural area per capita world-wide in hectare/person. Line. Average area Ribbon. Maximum and minimum values https://ourworldindata.org/	11
2	Infection of Apples with <i>A.tumefaciens</i> , three methods. A. agro-infiltration to the leaves B. micro-stabs to the leaf veins C. injection to the stem cut	18
3	Injection of <i>A.tumefaciens</i> into a stem cut of 4 apple Varieties. A. Injection into a stem cut B. Starking C. Pink Lady D. Golden Delicious E. Granny Smith	18
4	Injection of <i>A.tumefaciens</i> into a stem cut of 4 apple Varieties. A. Cotyledon like emerging from stem cut, two weeks post infection B. Shoot grow, three weeks post infection	21
5	Cas9 and actin validation using PCR. The products analysis by agarose gel electrophoresis. A. T-DNA cassette validation with Cas9 primers B. DNA validation with actin primers	21
6	Pink lady shoot regeneration. A. and B. Grafting and cotyledone like appearance	22
7	WOX1 expression by the RNA-seq analysis	23
8	WOX1 expression by the RT-qPCR with different normalizers. CV referse to the coefficient of variation and it represent a score for the amount in which the normalizer expressed consistently between the different tissues, measured as the standard deviation divided by the average expression. As the CV get smaller, the expression is more uniform.	24
9	Isolation of the WOX1 gene from PopAT shoot tip	25
10	Surgical removal of the axillary bud and injection of vector for de-novo shoot induction (Maher et al. 2020)	28
11	Abnormal shoot regeneration formation as a result of DR over-expression (Maher et al. 2020)	28
12	Agrobacterium-mediated in-planta transformation for Citrus maxima. a. Three to four week old <i>C. maxima</i> seedlings. b. Decapitated <i>C. maxima</i> seedlings. c. Agrobacterium infection. d. Agrobacterium infected seedlings with wounds wrapped with Parafilm. e. Dark incubation during co-culture. f. sprouted bud from newly formed callus. g. sprouted buds from xylem. h. regenerated shoots four weeks after transformation. i. regenerated shoots three months after transformation. (Zhang et al. 2017)	28

List of Tables

1	Composition of media for cultivation, transformation, selection and regeneration of hybrid poplar P.alba X P.glandulosa	13
2	Building blocks from Addgene	15
3	All primers	16
4	De-novo maristem induction constructs	17
5	List of genes when sorted by the expression specificity to shoot tip (First 10)	23

1 Abstract

Global environmental change undermines food security. In comparison to most crops, trees are resilient to temperature fluctuations and consequently offer vital insurance against famine. Crop improvement with available methods reaches a glass ceiling and genetic modification can contribute substantially to break through the barrier. Tissue culture is a required step in most genetic modification methods. Yet, tissue culture has its issues and the process is far from routine in most laboratories. In trees, because of long generation time, tissue culture issues become much more pronounced. In this work we are attempting to implement a novel gene editing method that doesn't require tissue culture such as de-novo meristem induction and transformation, on trees. In this work We have shown that over expression of the gene combination *WUS2-STM* and *WOX11-STM* were successful in invoking de-novo shoot regeneration in a young apple plants, in-planta.

2 Introduction

2.1 Trees and their vital role as source for food security

Food security is a fundamental necessity that kept mankind busy from the beginning of time. Many researchers today estimate that this need is under threat as a result of global environmental changes through land degradation, loss of biodiversity, changes in hydrology, and changes in climate patterns(Erickson et al. 2009). Moreover population growth is around 100 milion per year. Since the early 1990s, the number of extreme weather-related disasters has doubled(FAO 2020). Higher temperatures, water scarcity, extreme events like droughts and floods have already begun to impact staple crops around the world(Linden & Office 2015) and have reduced the yields of major crops like maize and wheat(Iglesius et al. 2001). According to the Food and Agriculture Organization of the United Nations, the climate variability has an impact of at least 80% to the unpredictable reduction in yield of cereal crops in semi-arid regions of the world such as the Sahel region of Africa(Shiferaw et al. 2014).

Fruit trees contribute in many ways to improving diets and combating hunger around the world(Vinceti et al. 2013). Trees are much more resilient to extreme weather-related disasters in comparison to most crops, and consequently they can offer vital insurance against famine during times of seasonal food shortages due to droughts, floods and heat/cold waves. This is the main reason behind the evergreen agriculture approach(Garrity et al. 2010). Trees resilient is due to their being perennial woody plants, which allows them to grow strong, with durable trunk and long roots. In addition, fruit trees are able to produce large yield on a given area resulting from their vertical growth. However, plant improvement methods raises a drawback since trees have a long generation time.

2.2 Classical plant improvement methods for trees on a human time scale

Since mankind developed the ability to grow plants for food, methods were developed to improve yield. The most ancient method is the selection and propagation of plants with preferred traits such as larger seeds. This method creates an artificial selection pressure that allows the highlighting of desirable traits in the plant. However this process is dependent on random mutations, making it slow. Further more, undesirable additional traits may appear.

The second method that man has developed for improving the crop is breeding. Breeding at its core is based on the idea of merging traits from differente lines to a single cultivar through crossbreeding. In most cases, there is an all ready main cultivar line that harbors a variety of desirable triats and probably used as a commertial line. That cultivar would be crossbred with some exotic line whose over all traits (or phynotype) are not at the commercial standart but withhold some favorable trait. The descendants of that cross would have equal parts of genetic matirial donated from each of the parents, and through the proccess of backcrossing with the commertial parent and selecting for offsprings based on thier phynotype,

it is possible to narrow in on a descendant with much of the commercial phenotype but that also keeps the desirable trait from the exotic line. This is why the commercial parent is often called the recurrent parent, and the exotic one is called the donor. Although this method is faster, this process takes many generations and even in annual plants takes a few years. Still, impressive results have been obtained in annual crops that have changed the fate of the human race to extremes. In trees, the time from pollination to a grown fruit bearing tree takes a few years in itself. As a result, to take the breeding process as is and apply it on trees takes decades. Therefore the realization of the potential in trees is not as high.

2.3 Genetic modification as a viable solution for trees improvement

With the understanding that the inherited genetic material is DNA, the ability was developed to utilize the acceleration of random mutations as a way of gaining genetic diversity and selecting based on the phenotypic outcome. By exposing seeds to chemicals or radiation we are able to increase the frequency of mutation events, some fraction of those mutations results with desirable traits. This process is called mutation breeding and plants created using mutagenesis are sometimes called mutagenic plants or mutagenic seeds. There are different kinds of mutagenic breeding, for instance such as chemical mutagens like ethyl-methanesulfonate and dimethyl-sulfate, or radiation such as gamma rays and X-rays(Schouten & Jacobsen 2007). Although this method increases the rate of random mutation formation, it still relies on random occurrences and offspring selection.

As a result of the genetic revolution, many genetic modification technologies were developed, some of them relevant for plants too. Those methods are more direct in their approach to manipulate DNA for an expected outcome. They can be divided into two groups, exogenous DNA delivery methods and site-specific endonucleases.

2.4 Exogenous DNA delivery methods

Techniques developed for the introduction of foreign DNA genes (exogenous) into a cell.

2.4.1 Biolistic particle delivery system or Gene gun

Gene gun or biolistic particle delivery system is a device used to deliver exogenous DNA (transgenes), RNA, or protein to cells. By coating particles of a heavy metal with a gene of interest and firing these micro-projectiles into cells using mechanical force, an integration of desired genetic information can be induced into cells. The technique involved with such micro-projectile delivery of DNA is often referred to as biolistics(O'Brien & Lummis 2011). This device is able to transform almost any type of cell and is not limited to the transformation of the nucleus, it can also transform organelles, including plastids and mitochondria(Rakoczy-Trojanowska 2002). Biolistics has proven to be a versatile method of genetic modification and it is generally preferred to engineer transformation-resistant crops, such as cereals. Notably, Bt maize is a product of biolistics. Biolistics introduces DNA randomly into the target cells. Thus the DNA may be transformed into whatever genomes are present in the cell, either nuclear, mitochondrial, plasmid or any others, in any combination, though proper construct design may mitigate this. The delivery and integration of multiple templates of the DNA construct is a distinct possibility, resulting in potential variable expression levels and copy numbers of the inserted gene(Shewry et al. 2008).

2.4.2 Protoplast transformation

Protoplast refers to the entire cell excluding the cell wall. Protoplasts can be generated by stripping the cell wall from plant, bacterial, or fungal cells by mechanical, chemical or enzymatic means(Davey et al. 2005). The advantages of using protoplast in comparison to a whole cell is the direct access to the cells membrane which result in increased uptake of DNA and increased transformation frequency. Treatment of

protoplast-plasmid mixtures with PEG and/or electroporation is the approach normally exploited to induce DNA into protoplasts. However, transformation frequencies typically remain low (ca. one in 10⁴ protoplasts giving stably transformed tissues)(Davey et al. 2005). Heat shock treatment and irradiation of recipient protoplasts enhance transformation frequency, probably by increasing the recombination of genomic DNA with incoming foreign DNA, or the initiation of repair mechanisms that favour DNA integration. Carrier DNA and the nature of the plant genome also affect transformation(Davey et al. 2005). DNA uptake into protoplasts has been especially important in transforming plants that are not amenable to other methods of gene delivery, particularly agrobacterium-mediated transformation. Many of such studies focused on cereals, particularly rice, once protoplast-to-plant systems became available for these crops(Rakoczy-Trojanowska 2002). However, protoplast regeneration into mature plants is hard to achieve in most plants, which is the major holdback of this approach.

2.4.3 Agrobacterium-mediated transformation

Agrobacterium is a genus of Gram-negative bacteria that uses horizontal gene transfer to cause tumors in plants. *Agrobacterium tumefaciens* is the most commonly studied species in this genus. Agrobacterium is well known for its ability to transfer DNA between itself and plants, and for this reason it has become an important tool for genetic engineering. The ability of Agrobacterium to transfer genes to plants and fungi is used in biotechnology, in particular, genetic engineering for plant improvement. Genomes of plants can be engineered by use of Agrobacterium for the delivery of sequences hosted in transfer of a DNA segment (T-DNA) binary vector. The essential parts of the T-DNA are its two small (25 base pair) border repeats, at least one of which is needed for plant transformation. The genes to be introduced into the plant are cloned into a plant binary vector that contains the T-DNA region, together with a selectable marker (such as antibiotic resistance) to enable selection for plants that have been successfully transformed. Plants are grown on media containing antibiotics following transformation, and those that do not have the T-DNA integrated into their genome will die(Mukeshimana et al. 2013). The most common methodology for introducing Agrobacterium to plant tissues is in liquid suspension of sectors of somatic tissue (explant), then co-culture on agar medium in the dark. Another method is Agroinfiltration, used to induce transient expression of genes in a plant. Agroinfiltration is performed by direct injection or by vacuum infiltration of suspended *Agrobacterium tumefaciens* into a plant leaf. The main benefit of agroinfiltration when compared to the more traditional plant transformation is speed and convenience, although yields of the recombinant protein in traditional methods are generally higher and more consistent(Undervisningsministeriet 2014). Floral dipping is another method that allows efficient plant agrobacterium-mediated transformation without need for tissue culture(Zhang et al. 2006).

2.5 Site-specific endonucleases

Site-specific endonucleases are enzymes that are capable of dissecting nucleic acid strands such as DNA or RNA at a specific target sequence. In contrast to restriction enzymes, this sequence can be engineered and hold longer sequences which increase it's specificity.

2.5.1 Zinc Finger Nucleases (ZFNs)

A zinc finger is a small protein structural motif that is characterized by the coordination of one or more zinc ions (Zn^{2+}) in order to stabilize the fold. The ability to engineer zinc fingers to have an affinity for a specific sequence of DNA, made them suitable for important applications such as zinc finger nucleases and zinc finger transcription factors. While significant progress has been made in ZFNs engineering capability, a barrier to their widespread adoption has been the challenge in engineering new DNA binding specificities(Chandrasegaran 1996).

2.5.2 Transcription Activator-like Effector Nucleases (TALENs)

Transcription activator-like effector nucleases are restriction enzymes that can be engineered to cut specific sequences of DNA. They are made by fusing a TAL effector DNA-binding domain to a DNA cleavage domain (a nuclease which cuts DNA strands). The simple relationship between amino acid sequence and DNA recognition of the TALE binding domain allows for the efficient engineering of proteins. That said, the failure of some custom TALENs suggests that yet unknown rules govern the assembly of functional repeat domains. For example, repeat composition may influence protein stability(Christian et al. 2010).

2.5.3 Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)

CRISPR systems are part of the adaptive immune system of bacteria and archaea, protecting them from invading viruses by cleaving their DNA in a sequence-dependent manner. The immunity is acquired by the integration of short fragments of the invading DNA known as spacers between two adjacent repeats at the proximal end of a CRISPR locus. The CRISPR arrays, including the spacers, are transcribed during subsequent encounters with invasive DNA and are processed into small interfering CRISPR RNAs (crRNAs) approximately 40 nt in length, which combine with the transactivating CRISPR RNA (tracrRNA) to activate and guide the CRISPR Assosiated 9 (Cas9) nuclease(Barrangou et al. 2007). A prerequisite for cleavage is the presence of a conserved protospacer-adjacent motif (PAM) downstream of the target DNA, which usually has the sequence 5'-NGG-3'(Gasiunas et al. 2012). Jinek et al., re-engineered the Cas9 endonuclease into a more manageable two-component system by fusing the two RNA molecules into a “single-guide RNA”(sgRNA) that, when combined with Cas9, could find and cut the DNA target specified by the guide RNA. By manipulating the nucleotide sequence of the guide RNA, the artificial Cas9 system could be programmed to target any DNA sequence for cleavage(Jinek et al. 2012). The Nobel Prize in Chemistry 2020 was awarded to Emmanuelle Charpentier and Jennifer A. Doudna for the development of the CRISPR/Cas9 enzyme as a genetic editing tool.

2.6 Genetic Editing bottleneck

Bottlenecks need to be overcome before the full potential of this technology is realized in plants. Plants distinguish themselves from most complex eukaryotes in the totipotency of their tissues(Indra & Vimla 1972). This has long allowed researchers to convert explant into a whole plants, with the use aseptic tissue culture growth medium. This somatic-germinal conversion (or regeneration) is the foundation of most plant transformation approaches. Transgenes are delivered to isolated somatic tissue followed by selection for the transgene and regeneration of the modified tissue into a whole, transgenic plant(Rasmussen et al. 2017). Despite many of these protocols being developed over decades, the process is far from routine in most laboratories. Further,success is often genotype dependent because specific growth medium need to be tuned for each new plant and the regenerated plants can have changes to their genome and epigenome(Kaeppler et al. 2000). In trees, because of long generation time, the issues of maintaining asceptic enviroment and assessing growth medium suitability in real time become much more pronounced.

Crop production globally is improving, but this trend seems to approach a plateau (Figure 1). Hence substantial changes in methods for agronomic processes and crop improvement are required(Tester, Mark and Langridge 2010).

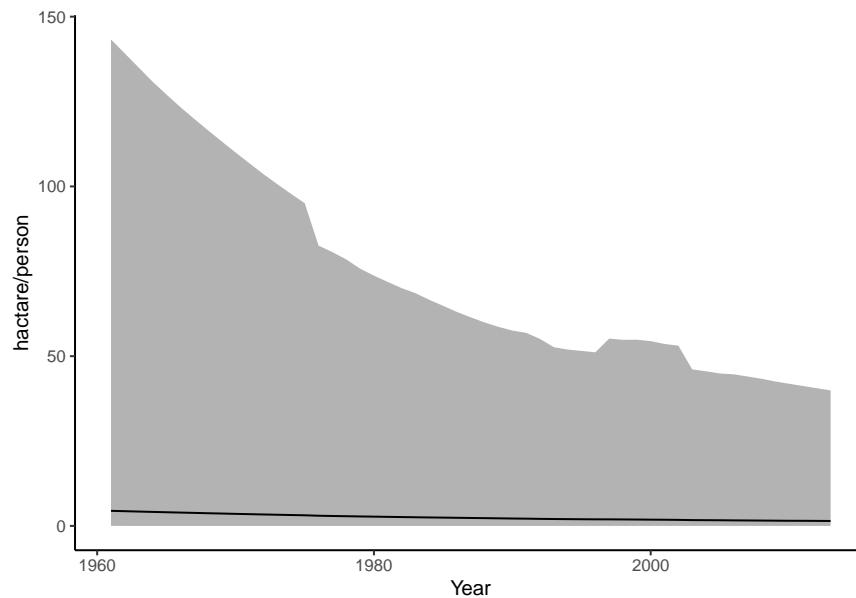


Figure 1: Agricultural area per capita world-wide in hectare/person.

Line. Average area **Ribbon.** Maximum and minimum values

<https://ourworldindata.org/>

2.7 Advance genetic Editing technologies

Most of the advanced methods of genetic editing are designed to help solve problems arising from the use of tissue culture, or to completely spare the need to use it. Tissue culture is designed to allow for the capture of rare cell-transformed events. This is done by providing a supportive environment for the explant, whose needed to expose as much as possible inner cells of the plant to the genetic manipulation and later allow for regeneration and differentiation to a new plant. For the most part, the regeneration begins with differentiation to a shoot meristem, and later to a full shoot. The route from shoot to a full plant through the induction of roots is much more approachable. The common way to induce the explant for meristem differentiation and shoot formation is with the use of plant hormones. Hormones can effect a vast array of genetic pathways, with the right hormone concentration balance at the right time it is possible to direct the tissue to any differentiation path we desire. The drawback of using hormones is the precise application needed and the broad spectrum of effects it have on the tissue such as epigenetic changes. In order to mitigate those drawbacks, researchers tried to induce regeneration with a less comprehensive effects on the plant by switching on specific genetic pathways with the use of transcription factors. those transcription factors are referred to as developmental regulators (DRs). It has been shown that by over expressing some DRs in the cell it would be possible to differentiate the cells in a more precise manner, similar to the work on induced pluripotent stem cells in humans. Some other work have been done to mitigate other drawbacks of tissue culture like precise growth medium and aseptic environment by utilizing the plant own environment to create a controlled micro environment in-planta. Lately Zhang et al. shown promising work on pomelo, in which they introduce a hybrid method of in-planta and tissue culture, where the transformation took place in a close environment around incision on soil grown plant, with the use of antibiotics as selection and hormones for development induction(Zhang et al. 2017). This method is revolutionary in that tissue that is still attached to the plant is much less susceptible to contaminations and most of its micronutrients can come from the neighboring tissues. Maher et al.(Maher et al. 2020) introduced the next step in independence from tissue culture by combining the two approaches of in-planta transformation and de-novo meristem induction. In that method developmental regulators (DRs), CRISPR/Cas9 and sgRNA are delivered to somatic cells of whole plants through Agroinfiltration. This induces meristems that produce shoots solely in cells that were infected successfully with T-DNA bearing CRISPR/Cas9 and sgRNA.

Examples of developmental regulators (DRs):

- Wuschel2 (*WUS2*) from *Zea mays* (Maize) is a transcription factor that plays a central role during early embryogenesis, organogenesis and flowering, probably by regulating expression of specific genes. Required to specify stem cell identity in meristems, such as shoot apical meristem (SAM)(Maher et al. 2020).
- Shoot meristemless (*STM*) from *Arabidopsis thaliana* appears to function in keeping central meristem cells undifferentiated, thus playing a major role in maintaining shoot and floral meristems(Maher et al. 2020).
- Isopentenyl transferase (*IPT*) from the Ti-plasmid of *Agrobacterium tumefaciens* is a key enzyme in cytokinin biosynthesis(Maher et al. 2020).
- Wuschel-related homeobox 11 (*WOX11*) from *Populus trichocarpa* (Poplar tree) acts as master regulator conducting the expression of key transcription factors to induce de novo shoot organogenesis in poplar(Liu et al. 2018).

DRs have a long history in plant development research and in recent years are utilized in transformation methods. WUSCHEL is the most known DR that is involved in the meristem cell identity. Several more DRs were identified in their capability to induce cell potency like the tumor induced gene IPT from *Agrobacterium tumefaciens*. Novel methods for the identification of new tissue specific transcription factors and their goal as a DRs in the cell became possible and commonly used, such as tissue specific transcriptome.

2.8 Green fluorescent protein (GFP) as a reporter gene for transformation

The green fluorescent protein is a protein that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range. In this study, it is used as a reporter gene for the agrobacterium transformation.

2.9 Poplar as a model organism for trees

Populus is a model system for some aspects of tree research for several reasons. It has a rapid growth rate compared to other trees, it is easy to propagate and transform and it has a relatively small genome (Taylor 2002), 45 time smaller than that of pine tree. The target gene that we choose for poplar is the Phytoene desaturase (PDS) gene, because it is a well known reporter gene for gene editing. PDS gene encodes one of the important enzymes in the carotenoid biosynthesis pathway. Knockout of PDS gene disrupt the carotenoid synthesis and results in susceptibility to photobleaching of the chloroplasts, which in turn results in albino and dwarf phenotype. Thus, it is a suitable target for evaluation of gene knockout occurrences.

2.10 Apple tree as a commertial crop

Apple trees are one of the most widely grown fruit tree in the world. In this study, apple plants are used as the commercial application for the transformation method. As a member of the Roseaceae plant family it carries the Self-incompatibility (SI) fertilization system. SI is a complex system, one out of several mechanisms that prevent plants from self-fertilizing mainly by rejection of the male gametophyte to maintain and increase the genetic variability. Plants have evolved two distinct SI systems, the sporophytic (SSI) and the gametophytic (GSI) systems. In *Malus domestica* (Apple), the GSI system requires the production of female determinants, known as S-RNases, which is produced in the style and penetrate the pollen tube to interact with the male determinants. The penetration of S-RNase into the pollen tube triggering a series of responses involving membrane proteins that inhibits the pollen-tube's growth process (Del Duca et al. 2019). Inactivation of S-RNase results in self-compatible plants (Goldway et al. 2012) hence preventing the need of cross pollination and allowing orchards of a single cultivar (in contrast to SI cultivars that requires cross pollination and at least two cultivars that pollinate each other). However, up to date non of apple cultivar are self compatible and following that we choose S-RNase as the target for gene editing.

2.11 Hypothesis

By infecting young plants with *Agrobacterium tumefaciens* harboring T-DNA that contains DRs, CRISPR/Cas9 and sgRNA, it is possible to achieve de-novo shoot regeneration with knockout at the target gene.

2.12 Milstones

- Contraction of vectors with mix of DRs for shoot regeneration examination.
- Achieve de-novo shoot regeneration as a result of the agro-infiltration.
- Observe mutation in the target sequence.

2.13 Goal

- Develop new method for in-planta trees transformation.

3 Materials and methods

The main goal of this work is to develope a protocol as the final product. For that purpose we use an array of already established protocols for the preperation and validation of our experiments. Some of the protocols use in this work were used as is from the source, but many others were changed to fit our specific use case.

3.1 Plant matirial

For the experiments in this work, we used two types of plant material. The first is *Populus Alba-Tremula* (PopAT) cultivar, which were reproduced by vegetative reproduction in tissue culture (Table 1). The second is *Malus domestica* (apple) cultivar, from seeds that were extracted from apple fruits.

Table 1: Composition of media for cultivation, tranformation, selection and regeneteration of hybrid poplar P.alba X P.glandulosa

Components	M1	M2	M3	M4
	(Plant Propagation and Pre-Culture)	(Co-Culture)	(Shoot Induction)	(Root Induction)
MS	4.43 g/L	4.43 g/L	4.43 g/L	2.215 g/L
Sucrose	30 g/L	30 g/L	30 g/L	30 g/L
Agar	5.8 g/L	5.8 g/L	5.8 g/L	5.8 g/L
NAA		0.05 mg/L	0.05 mg/L	0.02 mg/L
BAP		0.5 mg/L	0.5 mg/L	
IBA				0.05 mg/L
Kanamycin			30 mg/L	30 mg/L
Cefotaxime			200 mg/L	200 mg/L
Timentin			200 mg/L	200 mg/L
pH	5.8	5.8	5.8	5.8

3.2 Matrials

- Petri-dish (Greiner Bio-OneTM)
- MagentaTM vessel
- PARAFILM® (Sigma-AldrichTM)
- Lysogeny broth (LB from ForMediumTM)
- Agar
- Murashige & Skoog (MS from Sigma-AldrichTM)
- CTAB (Sigma-AldrichTM)
- Acetosyringone (Sigma-AldrichTM)

3.2.1 antibiotics

- Ampicillin (Sigma-AldrichTM)
- Kanamycin (Sigma-AldrichTM)
- Rifampicin (Sigma-AldrichTM)

3.2.2 Kits

Miniplsmid putification kit (PrestoTM)

3.2.3 Instruments

- MicroPulser Electroporator by Bio-Rad
- Microcentrifuge (PierceTM)
- PCR machine Biometra TRIO
- Power supply + Electrophoresis bath (PowerPacTM Basic Power Supply, Wide Mini ReadySub-Cell GT Horizontal Electrophoresis System)
- UV-light table (*UVDOC* HD2 by Uvitex Cambridge)

3.3 Protocols

- Gibson Assembly Cloning (Gibson et al. 2009): In 2009 Dr. Daniel Gibson and colleagues at the J. Craig Venter Institute developed a novel method for the easy assembly of multiple linear DNA fragments. Regardless of fragment length or end compatibility, multiple overlapping DNA fragments can be joined in a single isothermal reaction. With the activities of three different enzymes, the product of a Gibson Assembly is a fully ligated double-stranded DNA molecule. This has proven to be an efficient and effective method for the assembly of plasmids, and molecular biologists now use this method extensively.
- Golden-Gate cloning(Čermák et al. 2017): Golden Gate cloning technology relies on Type IIS restriction enzymes, first discovered in 1996. Type IIS restriction enzymes are unique from “traditional” restriction enzymes in that they cleave outside of their recognition sequence, creating four base flanking overhangs. Since these overhangs are not part of the recognition sequence, they can be customized to direct assembly of DNA fragments. When designed correctly, the recognition sites do not appear in the final construct, allowing for precise, scarless cloning.
- Gel agarose for DNA electrophoresis(Lee et al. 2012): Agarose gel electrophoresis is the most effective way of separating DNA fragments of varying sizes ranging from 100 bp to 25 kb. During gelation, agarose polymers associate non-covalently and form a network of bundles whose pore sizes determine a gel’s molecular sieving properties. To separate DNA using agarose gel electrophoresis, the DNA is loaded into pre-cast wells in the gel and a current applied. The phosphate backbone of the DNA

(and RNA) molecule is negatively charged, therefore when placed in an electric field, DNA fragments will migrate to the positively charged anode. Because DNA has a uniform mass/charge ratio, DNA molecules are separated by size within an agarose gel in a pattern such that the distance traveled is inversely proportional to the log of its molecular weight.

- E.coli Heat-shock competent preparation and transformation(Chang et al. 2017): Calcium chloride heat-shock transformation is a powerful molecular biology technique used to introduce foreign DNA into a host cell. The concept of the technique is to encourage bacterial cells to uptake DNA from the surrounding environment when stressed by heat shock by using CaCl₂ to render them more competent. This technique allows for introduction of plasmid for bacterial transformation.
- CTAB protocol for the isolating of DNA from plant tissues(Porebski et al. 1997): A relatively quick, inexpensive and consistent protocol for extraction of DNA from plant tissues. CTAB extraction, employing high salt concentrations to remove polysaccharides, the use of polyvinyl pyrrolidone (PVP) to remove polyphenols, an extended RNase treatment and a phenol-chloroform extraction.

4 Experiments scheme

4.1 Implementing De-novo maristem induction and transformation.

4.1.1 Construction of plasmids containing DRs, Cas9 and sgRNAs

Constructing plasmids with combinations of DRs for de-novo maristem induction for novel transformation protocol.

All the plasmid building blocks for the final vectors are bought from Addgene (Table 2), except for pMOD-B with S-RNase sgRNA array and pMOD-C with WOX11 from *Populus trichocarpa* which was assembled in-house.

Table 2: Building blocks from Addgene

Name	Purpose	BackBone	Insert	Species	Number
pTRANS_221	Empty Backbone with Cas9-csy4 gene and kana resistance	pCAMBIA	None		91115
pMOD_B2103	cassette for cloning multiple gRNA	pMOD_B2000	None		91061
pMOD_C'5014	Module C' with Pnos::WUS2	pMOD_C'4800EC	WUS2	Maize	127219
pMM107	Module C' with 35S::IPT	pMOD_C'5014	IPT	A.tumefaciens	127227
pRN110	Module D' with CmYLCV::STM	pMOD_D4800EC	STM	A.thaliana	127228

- Species column refers to the insert's origin species.

For the assembly and validation of the final vectors, costume primers were used as detailed in Table 3.

Table 3: All primers

Purpose	Name	Sequence	Direction
Gibson assembly	ptWOX11-pUC57 FWD	GAACACGGGGACTCCTGCAatggaaagataatcaaggcca	Fwd
	ptWOX11-pUC57 REV	TGGACAAGTCTAGGGCTCGAttatgctccagagatgattacc	Rev
Plasmid validation	pTRANS-R	CAGTCTCGTCAGGATTGCA	Rev
	pMOD-D STM	ATGGTCCGATGTGTCCTATG	Fwd
	pMOD-C WUS	AGCACATACGTCAGAAACCA	Fwd
	pMOD-C IPT	TGGCATATTATTCGCCACAA	Fwd
	pMOD-C WOX	GAACACGGGGACTCCTGCA	Fwd
	TC430	GTTGGATCTCTCTGCAGCA	Fwd
Cassette validation	Cas9 3f	CTCAGCTCCCTGGTGAGAAG	Fwd
	Cas9 2r	TAGCAGCGAGGAACAAATCA	Rev
Mutation validation	MD-S3-exon1 fwd	GTAATTAATCTGCCTCGCTGTTG	Fwd
	MD-S3-exon1 rev	CTAGGGACATCGATCAAATCTG	Rev
	MD-S2-exon1 fwd	GTAATTGATCTGCCTGCTCTTG	Fwd
	MD-S2-exon1 rev	TGTAATGTTGCACACGGCTGGC	Rev
RT-qPCR	PopAT WOX1 fwd2	TACAATGATAGTGGTGACTTCG	Fwd
	PopAT WOX1 rev2	ATCGGTACTATGAAGACGGC	Rev
	E3_ubiquitin fwd2	ATGTATGCCACAGATGCAAG	Fwd
	E3_ubiquitin rev2	AGCATTGACTTGGAAATACCAAG	Rev
	PP2A-4 fwd2	GCAGTTTCATGATCTGCAG	Fwd
	PP2A-4 rev2	TGATAGCGCACTTTCAATGC	Rev

The gene *WOX11* from *Populus trichocarpa* were synthesized into pUC57 plasmid as a service from GenScript(New Jersey, USA) and were assembled into pMOD-C backbone using Gibson Assembly Cloning protocol(Gibson et al. 2009) (Table 3).

The final plasmids were constructed into pTRANS backbone using Golden-Gate cloning(Čermák et al. 2017) (Table 4).

Table 4: De-novo maristem induction constructs

Target	BackBone	A	B	C	D
Apple	CmYLCV	35S::Cas9	S-RNase sgRNA		
	CmYLCV	35S::Cas9	S-RNase sgRNA	Pnos::WUS2	
	CmYLCV	35S::Cas9	S-RNase sgRNA	35S::IPT	
	CmYLCV	35S::Cas9	S-RNase sgRNA	35S::WOX11	
	CmYLCV	35S::Cas9	S-RNase sgRNA		YLCV::STM
	CmYLCV	35S::Cas9	S-RNase sgRNA	Pnos::WUS2	YLCV::STM
	CmYLCV	35S::Cas9	S-RNase sgRNA	35S::IPT	YLCV::STM
	CmYLCV	35S::Cas9	S-RNase sgRNA	35S::WOX11	YLCV::STM
	CmYLCV	35S::GFP	S-RNase sgRNA		
	CmYLCV	35S::GFP	S-RNase sgRNA	Pnos::WUS2	YLCV::STM
	CmYLCV	35S::GFP	S-RNase sgRNA	35S::WOX11	YLCV::STM
Poplar	CmYLCV	35S::Cas9	PopAT-PDS sgRNA		
	CmYLCV	35S::Cas9	PopAT-PDS sgRNA	Pnos::WUS2	YLCV::STM
	CmYLCV	35S::Cas9	PopAT-PDS sgRNA	35S::WOX11	YLCV::STM
	CmYLCV	35S::Cas9	PopAT-PDS sgRNA	Pnos::WUS2	YLCV::WOX1
	CmYLCV	35S::Cas9	PopAT-PDS sgRNA		YLCV::WOX1
	CmYLCV	35S::GFP	PopAT-PDS sgRNA		
	CmYLCV	35S::GFP	PopAT-PDS sgRNA	Pnos::WUS2	YLCV::STM
	CmYLCV	35S::GFP	PopAT-PDS sgRNA	35S::WOX11	YLCV::STM
	CmYLCV	35S::GFP	PopAT-PDS sgRNA	Pnos::WUS2	YLCV::WOX1
	CmYLCV	35S::GFP	PopAT-PDS sgRNA		YLCV::WOX1

Transformation of Top10 bacteria with constructs for replication was preformed using heat-shock transformation and growing on petri-dish containing Lysogeny broth medium with 1.1 % Agar 50 mg/L Ampicillin, at 37°C over-night. Evaluating constructs using colony PCR (Polymerase chain reaction) reaction (Table 3). Growing positive colonies in LB liquid-medium (containing 50 mg/L Ampicillin), shaking 200 rpm over-night at 37°C. Purifying constructs using miniplasmid purification kit.

Transformation of EHA-105 agrobacterium using electroporation MicroPulser Electroporator and growing on petri-dish containing LB-agar medium with 50 mg/L Ampicillin and 50 mg/L Rifampicin at 28°C for 2 days. Positive colonies are validated again using colony PCR.

Cultures of each positive EHA-105 agrobacterium are spread on new petri-dish containing LB-agar medium with 50 mg/L Ampicillin and 50 mg/L Rifampicin at 28°C for 4 days. At the day of plant transformation, the whole plate is picked using Drigalski spatula and transferred to 2 ml Microcentrifuge with a tip. The transferred colonies are weighed and elut in 2 μ l per mg Liquid medium containing 1/2 MS, 1 % sucrose and 200 μ M acetosyringone.

4.1.2 Infection methods experiment

The first experiment is designed for the assessment of different infection methods on the agro-infection effectiveness. Seeds are extracted from apple of the variety Pink-lady, that have been refrigerated for few months. The seeds are then grown in a germination tray (11X17 cells) for 1 month.

Three methods of Agro-infection was tested on the plants. The first is agro-infiltration to the leaves through pressure with needless syringe. The second is micro-stabs of concentrated bacteria culture into the leaf veins. The last is vertical cut of the stem as far as possible from the nearest axillary bud below and injection of concentrated bacteria culture into the cut (Figure 2). Each infection method got repetitions with each construct as follows: first 4, second 3, third 1.

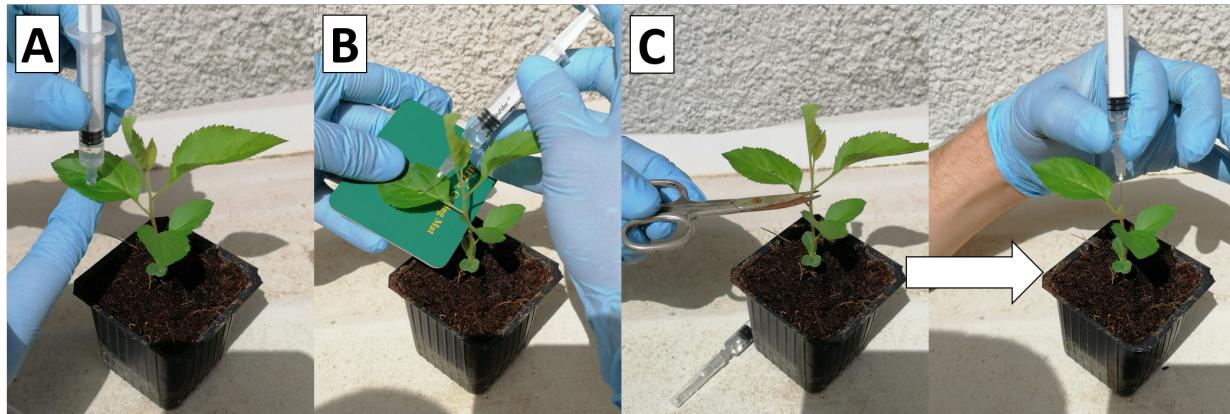


Figure 2: Infection of Apples with *A.tumefaciens*, three methods.

A. agro-infiltration to the leaves **B.** micro-stabs to the leaf veins **C.** injection to the stem cut

4.1.3 DRs experiment

The second experiment is designed for the assessment of different DRs on the de-novo shoot induction influence. Seeds are extracted from 4 varieties of apples that have been refrigerated for few months (Pink Lady, Granny Smith, Starking, Golden Delicious). The seeds are then grown in a germination tray (11X17 cells) for 1 month.

All plant are infected as described in method C (Figure 3). After injection, all stem cuts are covered with PARAFILM for 1 week. New shoots that grow from axillary buds are removed once a week.

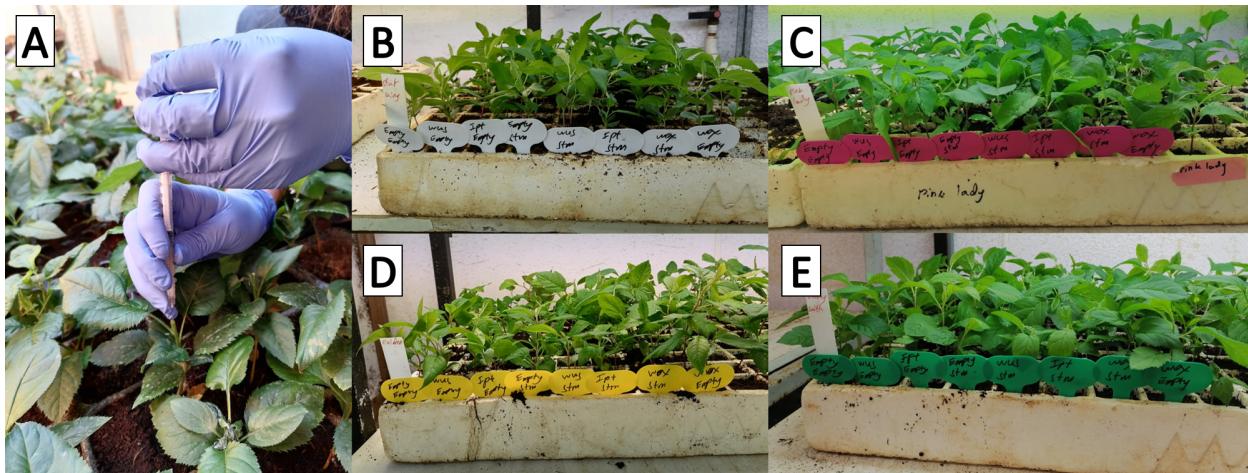


Figure 3: Injection of *A.tumefaciens* into a stem cut of 4 apple Varieties.

A. Injection into a stem cut **B.** Starking **C.** Pink Lady **D.** Golden Delicious **E.** Granny Smith

Every construct have 11 repetitions in each apple variety, 44 overall.

Validation of cassette integration in the genome by DNA extraction from old leaf in the base of the plant and new leaf in the shoot using CTAB protocol and sequence amplification from the T-DNA cassette that is part of the Cas9 gene using specific primers in PCR reaction. The PCR products are analyzed using agarose gel electrophoresis, and photographed on a UV-light table (Table 3).

Validation of mutation in the targeted sequence is achieved by amplification using specific primers in PCR reaction and sanger sequencing as a service from (Macrogen Europe B.V., Meibergdreef 57 1105 BA, Ams-

terdam, the Netherlands) (Table 3).

4.1.4 Scale-up experiment

The third experiment is designed for the assessment of de-novo shoot induction occurrences influenced by the two most promising DR combinations. Seeds are extracted from apples of the variety Pink-lady and Granny-smith, that have been refrigerated for few months. The seeds are then grown in a small pots (5X5 cm wide and 7 cm high) for 1 month. 800 seeds extracted of 2 varieties are infected with 2 most promising plasmids and 1 without DRs. Third of the plants are infected with the plasmid *WOX11-STM*, third with *WUS2-STM* and third with Empty-Empty. The plants are then sealed with parafilm and aluminum foil. The aluminum foil are covering for 2 days, and the parafilm for additional week.

4.1.5 High humidity experiment

Another experiment is designed to isolate as much of the variability in soil experiment by growing the seedlings in a semi-sterile and close environment. Than the plants can be infected in the same manner only without covering the infected stem cut.

4.1.6 Poplar infections

Similar experiments were performed on Poplar plants, the main difference was that the plant material was started from vegetative reproduction and not from seeds.

Another experiment was the infection of poplar ex-plant on sterile growth medium without the use of hormones and antibiotics for selection.

4.1.7 Identification of new transcription factors that are development regulators in poplar.

Tissue specific transcriptomes of the hybrid *Populus Alba-Tremula* (PopAT) were obtained from NCBI's SRA site. The tissues that were analyzed were shoot-tip, root-tip, bud, callus, xylem, leaf and bark. The transcripts were analyzed in order to identify new transcription factors that are most representative of specific tissue, and therefore suspected to have a strong role as a development regulators for that specific tissue in poplar. The transcripts were downloaded as an SRR files and was converted to FASTQ using the sratoolkit (version 2.11.0). Then the reads were processed using the FASTP tool. After processing, the reads from each tissue were aligned to a reference transcriptome from *Populus trichocarpa* (PopTri) from NCBI's Datasets site using Burrows-Wheeler Aligner (BWA, version 0.7.1), and was outputted as a SAM file. The alignment files were analyzed further in order to extract the frequency of each transcript occurrences per tissue. The mathematical way we chose to calculate the a score for the representativeness of transcript to a specific tissue was to divide his frequency in the tissue in question against the frequency in each tissue and then look only on the genes that were at least 1 order of magnitude greater in the tissue in question in comparison to all other tissues. Then we sort by the sum of all ratios.

All the analysis code is available on GitHub in <https://github.com/BenSiv/PopAT-expression-analysis>.

4.1.8 Validation of the RNA-seq analysis with real-time quantitative PCR (RT-qPCR)

For the validation of the RNA-seq analysis, RNA is extracted from the same tissues as the RNA-seq data (excluding callus). Then RT-qPCR is performed on the complementary DNA (cDNA) with specific primers for the gene that was identified as the most shoot-tip specific by the RNA-seq analysis. Several normalizer genes were used for the quantification of the expression between the different tissues.

5 Results

In this work we are attempting to implement a novel gene editing method in-planta on trees. We worked on two cultivars, PopAT from vegetative reproduction in tissue culture and apples from seeds. In this research we used PopAT plants as the best case scenario for gene editing in trees. With PopAT we could control and mitigate as much of the variability in our experiment, because there is no genetic diversity in the plants and as a model organism they are known to be susceptible to agroinfiltration. With apples it was a whole different story, the plants were grown from seeds and because the plants are known to be self incompatible, the seedlings are much more diverse in their genetic makeup. Although our research is based on some success of others, still there is no consensus about the best practice to approach in-planta transformation. On the one hand, we try new plants and new DRs, therefore it would be logical to go way back to expression in tissue culture for the assessment of those genes. On the other hand, we don't want to stray so far from our final goal of in-planta transformation and because of the complexity of the biological system, it is well understood that one can not deduce from tissue culture results to an in-planta experiment in a straightforward manner. Hence, our experiment scheme starts right away with in-planta experiment, but from the basics of that stage without taking much for granted. At first, we assess the best approach to facilitate the infiltration of the agrobacterium by different mechanical interventions. Later, we assess the influence of different combinations of DRs on the plant development. Finally, we scale up the best findings from the last two for statistical analysis purposes. In parallel to all that, we try to enhance our overall knowledge on the natural expression of genes in the different tissues and especially in the shoot-tip by analyzing tissue specific transcriptomes from PopAT.

5.1 Implementing De-novo maristem induction and transformation.

5.1.1 Construction of plasmids containing DRs, Cas9 and sgRNAs

This work is exploratory in nature, as a result a variety of plasmids was required to insure a wide range of DR combinations on both cultivars with different reporter genes. As a consequence, over 40 plasmids were planned and as much as 21 final plasmids were used. The construction of the plasmid was planned with the golden gate assembly protocol in mind. This assembly method is based on the compatibility of a backbone and 4 modules. Each module can either include a functional insert or not at all. The combination of the different building blocks is what gives us the diverse array of plasmids. Some of the building blocks were available to purchase ready made for use, but some needed to be constructed in house.

5.1.2 Infection methods experiment

Assessment of infection methods for the infiltration and infection of apple plants by agrobacterium, is performed with 3 methods (Figure 2). Infection method A is based on a well established protocol for *Nicotiana benthamiana* where the agrobacterium is pushed by pressure into the spaces between the cells in the leaf through the stoma (**Citation**). In contrast to *Nicotiana benthamiana*, apple leaves are stiff and can not absorb liquid as much. In Infection method B, the motivation to attack specifically the veins of the leaves is because in tissue culture it is clear that those cells are the first to regenerate. In Infection method C, we mimic in a way the natural growth of shoot tip, where if in fact there will be a transformation occurrence and a cell is regenerated by the over expression of the DRs to a meristem tissue, its possibility of growth increases by the help of the whole plant, like in grafting. In infection methods A and B, no phenotype was observed. In contrast in Infection method C, 2 newly formed shoots were observed from the stem cut site in plants that were infected with the constructs that included the genes *WOX11-STM* and *WUS2-STM* (Figure 4). In 1 of the cases, Cotyledon like emerged from the cut before the shoot.

DNA was extracted from old leaf in the base of the plant and new leaf in the shoot. A sequence from the T-DNA cassette that is part of the Cas9 gene was amplified with PCR reaction for the validation of T-DNA integration in the genome. The PCR products was loaded in agarose gel and run in electrophoresis bath. After that the gel was photographed on a UV-light table. For positive control the transformation plasmid

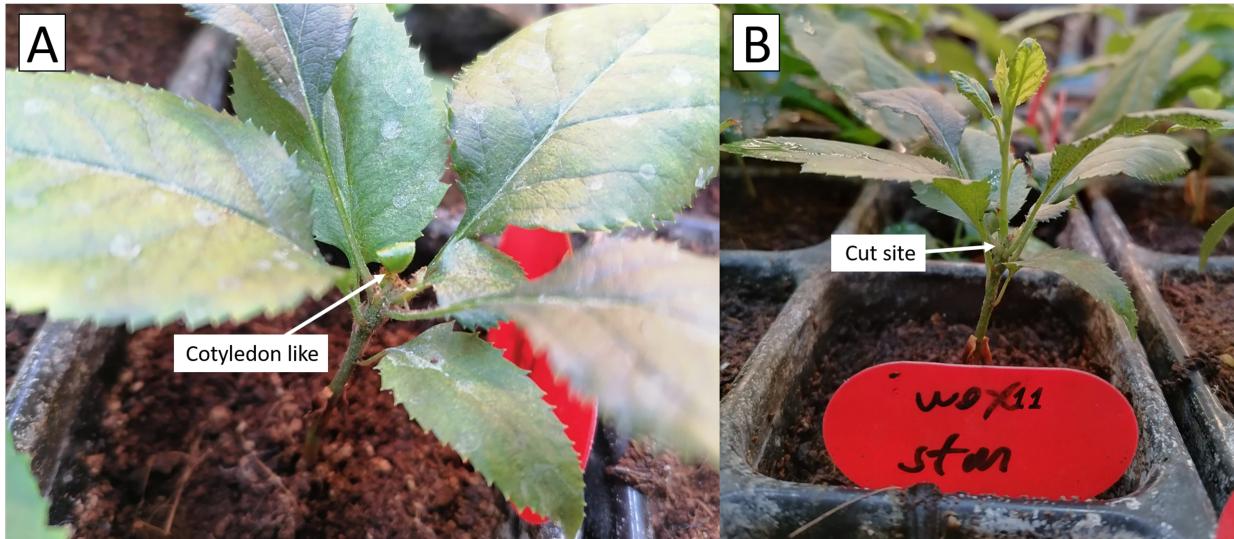


Figure 4: Injection of *A.tumefaciens* into a stem cut of 4 apple Varieties.

A. Cotyledon like emerging from stem cut, two weeks post infection **B.** Shoot grow, three weeks post infection

(pTRANS WOX11-STM) was used and for negative control the PCR stock without DNA (Cas9 stock) was used. A band was observed in the new leaf sample in the expected length, as seen in the positive control sample. To validate that the DNA extraction was successful, a sequence from the actin gene was amplified using PCR reaction and was analyzed too by agarose gel electrophoresis. For negative control the PCR stock without DNA (Actin stock) was used (Figure 5).

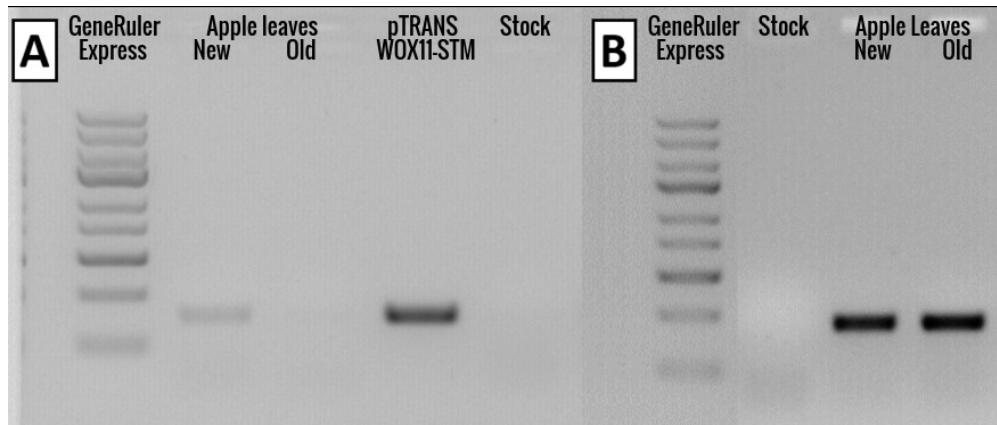


Figure 5: Cas9 and actin validation using PCR. The products analysis by agarose gel electrophoresis.

A. T-DNA cassette validation with Cas9 primers **B.** DNA validation with actin primers

Both alleles of the S-RNase gene (S2 and S3) was amplified and sent for sequencing for mutation analysis. No mutation was observed.

5.1.3 DRs experiment

Assessment of DRs for shoot induction in apple plants is preformed with all constructs (Table ??), on 4 apple varieties (Figure 3) and with 11 repetitions per construct. Out of 352 plants infected, 4 de-novo shoot regeneration was observed, 3 of them are Pink-lady variety and the 4'th is Starking. Shoot regeneration was observed after 1 week only. (Figure 6).

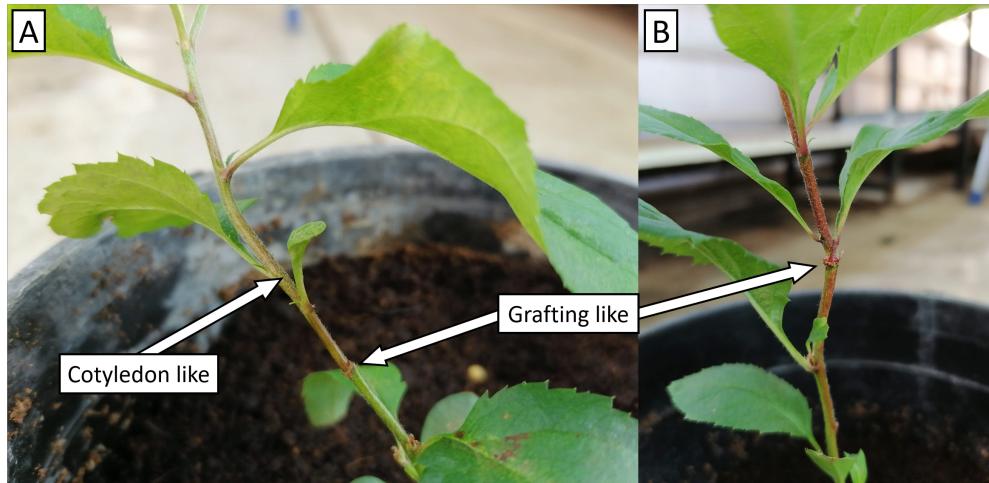


Figure 6: Pink lady shoot regeneration. **A.** and **B.** Grafting and cotyledone like appearance

5.1.4 Scale-up experiment

At the scale up experiment about 800 seeds were planted, and as much as 400 plants were infected with 2 of the most promising plasmids and 1 control plasmid. As a result of disease infection that was spread on the plants (Powdery mildew and Aphids), no phenotype as result of the treatment was observed.

5.1.5 High humidity experiment

At the high humidity experiment about 500 seeds were planted in a 300 ml containers on sterile soil. The objective is to exclude any variables from the environment and maintain moisture for the cells that exposed to air in the process. Unfortunately, no shoot regeneration was observed.

5.1.6 Poplar infections

Similar experiment were performed on PopAT as described above, unfortunately no phenotype as result of the treatment was observed. Although our initial assumption was that it would be easier to transform poplar compared to apples, we haven't taken into account the influence of the different growth stages which we act upon. Perhaps with seedlings of PopAT we could get different results.

5.1.7 Identification of new transcription factors that are development regulators in poplar

After several experiments in apples and poplar plants with the most known DRs, the results were mixed and insufficient for further analysis. We decided to try and identify new transcriptional factors that are more representative of the tissue in question. Therefore, tissue specific transcriptome data was analyzed including shoot-tip transcriptome. For each tissue, we mapped the reads onto transcriptome from *Populus trichocarpa* and extracted the read count per accession (gene transcript). After that we filtered the accessions to only those whose count was greater than at least an order of magnitude in shoot tip than any other tissue. After the filtration we were left with 85 accessions. We found that from the WUSHCEL-related gene family, WOX1 was by far the most expressed in shoot-tip in comparison to other tissues (Figure 7) and was found in the 8th place over all (Table 5).

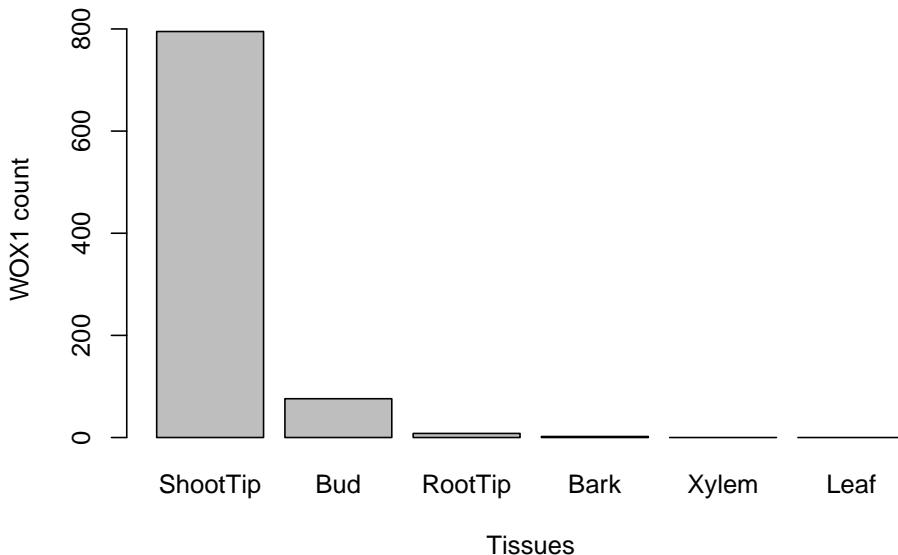


Figure 7: WOX1 expression by the RNA-seq analysis

Table 5: List of genes when sorted by the expression specificity to shoot tip (First 10)

Accession	Description
XM_024590559.1	glycine-rich protein 23-like (LOC112325069), mRNA
XM_024594880.1	O-glucosyltransferase rumi homolog (LOC7461760), mRNA
XM_024592723.1	UPF0481 protein At3g47200 (LOC18097220), transcript variant X1, mRNA
XM_024600744.1	uncharacterized LOC7476433 (LOC7476433), mRNA
XM_006371021.2	probable terpene synthase 6 (LOC18108167), mRNA
XM_024607000.1	glycine-rich cell wall structural protein-like (LOC112328447), mRNA
XM_024599796.1	uncharacterized LOC7470204 (LOC7470204), mRNA
XM_024582282.1	WUSCHEL-related homeobox 1 (LOC7493492), mRNA
XM_024588324.1	uncharacterized LOC7495787 (LOC7495787), transcript variant X4, mRNA
XM_024588316.1	uncharacterized LOC7495787 (LOC7495787), transcript variant X2, mRNA

5.1.8 Validation of the RNA-seq analysis with real-time quantitative PCR (RT-qPCR)

In most RT-qPCR experiments, the samples are from the same tissue and the only difference is a certain treatment (water salinity, gene expression etc.). Therefore it is easy to choose an house-keeping gene to act as a normalizer. In our case study, the samples are different tissues, which differ in many biological pathways. At first, we tried to use the most recognized house-keeping gene, Actin-7. The results showed too much variance in the expression, so we went and looked in the RNA-seq result for the most unchanged genes across all tissues and chose 2 genes, Serine/threonine-protein phosphatase PP2A-4 catalytic subunit and E3 ubiquitin ligase.

For further comparison, we analyzed the RT-qPCR results despite the normalizer inconsistency (Figures 8). The order, in which the tissues appeared when sorted by the *WOX1* gene expression remains consistent with the normalizers PP2A-4 and E3 against the RNA-seq analysis, the only tissue that moved was shoot

tip.

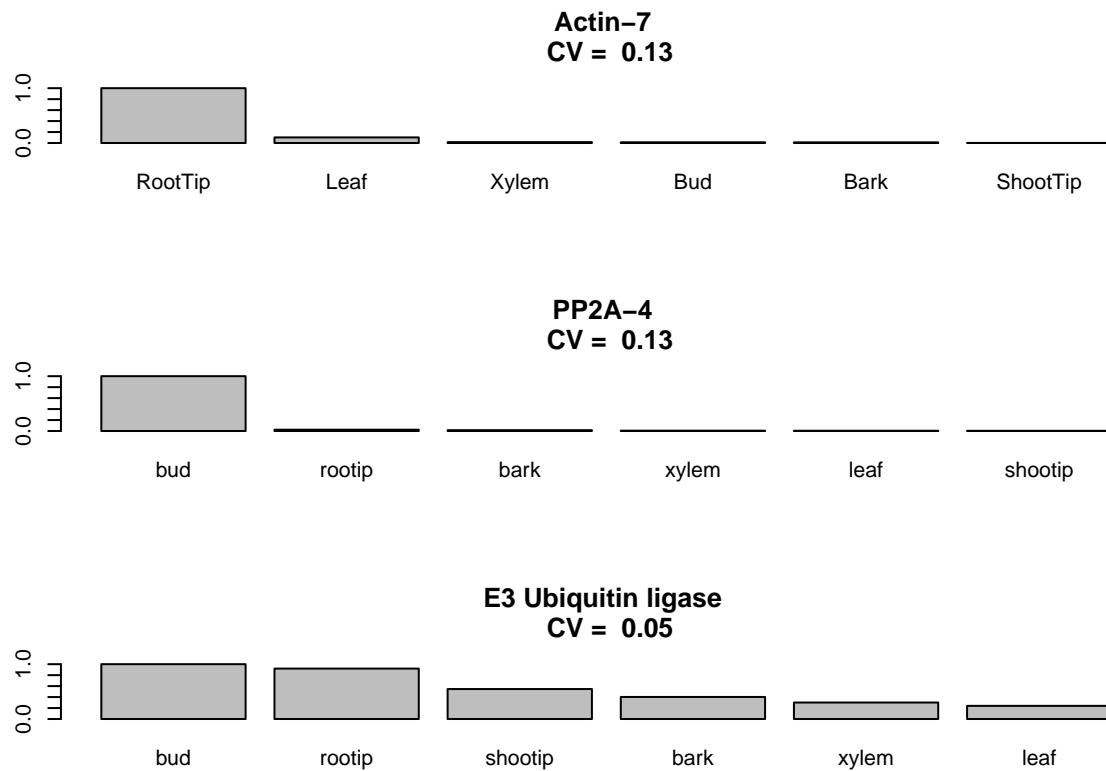


Figure 8: WOX1 expression by the RT-qPCR with different normalizers. CV referse to the coefficient of variation and it represent a score for the amount in which the normalizer expressed consistently between the different tissues, measured as the standard deviation divided by the average expression. As the CV get smaller, the expression is more uniform.

5.1.9 *WOX1* gene isolation from PopAT shoot tip

For the assembly of plasmids with the gene *WOX1* as a DR for shoot regeneration, DNA was extracted and purified from PopAT's shoot tip tissue. Then the gene was amplified using PCR reaction. The PCR product was analyzed using agarose gel electrophoresis and was found to be about 300 bp longer than expected, although it does not known to include introns. Consequently, we decide to try and isolate the gene from cDNA to see if the length would be as expected and we can deduce that in PopAT there is the presence of intron, or parphapse somthing else went wrong and the sequence we amplified was not the target. After extraction and purification of RNA from the tissue, cDNA was prepared, and *WOX1* was amplified. The band observed in the agarose gel was at the expected length, so it could be cleaned and be used in further assembly (Figures 9).

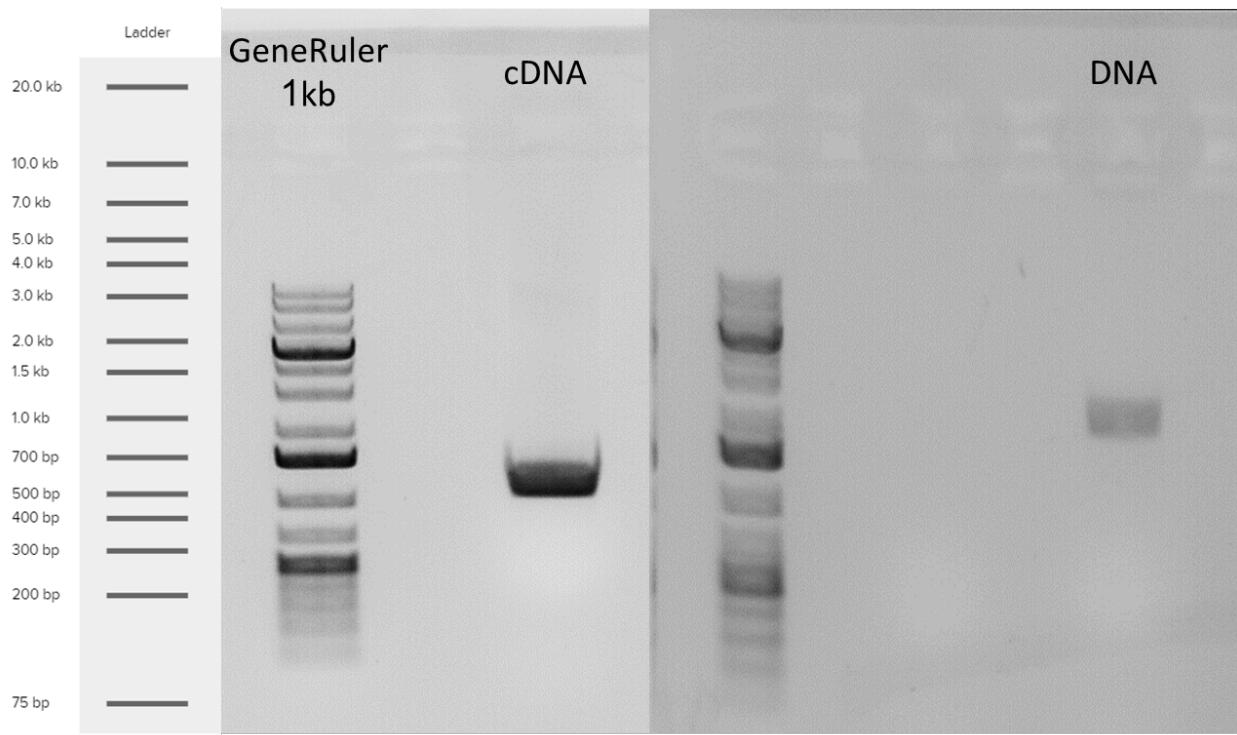


Figure 9: Isolation of the WOX1 gene from PopAT shoot tip

6 Conclusions

Food security is one of the major challenges facing humanity in the 21st century (FAO 2011), especially in light of forecasts regarding climate change and population growth. Cereals are staple crops and account for as much as 44% of all agriculture land use for food production (AREA USED FOR PRODUCTION (FAO 2017)). Cereal cultivars are annual plant and as such are more prone to be effected by change in irrigation and overall climate patterns(Shiferaw et al. 2014). In contrast, Trees, especially fruit trees in the context of food production, are more resilient to changes in the environment as a result of their strong and deep roots. Consequently trees serves a vital role as a source for food security. To date, fruit trees only serves a small part of the overall food production. For trees to reach their full potential as staple crop in many parts of the world, crop improvement methods need to be more robust. Genetic engineering and especially genome editing methods with the use of CRISPR/Cas9 technology have that potential. For those methods to be adopted, they need to fulfill certain criteria such as, speed, ease of execution, cheap and minimal preliminary knowledge required. The most common used method for crop improvement by genetic engineering is agrobacterium-mediated transformation that is performed on ex-plant in sterile environment on tissue culture medium. Many researchers combined that technology with CRISPR/Cas9 by integrating the Cas9 DNA sequence as part of the agrobacterium T-DNA cassette. This method takes several month at least to get from soil-grown wild type plant to soil-grown transformant one, it takes very skilled personal to perform and require preliminary knowledge in specific growth medium per cultivar.

In-planta transformation is much desirable goal. In pursuing that objective, Zhang et al.(Zhang et al. 2017) developed a new method for plant transformation, that mimicks the work in tissue culture, but in-planta. In that method, rather than grow the whole plant in a sterile environment on an agar medium, the researchers created micro environment on an inner tissue section of the plant. It was done by cutting a stem of a young pomelo plant, and enclosing it using a tube and parafilm. The tube is then filled with a liquid growth medium containing antibiotics and hormones (Figure 12). Few success was shown with that method, it holds several advantages over tissue culture methods such as needs less time and labor cost, requires less

equipments and less strict experiment conditions and less skilled personal. but as the design suggests, several of the drawbacks in tissue culture methods are present here too, like the need for hormones and antibiotics.

Maher et al.(Maher et al. 2020) developed novel method for plant gene editing by introducing DRs into the cell as well as CRISPR/Cas9 and sgRNA, with the use of agrobacterium on soil-grown plants. In that way, the phenotype formed by the transformation is de-novo shoot induction as well as knockout in the gene of interest. This method have the potential to remove the need entirely of tissue culture and enable gene editing in-planta. Furthermore, shoot regeneration is a direct result of the transformation, therefor no induction with hormones or selection with antibiotics is required in contrast to the work of Zhang et al.(Zhang et al. 2017). In their work, to show de-novo shoot induction, the vector was introduced to the location of axillary bud right after it was surgically removed (Figure 10). New shoot was formed when the right combination of DRs was introduced, but in most cases it was deformed as a result of the unregulated over expression of the DRs.

In this work we attempted to implement this novel transformation method, on apple and poplar plants as the first trees used for this method. The potential of this method is sound and in trees it is even more striking when compared with traditional methods. Poplar tree was chosen as a model tree and apple tree was chosen because of its commercially importance. Also, a major set back in apple plantation is its self-incompatibility, which means that new plantations need to be vegetative reproduced mainly by cuttings and grafts. In apples S-RNase is one of the key mechanisms that regulates self-incompatibility and as such can serve a suited target for gene editing (Del Duca et al. 2019). Therefor, the target gene of interest in this work is the apple's S-RNase, if knocked out will have notable commercially importance, as the first self-compatible apple variety. In poplar the target gene was PDS because it is a well established reporter gene for the verification of gene knockout.

In our work, we tried three different introduction of the agrobacterium to the plant, pressure injection to the leaf surface, injection to leaf veins and injection to stem cut. Similar to the method described by Zhang et al., our result was in favor of the latter (Figure 12). In comparison to Maher et al. that describe the work on *Nicotiana benthamiana* and also mentioned work on tomato, potato and grape, we attempted to implement the method on apple and poplar trees. Another result different in comparison to their work, the new shoot formed on the top of the stem was not deformed (Figure 11), possibly as a result of the nature of the growth, mimicking the natural shoot apical meristem, and the apical control with all its hormone flux involve in the process.

In the infection methods experiment only 1 plant was infected with each plasmid with the method described and later on shown promise results in de-novo shoot regeneration. Although regenerative plants was clearly observed, no mutation was found as a result of the Cas9 activity. Further investigation is required in the subject for more conclusive results, Therefor DRs experiment was preformed, with more repetitions and wider range of apple varieties.

In the DRs experiment 352 plants infected, out of 44 infected with *WOX11-STM*, 4 regenerate new shoot from the cut site. In the infection methods experiment, the cut site was not covered. After reviewing the work of (Zhang et al. 2017), a decision was made to use parafilm for better moister maintenance.

In the scale-up experiment, both parafilm and aluminum foil was used to cover the stem cut for kipping dark and moist environment. The Dark environment is crucial in the first 48 hours for the agrobacterium infection. Unfortunately, as a result of disease spread, no phenotype was observed.

To increase the possibility of infection success, the next seeds were grown under aseptic conditions, on soil. And were kept in a high humidity environment. No shoot regeneration formed in that case too.

In contrast to our first assumption that the biggest factor for success would be the chosen plant material, described as "the best case scenario" in the results chapter, it seems that it is more important to use plants in the correct growth stage i.e. seedlings and not cuttings. Hence we suggest that for future experiment to utilize this property of seedlings and all the other advantages of PopAT, it should be wise to experiment on PopAT seedlings.

To further attempt and increase the frequency of de-novo shoot formation, we pursue the identification of new transcription factors that are development regulators in trees and perhaps, under the right conditions,

would act as a master regulators. Fortunately, in 2017 the **DOE Joint Genome Institute** (Grigoriev et al. 2012) sequenced the *Populus tremula x alba* INRA717-IB4 transcriptome. With that dataset available, we could analyse the transcription profile per tissue, and highlight certain genes that holds strong correlation to the development of shoot. In our analysis we found that out of the WUSCHEL-related homeobox (WOX) gene family, *WOX1* had the strongest correlation for shoot formation. That finding correspond to the findings mentioned in Tvorogova et al's. review (Tvorogova et al. 2021), there *WOX1* found to regulate auxin response. This can be realized since many of the genes whose expression is affected by *WOX1* are involved in signaling pathways, transport, and synthesis of auxin. Furthermore, after narrowing the results only to those genes that are at least an order of magnitude shoot specific over all tissues, *WOX1* came in 8'th position out of 85 genes.

With strong correlation to our results of regeneration with the combination of genes *WOX11-STM* and *WUS2-STM*, It has been shown that in the regulation of SAM, STM and WUS act in parallel, and they are necessary for the normal expression of each other (Tvorogova et al. 2021). This insight can also explain the non-deformed shoot formation we observe in contrast to Maher et al's.(Maher et al. 2020) findings.

WOX11 known primarily as regulators of callus formation and development of adventitious roots, although it has been shown to be involved in other types of regeneration, such as shoot regeneration and somatic embryogenesis. For example, the positive effect of Pt WOX11 on shoot regeneration in *Populus alba x glandulosa* has been shown (Liu et al. 2018).

To validate our RNA-seq analysis, we extracted total RNA from similar tissues as the DOE's dataset (excluding callus, since it does not occur in natural growth). Then we run RT-qPCR analysis on the expression of *WOX1* with various normalizer genes. Since the expression profile of the different tissues vary massively, it has been hard to find an appropriate normalizer gene whose expression remains homogeneous between the tissues. Despite that, we can still see the overall trend in which it does seem that as the coefficient of variation (CV) get smaller, the results of the RT-qPCR become more similar to the RNA-seq results (Figure 8). When sorting the tissues by the expression of the gene *WOX1* in the RT-qPCR results and in the RNA-seq analysis, the only tissue moved in the order was shoot tip. This tissue is not well defined and the major variability in the results can be explained by the resolution of the isolation of the tissue. Shoot tip is a combination of SAM, stem and leaves among other tissues and it is hard to isolate, let alone define in a gene expression lens.

The quest to achieve an in-planta transformation in trees was a huge challenge. In this work we have built upon the success of legends in the field of plant manipulation and widen a lot the base knowledge and knowhow in this area. Although the final results could not reach the last milestone of in-planta gene editing, it has succeeded in constructing an array of vectors and manage to evoke de-novo shoot regeneration on an soil grown tree.

7 Acknowledgements

8 Supplementary information

Analysis code link.

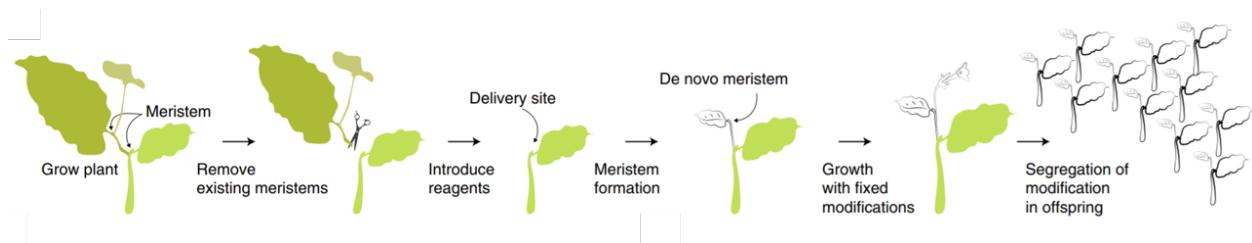


Figure 10: Surgical removal of the axillary bud and injection of vector for de-novo shoot induction (Maher et al. 2020)



Figure 11: Abnormal shoot regeneration formation as a result of DR over-expression (Maher et al. 2020)



8.1 Protocols

8.2 Gel agarose for DNA electrophoresis

Introduction:

Preperation protocol of gel agarose for DNA electrophoresis.

Materials:

- Tray + Comb (With enough places for samples + ladder)
- LE multi-purpose agarose (Usually 1-2%)
- TAE * 1 Buffer (volume depend on Container)
- Ethidium bromide solution (1 drop for every 50 ml of gel)
- loading dye (1 μ L loading dye per 5 μ L sample) *Taq ready mix already contain dye.

Procedure:

Gel mixture, loading and running

1. Measure agarose by weight.
2. Dissolves in TAE buffer.
3. Microwave until fully dissolved.
4. Add ethidium bromide.
5. Pour into tray + comb.
6. Wait until the gel polymerizes.
7. Carefully remove the comb to expose the sample wells.
8. Place the gel into the tank.
9. Pour TAE buffer into the tank high enough to cover the gel.
10. Add loading dye (if necessary).
11. load DNA sample.
12. Connect the tank to the power supply.
13. Set the voltage at ~110 V for 30 minutes *The passage of current will produce bubbles at the electrodes.

8.3 E.coli Heat-shock competent preperation (Chang et al. 2017)

Introduction:

Preperation of E.coli competent for Heat shock transformation.

Materials:

- Liquid nitrogen
- ice
- LB with sutable antibiotics
- 20 mM $CaCl_2$ + 15% glycerol sterile

Procedure:

Pre-Culture

1. Pick single colony into a 4 ml LB (antibiotick if needed)
2. Grow at $37^\circ C$, 150 rpm overnight culture

Culture

3. Add 0.5 ml of pre-culture to 50 ml LB, and Incubate until OD_{600} 0.5-1.
4. Chill culture on ice for 5-10 min.
5. transfer maximum 30 ml of the culture to a 50 ml tube.
6. Centrifuge for 10 min at 5000 rpm in $4^{\circ}C$.
7. Discard the supernatant and let the tube to dry inverted for 1 min.
8. Add 0.5 ml ice cold 20 mM $CaCl_2$ + 15% glycerol.
9. Aliquot 100 μl and freeze directly in liquid nitrogen.

8.4 E.coli heat-shock transformation (Chang et al. 2017)

Introduction:

Heat shock plasmid transformation to competent E.coli

Materials:

- Competent E.coli cells
- 50 μl For each DNA construct / 100 μl For ligation
- 50 ng of circular DNA
- Ice
- Water bath at $42^{\circ}C$
- 1.5 ml tube per sample (Eppendorf or similar)
- 1 ml of LB per sample (with no antibiotic added)
- LB + antibiotics plates 2 or 3 per sample for dilutions
- Drigalski spatula

Procedure:

Heat shock

1. Take competent E.coli cells from $-80^{\circ}C$ freezer. Use Top10 cells in most cases.
2. Turn on water bath to $42^{\circ}C$.
3. Put competent cells in a 1.5 ml tube (Eppendorf or similar). For transforming a DNA construct, use 50 μl of competent cells. For transforming a ligation, use 100 μl of competent cells. You may need more or less cells, depending how competent they are.
4. Keep tubes on ice.
5. Add 50 ng of circular DNA into E.coli cells. Incubate on ice for 20 min. to thaw competent cells.
6. Put tubes with DNA and E.coli into water bath at $42^{\circ}C$ for 1.5 min.
7. Put tubes back on ice for 5 minutes to reduce damage to the E.coli cells.
8. Add 1 ml of LB (with no antibiotic added). Incubate tubes for 1 hour at $37^{\circ}C$. (Can incubate tubes for 30 minutes, unless trying to grow DNA for ligation which is more sensitive. For ligation, leave tubes for 1 hour).
9. Spread about 100 μl of the resulting culture on LB plates (with appropriate antibiotic added). Grow overnight.
10. Pick colonies about 12-16 hours later.

8.5 CTAB protocol (Porebski et al. 1997)

Introduction:

CTAB protocol for the isolating of DNA from plant tissues

Materials:

- Liquid nitrogen
- Ice
- Pestles
- Stirer
- 65°C Bath
- Centrifuge
- CTAB buffer
- 2% cetyl trimethylammonium bromide, 1% polyvinyl pyrrolidone, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA
- 2-Mercaptoethanol
- Chloroform octanol (1:24)
- Sodium acetate (NaOAc)
- 100% Ethanol
- 70% Ethanol

Procedure:

1. Grind ~200 mg of plant tissue to a fine paste.
2. Add 700 μ l CTAB buffer pre-heated to 65°C + 7 μ l 2-Mercaptoethanol.
3. Vortex.
4. Incubation at 65°C for 30 min (Vortex few times).
5. Chill on ice.
6. Add 700ul Chloroform octanol (1:24).
7. Vortex.
8. Centrifuge 5 min 14,000 rpm.
9. Transfer the upper aqueous phase only (contains the DNA) to a clean tube (~550-600 μ l).
10. Add 400 μ l Chloroform octanol (1:24).
11. Centrifuge 5 min 14,000 rpm.
12. Transfer the upper aqueous phase only (contains the DNA) to a clean tube (~350 μ l).
13. Add 35 μ l Sodium acetate (NaOAc), (X0.1 of sample volume).
14. Add 875 μ l 100% Ethanol, (X2.5 sample volume).
15. Incubate at -80°C for Hour/over night.
16. Centrifuge 10 min 10,000 rpm.
17. Remove the supernatant.
18. Add 1 ml 70% Ethanol.
19. Centrifuge 10 min 10,000 rpm.
20. Remove the supernatant.
21. Air dry the pellet, invert on paper towel.
22. Resuspend the DNA in 50 μ l sterile DNase free water.

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