

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
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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**.

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), all that accompanying to population growth forecasts. 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 has 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 unpredictable reduction in yield of cereal crops in semi-arid regions of the world (like the Sahel region of Africa) is at least 80% the result of climate variability(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 for 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. This feature of the tree is its biggest drawback when it comes to breeding methods, as a result of the time required to select offspring with different traits.

2.2 Genetic modification as a viable solution for crop improvement and food security

Since mankind developed the ability to grow plants for food, they started to develop methods to improve the 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. This method mimics natural environmental selection pressure and is one of the main mechanisms of darwinian evolution for the adaptation of an organism to its environment. Despite this, this process is slow, dependent on additional mechanisms like random mutations and limited as a result of additional environmental conditioning that is beyond human control. Further more, additional traits are collected that are undesirable but unavoidable as a result of the mechanisms of genetic inheritance.

The second method that man has developed for improving the crop is breeding. Improvement by hybridizing different varieties of plants with different traits some of which are desirable. Then, a selection process is performed on the offspring similar to the first method in favor of isolating individuals with the desired traits. Although this method is faster, it still takes many generations to get an improved strain. In annual crops, impressive results have been obtained that have changed the fate of the human race to extremes. In trees,

as a result of a particularly long generation time, several years for fruiting from seed, the realization of the potential is not as high.

With the understanding that the inherited genetic material is DNA, the ability was developed to utilize another of the most important mechanisms in Darwinian evolution which is random mutations. 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. They can be divided into two groups, exogenous DNA delivery methods and site-specific endonucleases.

2.3 Exogenous DNA delivery methods

Exogenous DNA delivery methods are techniques developed for the introduction of foreign DNA genes (exogenous) into a cell. In this work the focus would be regarding only plant cells.

2.3.1 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).

2.3.2 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). Treatment of protoplast-plasmid mixtures with PEG and/or electroporation is the approach normally exploited to induce DNA uptake 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.

2.3.3 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 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). 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).

2.4 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.

2.4.1 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).

2.4.2 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).

2.4.3 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 simplicity and accessibility of the CRISPR/Cas9 technology platform provides many advantages over other genome editing methods, and this means that loss-of-function screening is now feasible and affordable on a genomic scale(Shalem et al. 2014). 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.

2.5 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 sectors of somatic tissue into 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, these issues become much more pronounced.

Crop production globally is improving, but this trend seems to approach a plateau as can be seen in the data from around the world regarding agricultural land area per capita, measured in hectares per person, (Figure 1). The scale of sustained increase in global food production needed is unprecedented and requires substantial changes in methods for agronomic processes and crop improvement(Tester, Mark and Langridge 2010). The new revolution, genetically modified crops could contribute to food production increases and higher food availability(Qaim & Kouser 2013).

Origin data for Figure 1 is available at <https://ourworldindata.org/>.

2.6 Advance genetic Editing technologies

2.6.1 De-novo meristem induction and transformation

De-novo meristem induction is a novel technology that aims to bypass the need for tissue culture. 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. This method was first introduced by Maher et al.(Maher et al. 2020).

Examples of developmental regulators (DRs):

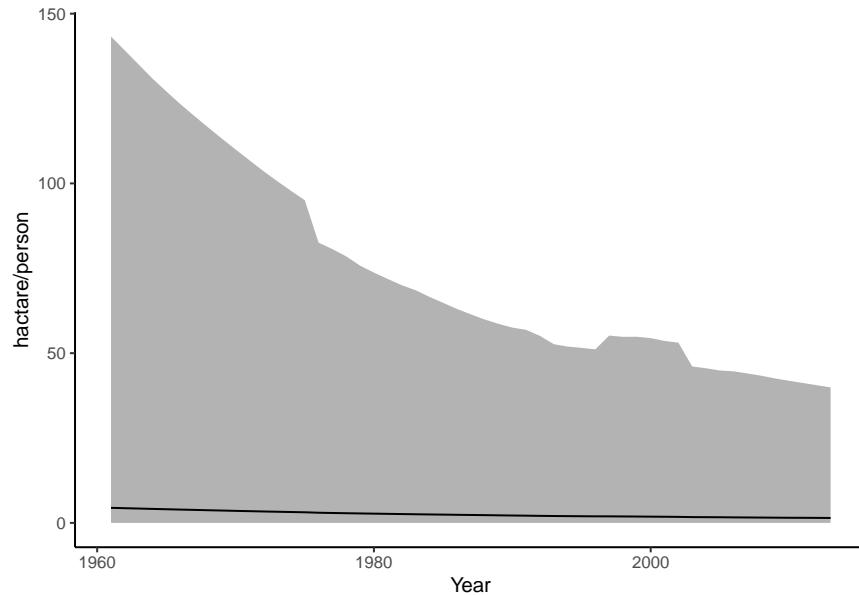


Figure 1: Agricultural area per capita world-wide in hectare/person.

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

- 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).
- 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.
- Isopentenyl transferase (*IPT*) from the Ti-plasmid of *Agrobacterium tumefaciens* is a key enzyme in cytokinin biosynthesis.
- 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, but only in recent years has started to make its way through to the field of 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.

2.7 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.

2.8 Poplar as a model organism for trees

Populus proved valuable as a model tree for several reasons. poplar have rapid growth rate when compared with other trees, it is also easy to propagate and transform and it has a relatively small genome (Taylor

2002), 450-550Mbp, only 5.5 time larger than that of *Arabidopsis thaliana*, and as much as 45 time smaller than that of pine tree. In this study a hybrid poplar clone of *Populus alba x tremula* is used.

2.9 Phytoene desaturase (PDS) gene as a reporter gene for gene editing target in poplar

phytoene desaturase 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.

2.11 Target gene S-RNase and it's commercial importance as a target for gene editing in apples

Self-incompatibility (SI) is a complex process, 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 penetrate the pollen tube to interact with the male determinants. The penetration of S-RNase into the pollen tube triggers a series of responses involving membrane proteins that inhibits the pollen-tube's growth process (Del Duca et al. 2019). Enciso-Rodriguez et al. has shown that self-compatibility can be achieved by knockout of S-RNase gene in potato (Enciso-Rodriguez et al. 2019). To this day, the self-incompatibility is managed in apple plantation by planting compatible varieties in parallel rows. Therefore, the Development of self-compatible variety can increase the plantation yield drastically by enabling plantation of mono-variety blocks, hence the commercial importance.

2.12 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.

2.13 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.

2.14 Goal

- Develop new method for in planta trees transformation.

3 Materials and methods

3.1 Plant material

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

3.2 Implementing De-novo maristem induction and transformation.

3.2.1 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 1), except for pMOD-B with S-RNase sgRNA array and pMOD-C with WOX11 from *Populus trichocarpa* which was assembled in-house.

Table 1: Building blocks from Addgene

Name	Purpose	BackBone	Insert	Species	Number
pTRANS_221	Empty Backbone	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 off the final vectors, costume primers were used as detailed in Table 2.

Table 2: 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	TACAATGATACTGGTGACTTCG	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 2).

The final plasmids were constructed into pTRANS backbone using Golden-Gate cloning(Čermák et al. 2017) (Table 3).

Table 3: 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 (Greiner Bio-One™) containing Lysogeny broth (LB from ForMedium™) medium with 1.1 % Agar 50 mg/L Ampicillin (Sigma-Aldrich™), at 37° C over-night. Evaluating constructs using colony PCR (Polymerase chain reaction) reaction (Table 2). 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 (Presto™).

Transformation of EHA-105 agrobacterium using electroporation MicroPulser Electroporator by Bio-Rad and growing on petri-dish containing LB-agar medium with 50 mg/L Ampicillin and 50 mg/L Rifampicin (Sigma-Aldrich™) 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 (Pierce™) with a tip. The transferred colonies are weighed and elut in 2 μ l per mg Liquid medium containing 1/2 Murashige & Skoog (MS from Sigma-Aldrich™), 1 % sucrose and 200 μ M acetosyringone (Sigma-Aldrich™).

3.2.2 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 needleless 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.

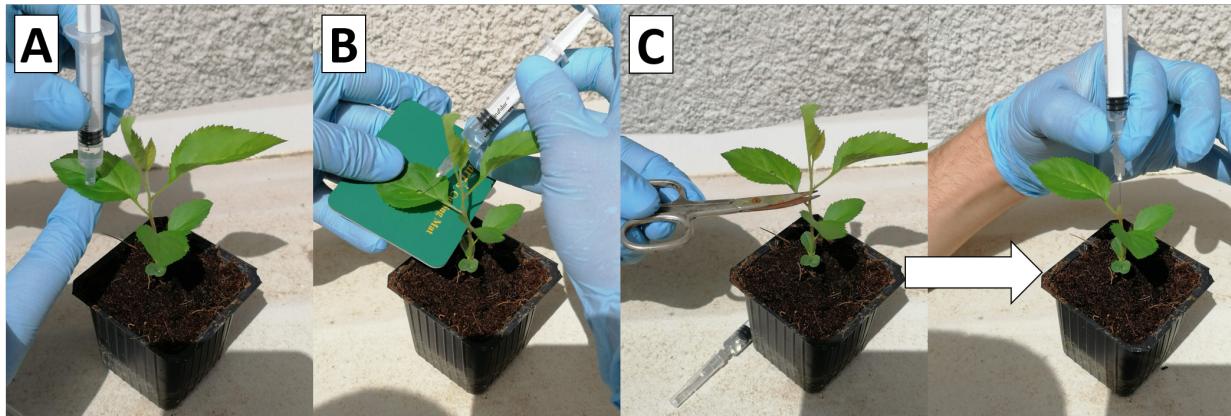


Figure 2: Infection of Apples with *A.tumefaciens*, three methods.

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

3.2.3 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® (Sigma-Aldrich™) 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 (Sigma-Aldrich™) 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 (*UVDOC* HD2 by Uvitex Cambridge) (Table 2).

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, Amsterdam, the Netherlands) (Table 2).

3.2.4 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.

3.2.5 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.

3.2.6 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.

3.2.7 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>.

3.2.8 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 cDNA with specific primers for the gene that was identified as the most shoot-tip specific by the RNA-seq analysis.

4 Results

4.1 Implementing De-novo maristem induction and transformation.

4.1.1 Infection methods experiment

Assessment of infection method for the infiltration and infection of apple plants by agrobacterium, is performed with 3 methods (Figure 2). In infection methods A and B, no phenotype was observed. In contrast, 2 newly formed shoots was observed from the stem cut site in plants that was 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.

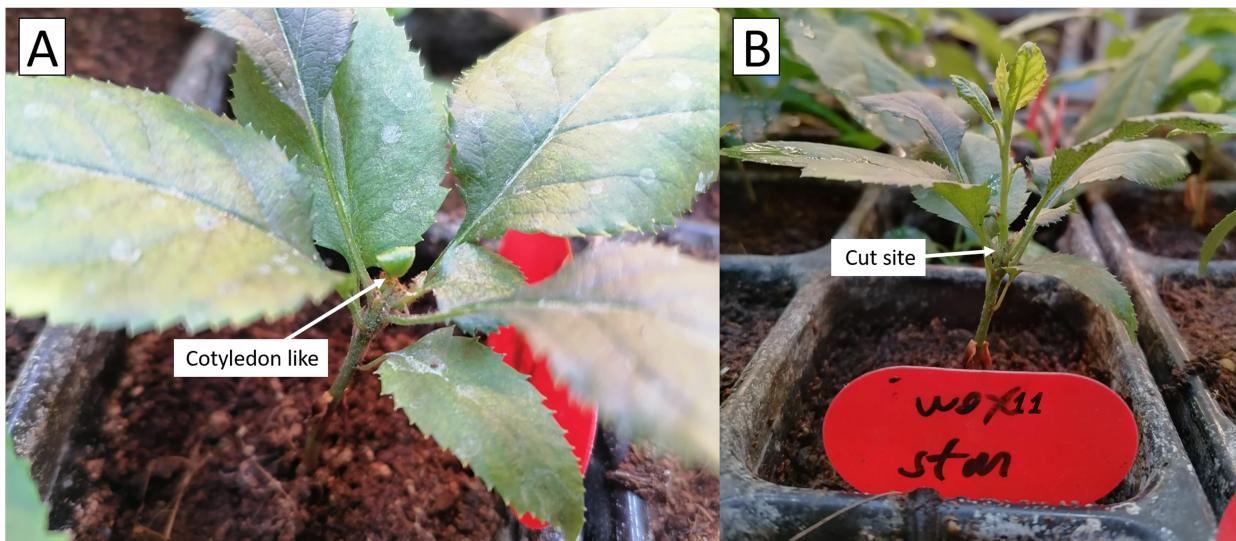


Figure 4: Injection of *A.tumefaciens* into a stem cut of 4 apple Varieties.

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

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 (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).

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

4.1.2 DRs experiment

Assessment of DRs for shoot induction in apple plants is performed 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).

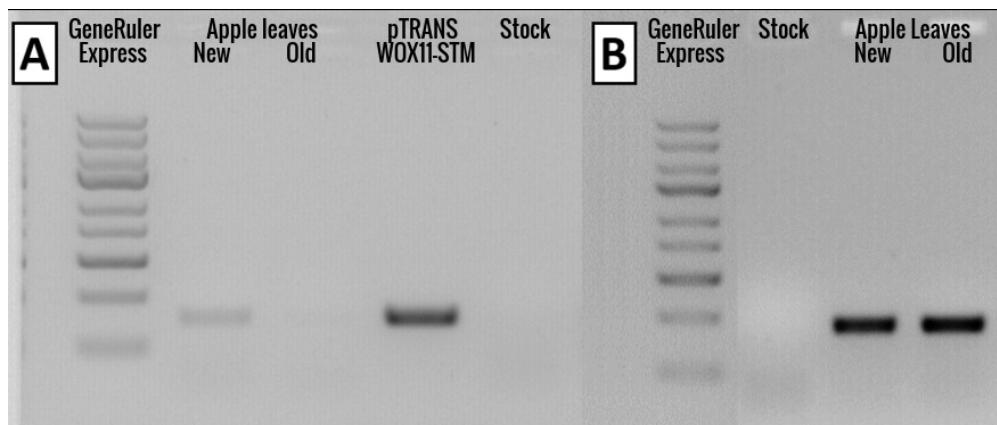


Figure 5: Cas9 and actin validation using PCR. The products analysis by agarose gel electrophoresis.
A. T-DNA cassette validation with Cas9 primers **B.** DNA validation with actin primers

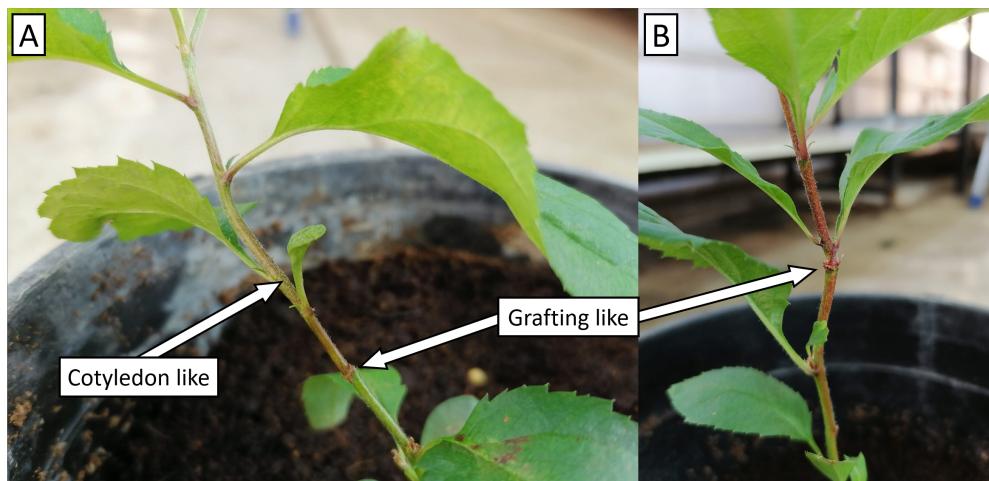


Figure 6: Pink lady shoot regeneration. **A.** and **B.** Grafting and cotyledone like appearance

4.1.3 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.

4.1.4 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.

4.1.5 Poplar infections

No phenotype as result of the treatment was observed.

4.1.6 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, shoot development. Therefore, tissue specific transcriptome data was analyzed including shoot-tip transcriptome. We found that from the WUSHCEL-related family, WOX1 was by far the most expressed in shoot-tip in comparison to other tissues (Figure 7).

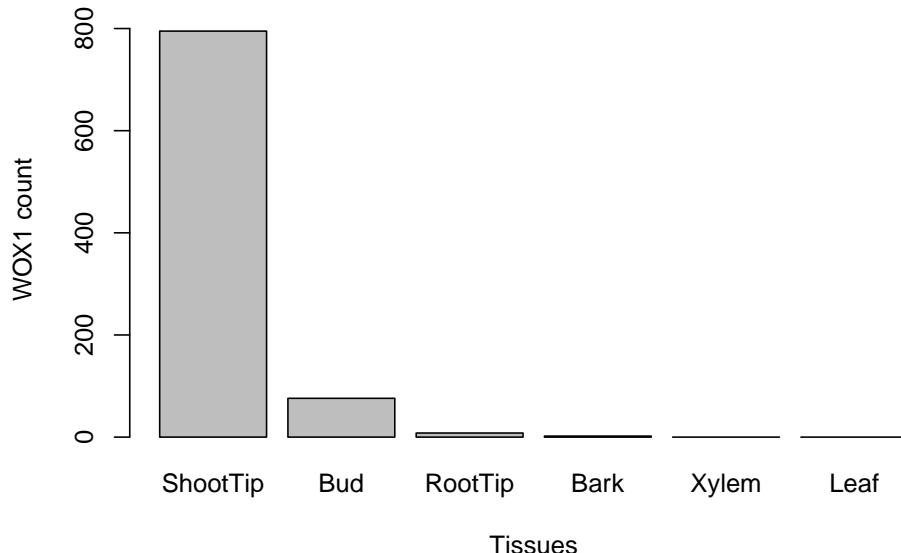


Figure 7: WOX1 expression by the RNA-seq analysis

4.1.7 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 a 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. Further search in similar studies lead us to investigate the genes PP2A-2 and U6 snRNA.

For further comparison, we analyzed the RT-qPCR results despite the normalizer inconsistency. (Figures 8))

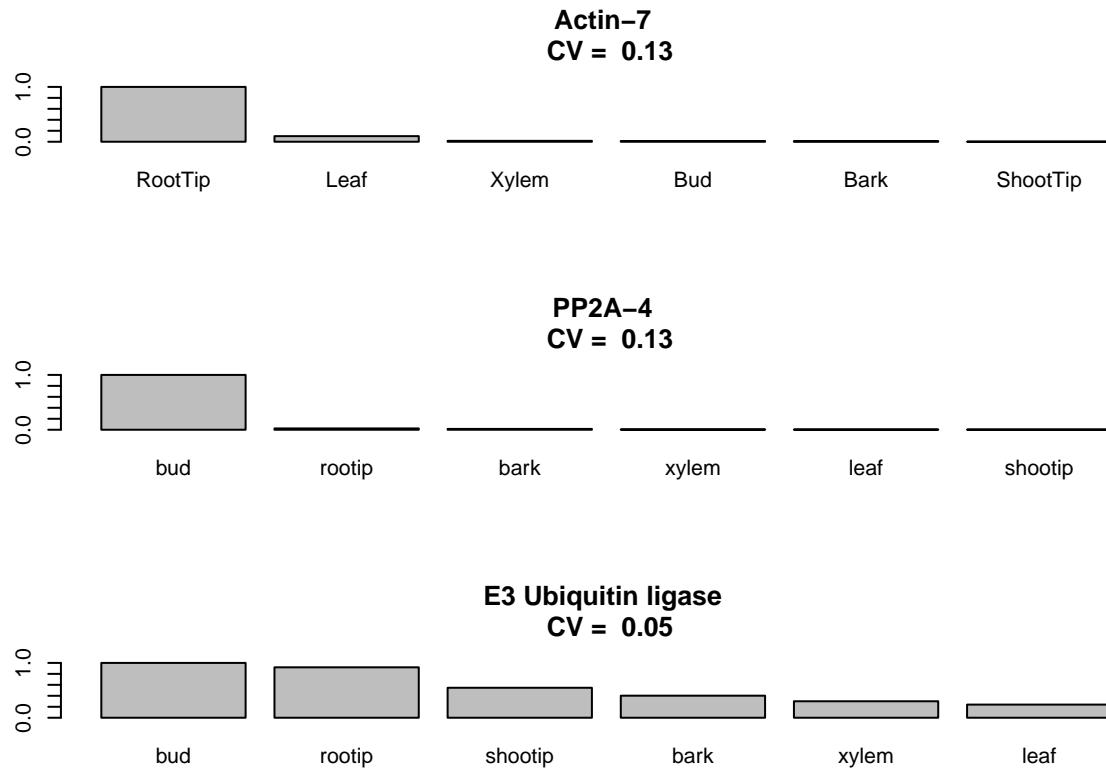


Figure 8: WOX1 expression by the RT-qPCR with different normalizers. CV referse to the coefficient of variation and it represent a score for the amount in which the normalizer expressed consistently between the different tissues, measured as the standard deviation divided by the average expression. As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

5 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 preformed 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 preform 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 envoriment on an agar medium, the researchers created micro enviroment on an inner tissue section of the plant. It was done by cutting a stam of a young pommelo plant, and enclosing it using a tube and parafilm. The tube is than filled with a liquid growth medium containing antibiotics and hormones (Figure 11). Few secess was shown with that method, it holds several advantages over tissue colture 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 colture methods are present here too, like the need for hormones and antibiotiotics.

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 9). 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 vains and injection to stem cut. Similar to the method described by Zhang et al., our result was in favor of the latter (Figure 11). 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 10), 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.

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 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 hold 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 corresponds to the findings mentioned in Tvorogova et al's. review (Tvorogova et al. 2021), where *WOX1* was 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 8th position out of 85 genes.

With strong correlation to our results of regeneration with the combination of genes *WOX11-STM* and *WUS-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 significantly, 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).

6 Acknowledgements

7 Supplementary information

Analysis code link.

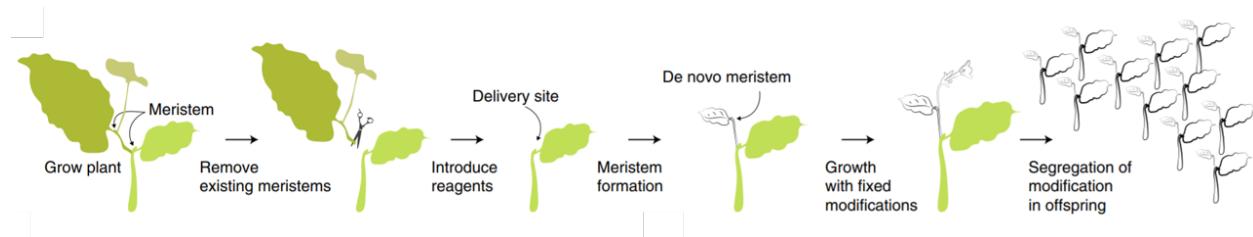


Figure 9: Surgical removal of the axillary bud and injection of vector for de-novo shoot induction (Maher et al. 2020)



Figure 10: Abnormal shoot regeneration formation as a result of DR over-expression (Maher et al. 2020)

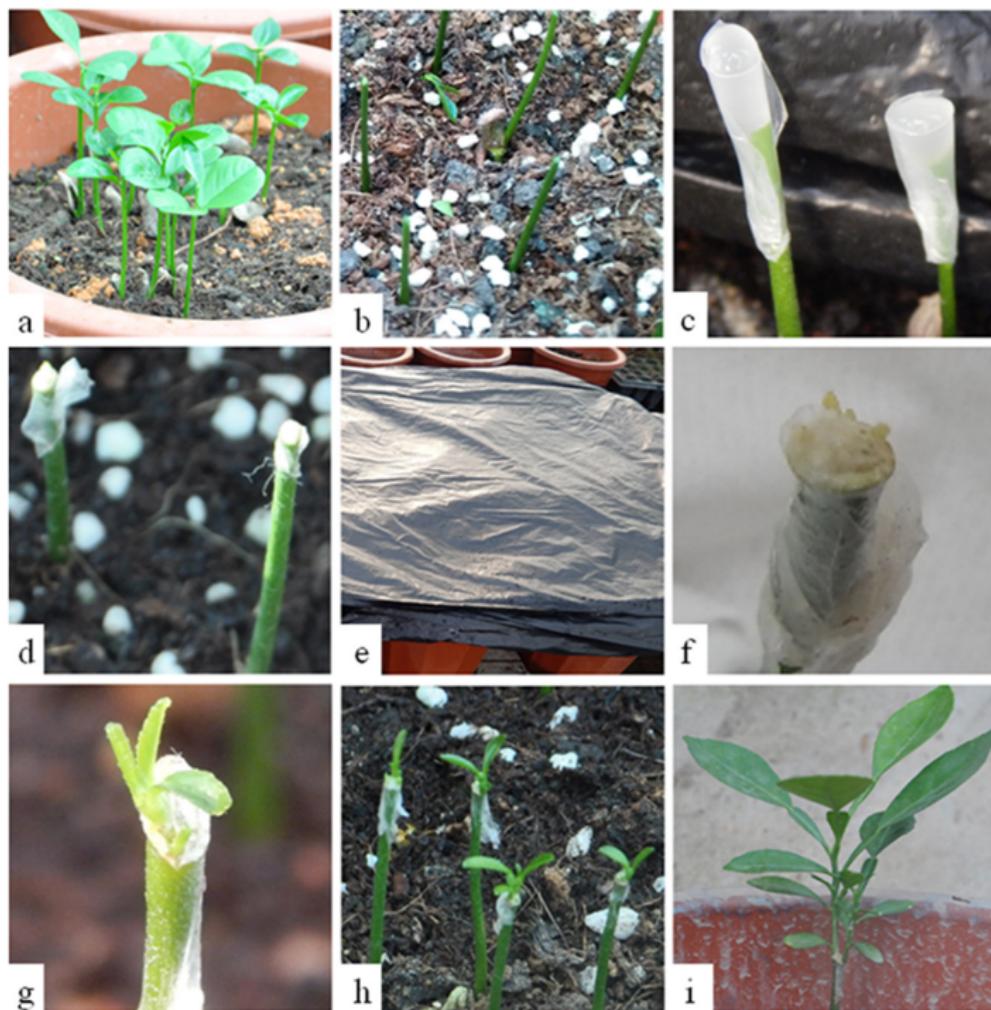


Figure 11: Agrobacterium-mediated in planta transformation for *Citrus maxima*. **a.** Three to four week old *C. maxima* seedlings. **b.** Decapitated *C. maxima* seedlings. **c.** Agrobacterium infection. **d.** Agrobacterium infected seedlings with wounds wrapped with Parafilm. **e.** Dark incubation 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)

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