May the Yeast Whisper Secrets of Trees

Designing Yeast Libraries with Potential Transcription Factors of *PaSPL1* and Validating Transformation Methods for Norway Spruce (*Picea abies*) Calli and Seedlings

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Abstract

The Norway spruce (*Picea abies*) stands as one of Sweden's most valuable natural resources, yet its slow reproductive cycle poses challenges for swift breeding in response to climate change, spanning 20-25 years to reach reproductive maturity. The discovery of the natural early cone-setting variant, *acrocona*, offers insights into the genetic mechanisms governing cone development timing. Notably, the *SQUAMOSA-PROMOTER BINDING PROTEIN-like-1* (*PaSPL1*) gene has been linked to this trait through a mutation in a micro-RNA-regulated region.

This study focused on creating yeast libraries containing potential transcription factors (TFs) of *PaSPL1*, intended for Yeast 1-hybrid mating experiments. These libraries will be complemented by yeast libraries containing *PaSPL1* promoters from genotypes with irregular cone-setting phenotypes and will help elucidate the regulatory network of *PaSPL1*. As *acrocona* only produces early female cones, we also aimed to develop entry clones carrying putative B-class ("male-forming") genes *DEFICIENT AGAMOUS LIKE12* (*DAL12*) and *DAL13*, that will be used for transforming *acrocona* calli and evaluation of their role in cone development. Lastly, protocols for stable calli transformation, alongside novel methods for transient transformation of seedlings with RUBY vectors, were established and evaluated.

Keywords: Norway spruce, *Picea abies*, cone-setting, *PaSPL1*, *DAL12*, *DAL13*, yeast 1-hybrid, calli transformation, transient transformation

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1. Background

1.1 Norway spruce: Importance, breeding, and issues

Over the past 14,500 years, Norway spruce (*Picea abies*) has proliferated throughout the Scandinavian Peninsula, becoming one of the predominant tree species in Sweden (Nota et al., 2022). Its wood is highly valued for construction, pulp, and paper, and the trees gain mass relatively quickly, making spruce one of the most economically important tree species in Swedish forests (Fugeray-Scarbel et al., 2023). Given the immense economic importance of spruce, the maintenance of healthy tree populations is imperative.

As the world faces climate change, coniferous forests will likely be among the most impacted ecosystems due to their sensitivity to extreme weather. This type of stress often makes the trees susceptible to another kind of abiotic or biotic stress, and the combinations are frequently detrimental (Seidl et al., 2017). For example, higher temperatures in combination with drought promote the susceptibility of the spruce trees to bark-beetle outbreaks (Pettit et al., 2020). Thus, we must counteract these issues quickly and efficiently via breeding. One of the main barriers to spruce's fast and efficient breeding is the very long time it takes to transition into the adult reproductive stage. The transition typically happens around 20-25th year, but some trees in Sweden only set cones once they reach 40-50 years old (Lindgren et al., 1977). Understanding the molecular mechanisms responsible for cone-setting is highly interesting if we want to shorten the generation time. However, this task is not easy as the transition is coordinated by both internal (hormonal pathway, age-dependent pathway, autonomous pathway) and environmental (vernalisation pathway, photoperiod pathway) cues that are intertwined in a complex manner (Ma et al., 2022). In such cases, applying a forward genetics approach and identifying genes responsible for the observed phenotype is often beneficial. Notably, a natural variant of Norway spruce with an unusual cone-setting phenotype was described near Uppsala in 1890 (Fries) and was named *acrocona* (*Picea abies* var. *acrocona*).

1.2 *Acrocona* and *PaSPL1*

The name *acrocona* comes from Greek (*ákro* "highest point" + *kónos* "cone") and refers to the tree's ability to set cones on the top positions of branches (Fries, 1890). These apical cones are also abnormal in their anatomy as they have needles typical for wild-type vegetative shoots at the base, which then gradually take the form of bracts and ovuliferous scale-like structures towards the tip, which corresponds to the wild-type female cone anatomy (Akhter et al., 2022; Uddenberg et al., 2013). *Acrocona* phenotype can be further characterised by a bushy appearance and frequent cone-setting (Uddenberg et al., 2013). Inbred crossing experiments of *acrocona* carried out in recent decades have shown that 25% (19 out of 75 plants) of the offspring can set cones already during the second growth cycle (Uddenberg et al., 2013). This unusually early cone-setting phenotype became highly interesting among researchers, and identifying its molecular mechanisms could be a key element in breeding more efficiently.

Comparative analysis of *acrocona* and WT transcripts uncovered that *Picea abies SQUAMOSA-PROMOTER BINDING PROTEIN-like-1* (*PaSPL1*) is highly upregulated in *acrocona* (Akhter et al., 2022). This motivated researchers from our group to examine this gene further; and sequencing revealed that it has a single nucleotide polymorphism (SNP) mutation at position 1421, where the guanine (G) is replaced by adenine (A) in *acrocona*. This mutation is located in a site that is important for binding regulatory micro RNAs (miRNA), *miRNA156* and *miRNA529*. The lack of regulation results in increased levels of PaSPL1, and thus, it likely promotes the phenotypic changes associated with *acrocona* (Akhter et al., 2022).

The binding of said miRNAs to mRNA of *SPL* genes is a highly conserved regulatory element in all land plants, and it affects many developmental and physiological functions. For instance, in *Arabidopsis*, *miRNA156* regulates *AtSPL2*, *AtSPL9*, *AtSPL10*, and *AtSPL15,* and through this, it promotes vegetative phase change and flowering and inhibits branching and leaf initiation (Wang & Wang, 2015).

1.3 Potential transcription factors of *PaSPL1*

As *PaSPL1* appears to play a crucial role in the transition to the reproductive stage, we should also understand how its expression is regulated at the transcription level and post-transcriptionally. The group has recently identified several genotypes that showcased differentially expressed genes (DEGs), and they also differ in the *PaSPL1* promoter sequences in regions where transcription factor binding can occur (Mishra et al., n.d.). For that reason, one of the objectives of this thesis is to design yeast libraries containing potential transcription factors (TFs) of *PaSPL1*. These libraries will be used in a yeast 1-hybrid (Y1H) experiment, where the yeast

containing the TFs will be mated with yeast that contain *PaSPL1* promoters from different genotypes.

The group members selected the TFs for the Y1H experiment (Table 1) in the following way. Firstly, differentially expressed genes (DEGs) analysis revealed different mRNA levels of certain genes in the buds of genotypes that differ in cone-setting phenotype when treated with gibberellic acid (GA): "703" produces few cones, "171" medium amount, and "735" large amount (Mishra et al., n.d.). DEGs previously identified by Akhter et al. (2022) that were differentially expressed in *acrocona* and wild-type were also included.

Consequently, from this list of DEGs, the group selected the ones that code for TFs that contain binding domains that match motifs found on the *PaSPL1* promoters of several genotypes: "735" allele 1, "735" allele 2, "698", "703", "402", and *acrocona* (Mishra et al., n.d., Table 1). In addition, *PaNLY* (*P. abies NEEDLY*) and *PaLFY* (*P. abies LEAFY*) were also added to the list, although they were not differentially expressed, as their homologue *LFY* is known to interact with *SPL* genes and is a part of GA signaling pathway in *Arabidopsis* (Teotia & Tang, 2015, Table 1).

Table 1: **List of potential transcription factors of PaSPL1 identified by the group (Mishra et al., n.d.).** IDs are from Plantgen ie (*), GenBank (**), or European Nucleotide Archive (***). Names that are in bold are well-established based on previous studies. Other names (PaXXX-like-1) are only used in this study and are derived from homology with *Arabidopsis* genes and the non-abbreviated names are described in Table 5.

ID	Gene name used in this study	First record of cDNA Cloning	Reason for selection
MA_8147g0020*	*PaR2R3-like-1*	-	DEG (Mishra et al., n.d.)
MA_55123g0010*	*PaMYB31-like-1*	-	DEG (Mishra et al., n.d.)
MA_10208000g0010*	*PaGAMYB-like-1*	-	DEG (Mishra et al., n.d.)
MA_14452g0010*	*PaTT2-like-1*	-	DEG (Mishra et al., n.d.)
MA_97319g0010*	*PaAP2 ERF-like-1*	-	DEG (Mishra et al., n.d.)
MA_2193g0020*	*PaAP2-like-1*	-	DEG (Mishra et al., n.d.)
MA_16120g0010*	***DAL19***	Uddenberg et al., 2013	DEG (Mishra et al., n.d.)
AF064080.1**	***DAL10***	Carlsbecker et al., 2003	DEG (Akhter et al., 2022)
X80902.1**	***DAL1***	Tandre et al., 1995	DEG (Akhter et al., 2022)
MA_9455802g0010*	*PaTIFY10A-like-1*	-	DEG (Mishra et al., n.d.)
MA_10433497g0010*	*PaTIFY10B-like-1*	-	DEG (Mishra et al., n.d.)
AY701762.1**	***PaNLY***	Carlsbecker et al., 2004	Homology to *LFY*

AY701763.1**	***PaLFY***	Carlsbecker et al., 2004	Homology to *LFY*
MA_41006g0010*	*PaHY5-like-1*	-	DEG (Mishra et al., n.d.)
MA_11267g0020*	*PaLHY-like-1*	-	DEG (Mishra et al., n.d.)
MA_115536g0010*	***PaCCA1***	Karlgren et al., 2013	DEG (Mishra et al., n.d.)
OU384166.1***	***PaSPL1***	Akhter et al., 2022	DEG (Akhter et al., 2022)

1.4 Putative B-class genes *DAL11, DAL12,* and *DAL13*

If successful, our efforts will elucidate the molecular basis underlying the *PaSPL1* regulatory network, identifying genes pivotal in determining the timing and frequency of seed cone formation. It's important to note that the early cone-setting *acrocona* phenotype specifically pertains to seed cones, so pollen cone initiation appears to be governed by distinct mechanisms (Uddenberg et al., 2013; Sundström et al, 1999). Therefore, we must also identify male identity genes to facilitate efficient spruce breeding.

In angiosperms, the formation of the floral organs - sepals, petals, stamens (male organs), and carpels (female organs) - is regulated by MADS-box-containing TFs that are differentially active between the organs. Based on the differential activity, the TFs were then divided into three classes (A-class, B-class, and C-class) and ABC model of floral development was proposed (Coen and Meyerowitz, 1991). In this model, A-class TFs (in *Arabidopsis* APETALA 1) are responsible for formation of sepals and in combination with B-class TFs (in *Arabidopsis* PISTALLATA and APETALA 3) for formation of petals. Stamens are formed when B-class TFs are present along with C-class TFs (in *Arabidopsis* AGAMOUS). Finally, the formation of carpel is regulated by C-class genes (Coen and Meyerowitz, 1991). Subsequently, researchers also discovered D-class TFs (in *Arabidopsis* SHATTERPROOF 1 and 2, and SEEDSTICK) required for development of ovules (Favaro et al., 2003); and E-class genes (in *Arabidopsis* SEPALLATA 1, 2, 3, and 4) that must be present in all floral organs (Ditta et al., 2004). In Norway spruce, a functional analysis of *AGAMOUS* homologue *DEFICIENT AGAMOUS LIKE 2* (*DAL2*) uncovered that it also seems to have the C-class ("female-parts-forming") function (Teandre et al., 1998). This discovery motivated researchers to also look for the B-class ("male-parts-forming") genes.

Contrary to *acrocona* and regular female cones, male cones of wild-type Norway spruce individuals have elevated expression levels of *DAL11*, *DAL12*, and *DAL13* transcripts in the meristematic tissues, and these genes were thus flagged as putative B-class genes (Sundstöm et al., 1999). It was suggested that *DAL11* and *DAL12* are particularly important for the establishment and maintenance of male identity of the

meristematic tissue of the cones, whereas *DAL13* is specifically expressed in cells that will, in later stages, form microsporophylls (= reduced leaves that carry microsporangia with pollen) (Sundström et al., 1999).

These roles remained putative for the past 25 years and could finally be determined by overexpression experiments performed on spruce wild-type and *acrocona* embryogenic cell lines. If *DAL11*, *DAL12*, and *DAL13* were enough to establish the male cones in plants recovered from these calli, their B-class function would be confirmed. In this study, we will focus on *DAL12* and *DAL13* as their expression patterns are more distinct and their mRNA might be easier to isolate from the available materials.

1.5 RUBY vectors

Not all Norway spruce embryonic cell lines are easily transformable, and it is thus beneficial to evaluate their transformability before we begin transformation efforts with the *DAL12* and *DAL13* genes. Such evaluation can be performed by introducing a reporter gene that can easily visualise successful transformation events.

Reporter genes are widely used in molecular biology for various purposes. Typically, the detection of expression of such genes requires chemical activation, like in the case of β-glucuronidase (GUS) (Jefferson et al., 1987) or UV light for visualisation of fluorescent proteins (Chalfie et al., 1994). To simplify the detection process, He et al. (2020) developed a vector called RUBY, which contains three genes that encode enzymes responsible for converting tyrosine to betalains. For example, these compounds can be found in *Beta vulgaris* (beetroot) and are responsible for the bright red colour. The detection of betalains can be thus performed simply by the naked eye and does not require the addition of any chemicals or exposure to UV light. The cleverness of this reporter's design also lies in how it is constructed. The three enzyme-encoding genes, *P450 oxygenase CYP76AD1* (*CYP76AD1*), *L-DOPA 4,5-dioxygenase* (*DODA*), and *glucosyltransferase* (*GT*), are linked with short sequences that encode 2A peptides that undergo self-cleavage and the enzymes are consequently separated post-translationally. The 2A peptides are 18-22 amino-acid-long oligopeptides that are derived from viral DNA (Ahier et al., 2014). Due to its design, the 2A-linked system can be under the control of a single promoter.

In this study, we utilise vectors where the 2A-linked system is under the control of the *35S cauliflower mosaic virus* (*CaMV 35S*, further just *35S*) promoter or maize *UBIQUITIN* (*UBQ*) promoter. Both of these constructs were designed by He et al. (2020) and tested on stable transformants of *Arabidopsis thaliana,* and 35S:RUBY was also transiently expressed in tobacco. In all cases, the transformed plants displayed a visible red colour. The researchers also successfully transformed rice

calli with RUBY under the control of an artificial *DR5* promoter, which is auxin responsive.

As *35S* and *UBQ* promoters have been widely and successfully used to transform conifers' calli (Zhao et al., 2024), we chose to use them instead of the *DR5* promoter for the calli transformation.

1.6 *In-planta* transient transformation of Norway spruce seedlings

As the presence of the putative B-class *DAL* genes might be required only for a short time to establish the male identity, it could be easier to study their function by directly introducing them to the tissue that will later form cones. This could be accomplished by *in-planta* transient transformation mediated by *Agrobacterium tumefaciens* (*Agrobacterium*). Currently, there are no established protocols for this exact purpose. Nevertheless, researchers have previously used various methods for different species and tissues that could be utilised.

Liu et al. (2020) developed a protocol for the transformation of hypocotyls and needles of *Pinus tabuliformis* by adding a sonication step to the standard infiltration protocol. They tested two options: sonication followed by infiltration and infiltration followed by sonication. Both options yielded transformants, and GUS expression was observed.

Salazar-Gonzáles et al. (2023) developed another method. This group successfully performed *in-planta* transient expression of the 35S:RUBY vector in leaves of *Persea americana* (avocado) by introducing micro-wounding and conducting the infiltration under conditions with decreased atmospheric pressure (~ 300 bar).

2. Aims

This project aims to fulfil the following:

- The primary goal is to establish a Y1H protocol for the group and create yeast libraries that contain potential TFs of *PaSPL1*. In the future, the TF-containing yeasts will be mated with yeasts containing the selected *PaSPL1* promoters. If the TF and the promoter interact, a reporter gene will be activated
- This thesis also aims to validate the existing calli transformation protocol and determine the transformability of Norway spruce calli derived from different genotypes using the RUBY vectors.
- Another aspiration of this work is to generate vectors that contain *DAL12* and *DAL13* that can later be used for calli agro-transformation.
- Lastly, we want to test 4 different methods designed for *in-planta* transient transformation of apical shoots of spruce seedlings.

As evident, this thesis is primarily methodology-oriented, focusing more on experimental set-up and validation than on the generation of "final results." Thanks to the foundation established in this thesis project, other members of the group will later produce these.

3. Materials

3.1 Machines

CFX Duet Real-Time PCR System (BIO-RAD, Hercules, CA, USA), NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA), T100™ Thermal Cycler (BIO-RAD, Hercules, CA, USA), Ultrasonic liquid processor (Misonix, Inc., Farmingdale, New York)

3.2 Buffers

- **Basic RNA extraction buffer** - Tris-HCl (100 mM, pH 8, VWR International, Radnor, PA, USA), NaCl (2 M, VWR International, Radnor, PA, USA), RNase-free CTAB (2%, Sigma-Aldrich, St. Louis, MO, USA), EDTA (30 mM Merck KGaA, Darmstadt, Germany), DEPC water
- **Basic DNA extraction buffer** - Tris-HCl (100 mM, pH 8, VWR International, Radnor, PA, USA), NaCl (2 M, VWR International, Radnor, PA, USA), RNase-free CTAB (2 g, Sigma-Aldrich, St. Louis, MO, USA), EDTA (25 mM Merck KGaA, Darmstadt, Germany), water. Autoclave
- **CHISAM** – Chloroform (Merck KGaA, Darmstadt, Germany) : Isoamyl alcohol (Merck KGaA, Darmstadt, Germany) (24: 1)
- **Final RNA extraction buffer** - to 5 ml of Basic RNA extraction buffer, add Polyvinylpolypyrrolidone (0.1 g, Sigma-Aldrich, St. Louis, MO, USA), Proteinase K (0.375 ml of 20 mg/ml, Roche, Basel, Switzerland), β-Mercapto ethanol (0.1 ml, Sigma-Aldrich, St. Louis, MO, USA)
- **Final DNA extraction buffer -** to 5 ml of Basic DNA extraction buffer, add β-Mercapto ethanol (0.1 ml, Sigma-Aldrich, St. Louis, MO, USA), Polyvinylpolypyrrolidone (0.1 g, Sigma-Aldrich, St. Louis, MO, USA)
- **Infiltration buffer** - per 1 L: MgCl2 (10 ml of 1 M, Merck KGaA, Darmstadt, Germany), MES (pH 5.5, 10 ml of 1 M, Merck KGaA, Darmstadt, Germany), 100% Tween-20 (50 µl, Merck KGaA, Darmstadt, Germany)
- **Metabolomic extraction buffer** - methanol (50%, VWR International, Radnor, PA, USA), ascorbic acid (1 mM, Duchefa Biochemie, Haarlem, the Netherlands), formic acid (0.5%, Merck KGaA, Darmstadt, Germany)

3.3 Media

- **Minimal solid yeast media with tryptophan dropout** - per 1 L: Yeast nitrogen base without amino acids (1.7 g, Thermo Fisher Scientific, Waltham, MA, USA), ammonium sulfate (5 g, Merck KGaA, Darmstadt, Germany), tryptophan dropout supplement (1.92 g, Sigma-Aldrich, St. Louis, MO, USA), Bacto agar (40 g, Saveen Werner, Limhamn, Sweden); pH 5.9
- **liquid YPDA** (Takara Bio, Shiga, Japan)
- **solid YPD** (Duchefa Biochemie, Haarlem, the Netherlands)
- **LB (High Salt)** (Duchefa Biochemie, Haarlem, the Netherlands)
- **Low salt LB -** per 1 L: Tryptone (10 g, Duchefa Biochemie, Haarlem, the Netherlands), NaCl (5 g, VWR International, Radnor, PA, USA), Yeast Extract (5 g, Gibco, Grand Island, NY, USA) => pH adjusted to 7.5 => Bacto agar added (15 g, Saveen Werner, Limhamn, Sweden) => autoclaved
- **SOC** (Thermo Fisher Scientific, Waltham, MA, USA)
- **½ LP solid media** - per 1 L: ½ LP powder (2.08 g, Duchefa Biochemie, Haarlem, the Netherlands), sucrose (10 g, Duchefa Biochemie, Haarlem, the Netherlands), 2,4-Dichlorophenoxyacetic acid (10 µl of 1 M*), 6-Benzylaminopurine (40 µl of 111 mM*), 1000x sugar stock (1 ml*) => pH adjusted to 5.8 => 3.5 g of gelrite (Duchefa Biochemie, Haarlem, the Netherlands) added => autoclaved => filter-sterilized L-glutamine added (20 ml of 17.6 mg/ml, Duchefa Biochemie, Haarlem, the Netherlands)
- **Solid plant infection media -** Identical with ½ LP solid media, filter-sterilized acetosyringone (Merck KGaA, Darmstadt, Germany) is added after autoclaving (2 ml of 100 mM).
- **Liquid plant infection media -** Identical with the Solid plant infection media, but gelrite is not added.
- **Liquid bacterial infection media** - per 1 L: ½ LP powder (1.9 g, Duchefa Biochemie, Haarlem, the Netherlands), sucrose (68.4 g, Duchefa Biochemie, Haarlem, the Netherlands), glucose (39.6 g, Merck KGaA, Darmstadt, Germany), 2,4-Dichlorophenoxyacetic acid (10 µl of 1 M*), 6-Benzylaminopurine (40 µl of 111 mM*), Casamino acid (1 g, Difco Laboratories, Detroit, MI, USA) => pH adjusted to 5.2 => filter sterilized => acetosyringone added (2 ml of 100 mM, Merck KGaA, Darmstadt, Germany)

- **NS-Co-culture media** - Identical with ½ LP solid media, but 1000x sμgar stock is not added, and filter-sterilized acetosyringone is added after autoclaving (1 ml of 100 mM, Merck KGaA, Darmstadt, Germany).
- **NS-Recovery media** - Identical with ½ LP solid media, but Timentin (2.5 ml of 160 mg/ml, Duchefa Biochemie, Haarlem, the Netherlands) is supplemented, and L-glutamine is not added

Chemicals marked with an asterisk (*) are in-house stock solutions without specified manufacturer.

3.4 Kits

- SuperScript™ IV First-Strand cDNA Synthesis Reaction (#18091050, Thermo Fisher Scientific, Waltham, MA, USA)
- RNeasy Mini kit (#74104, Qiagen, Hilden, Germany)
- Zymoclean Gel DNA Recovery Kit (#D4002, Zymo Research, Irvine, CA, USA)
- pENTR™/D-TOPO™ Cloning Kit, with One Shot™ TOP10 Chemically Competent *E. coli* (#K240020, Thermo Fisher Scientific, Waltham, MA, USA)
- Phusion™ High-Fidelity DNA Polymerase (2 U/μL) (#F530S, Thermo Fisher Scientific, Waltham, MA, USA)
- DreamTaq Green Master Mix (2X) (#K1081, Thermo Fisher Scientific, Waltham, MA, USA)
- GeneJET Plasmid Miniprep Kit (#K0502, Thermo Fisher Scientific, Waltham, MA, USA)
- Gateway™ LR Clonase™ II Enzyme mix (#11791100, Thermo Fisher Scientific, Waltham, MA, USA)
- Frozen-EZ Yeast Transformation II™ (#T2001, Zymo Research, Irvine, CA, USA)
- Phire Plant Direct PCR Master Mix (#F160S, Thermo Fisher Scientific, Waltham, MA, USA)
- Phusion High-Fidelity PCR Master Mix with HF Buffer (#F531L, Thermo Fisher Scientific, Waltham, MA, USA)
- Gateway™ BP Clonase™ II Enzyme mix (#11789020, Thermo Fisher Scientific, Waltham, MA, USA)

- SYBR™ Green PCR Master Mix (#4309155, Thermo Fisher Scientific, Waltham, MA, USA)

3.5 Organisms, vectors, and primers

Organisms:
Picea abies
Picea abies var. acrocona
Escherichia coli (TOP10, Thermo Fisher Scientific, Waltham, MA, USA)
Agrobacterium tumefaciens (GV3101, in-house stock)
Saccharomyces cerevisiae (Yα1867, in-house stock)
Beta vulgaris

Vectors:
pENTR™/D-TOPO™
(https://www.snapgene.com/plasmids/topo_cloning_vectors/pENTR_D-TOPO)
pDONR™/Zeo
(https://www.snapgene.com/plasmids/gateway_cloning_vectors/pDONR_Zeo)
pDEST-AD-2u
(Figure 6)

Primers used for DNA amplification are described in Tables 5 and 6 in the appendix and were manufactured by TAG Copenhagen (Copenhagen, Denmark). Primers used for sequencing were manufactured by Eurofins Genomics (Ebersberg, Germany)

4. Methods

4.1 Yeast 1-hybrid (Y1H) - Prey transcription factor cloning

The Y1H assay is a method aimed at identifying TFs that interact with promoters, thereby enabling researchers to elucidate regulatory networks. In our study, this method will be utilized to assess interactions between the TFs outlined in Table 1 and specific *PaSPL1* promoters, namely 735 allele 1, 735 allele 2, 698, 703, 402, and *acrocona* (Mishra et al., n.d.). This approach will provide insights into how these TFs potentially regulate the expression of *PaSPL1* and influence downstream biological processes. The experimental setup used in this thesis was based on a protocol published by Gaudinier et al. (2017). Figure 1 illustrates the entire process leading to the Y1H assay. Steps 1-14a describe the steps towards creating TF-containing yeast libraries. The future perspectives section discusses subsequent steps (14b, 15-17). Please note that while the term "yeast libraries" typically refers to a larger collection of TF-containing yeast stocks than what we created in this project, we will use this term further on to maintain consistency with the terminology used in similar projects.

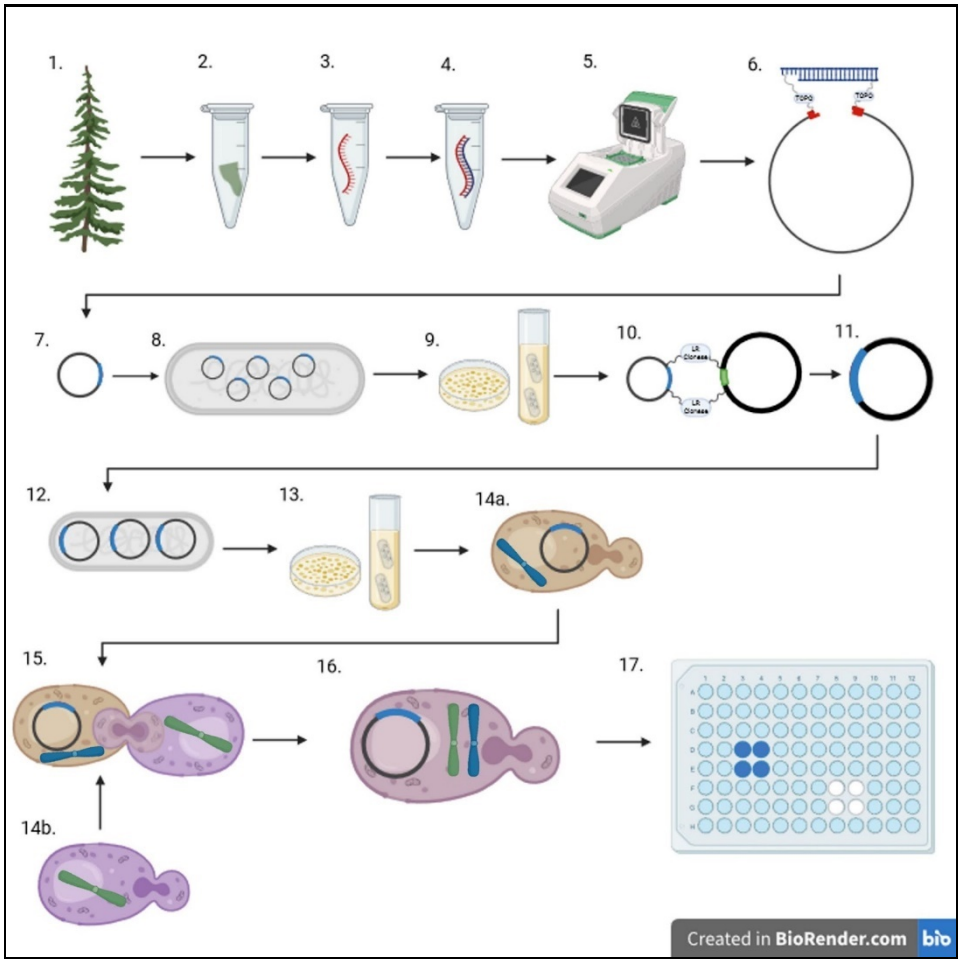


Figure 1: **Step-by-step overview of the Y1H experiment from the TF-containing yeast libraries perspective.** Created in BioRender.com.

4.1.1 Collection of the spruce buds

The female, male, and vegetative cones used for RNA extractions were collected on the 18th of August, 2016 (unspecific time). However, only some of the genes could be PCR quantified from the cDNA synthesised from these samples, and for that reason, the collection was repeated on the 11th of April 2024 (afternoon) and the 16th of April 2024 (morning). As some genes were still hard to obtain, we also utilised RNA extracted from vegetative buds of the genotypes 171, 703, and 735 that were collected in June 2023 (Figure 1, steps 1-2). The collection procedure was as follows: a small branch was excised from the tree, and buds were categorized based on their morphology and size as male, female, or vegetative. The buds were frozen on dry ice shortly after collection (5 - 100 minutes) and stored at -80°C until further processing.

4.1.2 RNA extraction from the buds

RNA from the collected buds was extracted according to the protocol constructed by Azevedo et al. (2003) (Figure 1, step 3). Briefly, the frozen tissue was homogenised in a tissue lyzer, and 1.2 ml of the final RNA extraction buffer was added. The samples were kept in a water bath at 42°C for 90 minutes and shaken manually several times. After the incubation, 1 ml of CHISAM was added, and the aqueous phase containing RNA was separated by centrifugation (13 000 RPM, 15 minutes., 4°C). This wash step was repeated one more time. Subsequently, 0.7 volume of isopropanol (Merck KGaA, Darmstadt, Germany) was added, and samples were precipitated overnight at -20°C. The next day, samples were centrifuged (14 000 RPM, 20 minutes, 4°C) and collected RNA was resuspended and purified with an RNeasy Mini kit. Pure RNA was eluted with RNase-free water. Concentration and purity were measured on NanoDrop. Only pure samples (260/280 $\geq$ 2.0) were used for further processing.

4.1.3 cDNA synthesis

Once we obtained the purified RNA, we used the SuperScript™ IV First-Strand cDNA Synthesis Reaction kit to reverse transcribe cDNA (Figure 1, step 4). 500 ng of purified RNA were mixed with 50 ng of random hexamers primers, 1 µl of 10 mM dNTPs, and filled to 13 µl with DEPC-treated water. The reaction was mixed and incubated at 65°C for 5 minutes and then on ice for 1 min. Furthermore, we added 5× SSIV Buffer (4 µl), 100 mM DTT (1 µl), Ribonuclease Inhibitor (1 µl), and SuperScript™ IV Reverse Transcriptase 200 U/µL (1 µl) to the reaction tube. The reaction was mixed and incubated at 23°C for 10 minutes and then at 55°C for 10 minutes. The reaction was finally inactivated by incubation at 80°C for 10 minutes. The obtained cDNA was stored at -20°C. The quality of the cDNA was validated by semi-quantitative PCR by determining the expression of the constitutively expressed polyubiquitin gene.

4.1.4 PCR Amplification and Purification of TF-encoding sequences

The first step of PCR amplification of the TF-encoding sequences from the cDNA (Figure 1, step 5.) is the reaction setup. On the ice, 2-4 µl of cDNA were mixed with 1 µl dNTPs, 10 µl of 5X Phusion HF Buffer, 0.5 µl of Phusion polymerase, 2.5 µl of forward primer with CACC overhang (0.5 µM), and 2.5 µl of reverse primer (0.5 µM) (Sequences in appendix Table 5). The reaction was filled to 50 µl with MQ and placed in a thermal cycler. The PCR settings used are detailed in the appendix Table 4. Products were loaded on 1% agarose gel with Midori Green dye

(Nippon Genetics Europe, Düren, Germany), and bands at the expected position were carefully cut out and purified immediately or stored at -20°C.

Samples were purified with a Zymoclean Gel DNA Recovery Kit per the manufacturer's protocol. The purified product was eluted in 6-8 µl of the elution buffer, and the concentration of DNA was measured using NanoDrop.

4.1.5 D-TOPO™ reaction, TOP10 transformation, selection, and isolation of plasmids

Once the TF-encoding sequences were amplified and purified, a D-TOPO™ reaction was initiated (Figure 1, steps 6-7). The quantity of the PCR product per reaction recommended by the manufacturer (Thermo Fisher Scientific) was 1-5 ng of product up to 1kb and 5-10 ng above 1 kb. Nevertheless, our experiments were unsuccessful when run with these concentrations of products, and we determined that using 50-100 ng works better in this case. The recommended reaction time was 5 mins at RT. However, in our case, 2 hrs at RT or 16-20 hrs at 8°C yielded positive colonies more frequently.

Therefore, our modified standard protocol involved adding 0.5-4 µL of the purified, concentrated PCR product (50-100 ng of DNA per reaction) to an Eppendorf tube. To this, we added 1 µL of salt solution (1.2 M NaCl, 60 mM $MgCl_2$) from the kit and 1 µL of pENTR™/D-TOPO™ vector (15-20 ng/µL). The ligation reaction was then filled to 6 µL with sterile water, mixed thoroughly, and incubated either at RT for 0.5-2 hours or at 4°C overnight (Figure 2).

After the ligation, 2-3 µl of the reaction were pipetted on chemically competent TOP10 *Escherichia coli* (*E.coli*) cells thawed on ice for 2-5 minutes. The cells with vector were flicked with a finger a few times and kept on ice for 30 minutes. After that, the cells were heat-shocked in a thermal block at 42°C for 30 sec. Samples were again placed on ice for 3-5 minutes, and 250 µl of SOC media (RT) was added. The culture was grown at 37°C, 220 RPM for 1 hr and consequently plated on LB media supplemented with 50 µg/ml kanamycin (Duchefa Biochemie, Haarlem, the Netherlands) (Figure 1, steps 8-9). Plates were then incubated overnight at 37°C. The following day (after 16-18 hrs), plates were collected, and well-separated individual colonies were sampled for colony PCR. A stab from each colony was resuspended in 50 µl of MQ water, and 1 µl was added to the PCR tube with 7.6 µl of DreamTaq Green Master Mix (2X), 0.15 µl of forward primer, 0.15 of reverse primer and 6.1 µl MQ. The settings used can be seen in the appendix. Positive colonies were inoculated in 7 ml LB with 50 µg/ml kanamycin at 37°C and 220 RPM for 18 hours. The next day, cells were collected, and plasmids were extracted with GeneJET Plasmid Miniprep Kit per the manufacturer's protocol. Plasmids were eluted in 50 µl of the elution buffer, and concentration and purity were measured using NanoDrop. The isolated plasmids (diluted to a concentration of 50-150 ng/µl) were subsequently sent for sequencing with vector-specific M13 primers

(M13uni-21: TGTAAAACGACGGCCAGT, M13rev-29: CAGGAAACAGCT ATGACC).

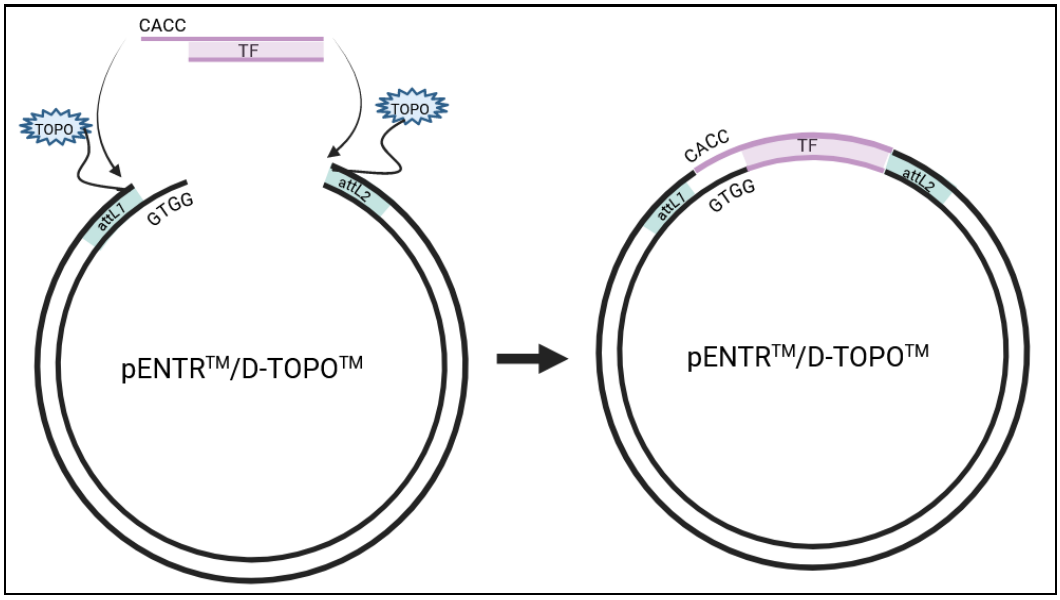


Figure 2: **D-TOPO™ rection.** Created in BioRender.com and adapted from the pENTR™/D-TOPO™ manual.

4.1.6 LR reaction, TOP10 transformation, selection, and isolation of plasmids

When the presence of TF-encoding sequence in the pENTR™/D-TOPO™ was confirmed by sequencing, we proceeded with the LR reaction (Figure 1, steps 10-13). A volume of 1-6 µl (50-150 ng) of the TF-containing pENTR™/D-TOPO™ vector (=entry clone) was supplemented with 1 µl of pDEST-AD-2u (=destination vector; 150 ng/µl) and with 1-6 µl of TE buffer (pH 8, Thermo Fisher Scientific, Waltham, MA, USA) in the final volume of 8 µl. 2 µl of Gateway™ LR Clonase™ II Enzyme mix was added. The reaction was mixed and incubated at 25°C for 1 hr (Figure 3). Afterwards, 1 µl of Protease K (from the LR kit) was mixed in, and the LR reaction was inhibited at 37°C for 10 minutes. The ligated expression vector was then kept on ice, and from this point, TOP10 cells were transformed and selected, and plasmids were isolated and sequenced in the same manner as in section 4.1.4. The only differences are the selective antibiotics for the pDEST-AD-2u vector (ampicillin, 100 µg/ml, Duchefa Biochemie, Haarlem, the Netherlands) and the primer used for sequencing: pDEST-R-1704: TTGACCAAACCTCTGGCGAA

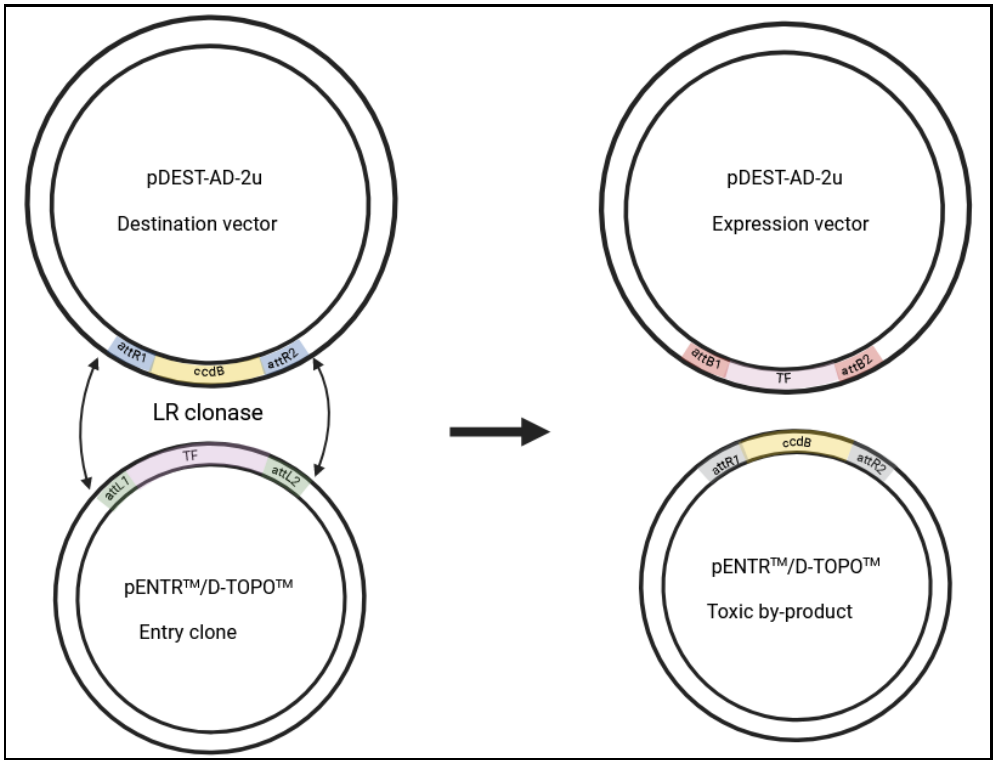


Figure 3: LR reaction. Created in BioRender.com and adapted from the Gateway™ manual.

4.1.7 Yeast competent cells preparation, transformation, and selection

Once we have confirmed that the TF has been successfully recombined into the expression vector with sequencing, we began the yeast transformation process (Figure 1, step 14a). Yeast strain Yα1867 was grown at YPD plate for 3-4 days at 28°C. Several colonies were collected and resuspended in 16 ml of YPDA media (pH 6.2) to reach a starting OD600 of 0.2-0.3. The culture was then grown for 8.5 hrs. at 28°C and 220 RPM until OD600 of 0.9. The cells were collected at 500 xg for 4 minutes. In the next step, the yeast cells were resuspended in 16 ml of Frozen-EZ Solution 1 (from Frozen-EZ Yeast Transformation II™ kit) and again collected at 500 xg for 4 minutes. The cell pellet was then resuspended in 1.6 ml of Frozen-EZ Solution 2, and the aliquots were wrapped in a paper towel to decelerate the freezing process and placed at -70°C.

50 µl of competent Yα1867 was mixed with 600-900 ng of pDEST-AD-2u TF-containing expression vector and 500 µl of Frozen-EZ Solution 3 was supplemented. The reaction was incubated at 30°C for 2.5 hrs. and occasionally inverted. Transformed yeast cells were plated on minimal solid yeast media with tryptophan dropout (pH 5.9) and grown for 7-10 days. Separated colonies were picked and resuspended in 25 µl of 2X Phire Tissue Direct PCR Master Mix, 7 µl of gene-specific forward primer (0.7 µM), 7 µl of gene-specific reverse primer (0.7 µM), and 18 µl of MQ. The settings used for PCR are described in the appendix

(Table 4). Positive colonies were restreaked and stored as cryostocks, ready to be used for the Y1H mating experiment.

4.2 Transformation of spruce calli

Spruce embryonic calli derived from different trees were first genotyped to identify the zygosity of other cell lines (WT homozygote, *acrocona* homozygote, or heterozygote). Based on internal protocols provided by B. Dedicova and S. Johansson from Umea Plant Science Center, various methods were tested to transform spruce calli via agro-infiltration using RUBY vectors with different promoters.

4.2.1 Calli genotyping

Each callus' 150-200 mg mass was snap-frozen in liquid nitrogen and ground with mortar and pestle. The ground tissue was supplemented with 700 µl of pre-warmed Extraction buffer and 4 µl of RNase A (4 mg/ml, QIAGEN, Hilden, Germany) and vortexed. The samples were then incubated at 65°C for 30 minutes and occasionally inverted. 700 µl of CHISAM was added to the mix, and the reaction was centrifµged at 13 000 RPM for 10 minutes. The upper aqueous phase was transferred into a new tube, and the CHISAM wash step was repeated. The aqueous phase was again separated, mixed with 700 µl of isopropanol (Merck KGaA, Darmstadt, Germany), and incubated at -20°C overnight. On the succeeding day, precipitated DNA was collected by centrifµgation (13 000 RPM, 20 minutes) at 4°C. The supernatant was discarded, and the DNA was washed with 70% ethanol and dried at 37°C for 40 minutes. The purified DNA was resuspended in 35 µl of sterile water, and concentration was measured on NanoDrop. 2.5 µl of the diluted DNA (50-150 ng/µl) were mixed with 25 µl of Phusion master mix, 5 µl of primers (Forward: 0.25 µM, Reverse: 0.25 µM), and 17.5 µl of MQ. PCR program settings are in the appendix in Table 4. Products with single bands were sent unpurified for sequencing, and zygosity was determined based on the nucleotide identity at position 1421 of *PaSPL1* (G = Wild-type; A = *acrocona*; G and A = heterozygote) (Akhter et al., 2022).

4.2.2 Transformation of *Agrobacterium*

35S: RUBY and UBQ: RUBY were obtained in *E. coli* as agar stabs (Addgene: #160908 and #160909; He et al., 2020). Cells were plated on selective media with Streptomycin (50 µg/µl, Duchefa Biochemie, Haarlem, the Netherlands), grown for 16-18 hrs. at 37°C, and plasmids were isolated in the same way as in section 4.1.5. The identities of vectors were confirmed with sequencing (primer pGEX 3': CCGGGAGCTGCATGTGTCAGAGG).

Agrobacterium GV3101 competent cells containing pTok47, a vector for increased virµlence, were thawed on ice for 10-15 minutes. 500 ng of RUBY vector were supplemented with 50 µl of the cells and mixed by tapping 5x. The reaction was incubated on ice for 5 min, transferred to liquid nitrogen for 5 min, and then heat-shocked at 37°C for 5 min. 950 µl of LB was added, and the culture was grown at 28°C, at 220 RPM, for 3 hours. Succeedingly, 100-200 µl of the culture was plated on LB plate containing gentamycin (25 µg/ml, selection for GV3101, Duchefa Biochemie, Haarlem, the Netherlands), rifampicin (50 µg/ml, selection for GV3101, Duchefa Biochemie, Haarlem, the Netherlands), tetracycline (5 µg/ml, selection for pTok47, Duchefa Biochemie, Haarlem, the Netherlands), and spectinomycin (50 µg/ml, selection for RUBY vector, Duchefa Biochemie, Haarlem, the Netherlands) and grown for 2-3 days at 28°C.

Separated colonies were picked and inoculated, and plasmids were extracted in the same way as from *E. coli* in section 4.1.5. The extracted plasmids were then confirmed by sequencing (again with primer pGEX 3').

4.2.3 Spruce calli preparation

Calli were maintained on ½ LP media (pH 5.8) at 22°C in the dark and subcultured every 2-3 weeks. 3-week-old calli that displayed healthy growth (translucent/white) were picked and subcultured in one of the two ways:

- Liquid plant infection media - calli were submerged in 20-25 ml of the infiltration media and placed on a shaker (100 RPM) in the dark for three days at 22°C.
- On solid plant infection media - calli were segmented into 2-3 mm cell clusters and placed on the media for three days in the dark at 22°C.

4.2.4 Infiltration of calli

Several colonies derived from previously confirmed *Agrobacterium* colonies were collected from a selection plate and inoculated overnight (220 RPM, 28°C) in 50 ml LB media with appropriate antibiotics (25 µg/ml gentamycin, 50 µg/ml rifampicin, 5 µg/ml tetracycline, and 50 µg/ml spectinomycin) collected after 18 hrs (15 min, 3000 RPM). The pelleted cells were resuspended in a liquid bacterial infection medium (pH 5.2), and OD600 was adjusted to 1. The suspension was placed on a shaker (100 RPM) for 3 hrs at 22°C in the dark. The calli were then infected in the following ways:

- Calli in liquid plant infection media — *Agrobacterium* in suspension was added to the calli medium so that the final OD600 was 0.5. The co-culture was incubated for 1 hr at 22°C, 100 RPM in the dark. The calli were then placed on filter paper on NS-Co-culture media (pH 5.8) and grown at 22°C in the dark for 4 days.
- Calli on Solid plant infection media - 10 - 50 µl of *Agrobacterium* suspension (OD600 = 1) were pipetted on the top of the cell clusters, and the plates were incubated at 22°C in the dark for 1 hr. Afterwards, excess *Agrobacterium* suspension was removed, and plates were grown at 22°C in the dark for four days.

4.2.5 Post-cultivation recovery

Four days after the co-cultivation, the calli were transferred on filter paper and placed into plates with sterile water and ticarcillin disodium / clavulanate potassium (400 µg/ml, Duchefa Biochemie, Haarlem, the Netherlands) for 30-60 min. The calli on filter paper were transferred to a Büchner funnel connected to a vacuum pump, and excess liquids were drained. The filter paper with calli was then transferred to ticarcillin disodium / clavulanate potassium-supplemented NS-Recovery media (pH 5.8), placed in the dark at 22°C, and monitored regularly for RUBY expression.

4.3 Tests of *in-planta* transient expression in apical meristem spruce seedlings

4.3.1 Spruce seedlings' cultivation

The approximately 2-year-old spruce seedlings were obtained from a breeding company Skogsplantor in a "dormant" state. They were kept in the dark at 8°C for two weeks, then transferred outside for four days when temperatures were around 15°C. Finally, the trees were grown in the greenhouse until their buds started developing into shoots.

4.3.2 Agro-infiltration suspension preparation

An overnight culture was started with 2.5 ml of *Agrobacterium* cryo-stock and 250 ml of LB (pH 7). The next day, after 16-18 hours, the culture was spun down at 3000 rpm for 10-15 minutes at room temperature. The LB was decanted, and the pellet was resuspended in the infiltration buffer. The resuspended pellet was spun down again at 3000 rpm for 10 minutes at room temperature, and the liquid was decanted, repeating this process twice. The pellet was then resuspended in the Infiltration buffer again, and the OD was adjusted to 0.7. Acetosyringone was added

to a final concentration of 150 µM. The suspension was kept at RT for 3 hours, with occasional inversion, to allow the cells to acclimate before agro-infiltration.

4.3.3 Infiltration methods

The protocols used for infiltration were developed based on methods described by Liu et al. (2020) (Method 1 and 2) and Salazar-Gonzáles et al. (2023) (Method 3 and 4).

Method 1: Sonication followed by infiltration (SI)

The top part of the seedling was submerged in deionised water and sonicated with a microprobe for 15 minutes (50-70W). Subsequently, the top 10-15 cm of the seedlings were incubated in 25-30 ml of the infiltration buffer (in a 50 ml falcon tube) for 30 minutes. The seedling was rinsed with sterile water twice. The infected part was covered with aluminium foil for one day. Observations and collections were made after 13 days post-infiltration (DPI). The apical shoot tissue was cut off, placed into a 15 ml falcon tube, frozen in liquid nitrogen, and stored at -70°C.

Method 2: Infiltration followed by sonication (IS)

The same protocol was followed as method 1, but infiltration and sonication were in the opposite order.

Method 3: Vacuum infiltration

The tree tip was placed in a falcon tube with 25-35 ml of the infiltration buffer inside the vacuum pot and incubated for 15-40 minutes while the pot was wrapped in parafilm. Subsequently, the vacuum pump was activated, pressure decreased to 150-400 mbar, and seedlings were left inside for 5 minutes with 2-3 vacuum breaks. The seedling was rinsed with sterile water. The infected part was covered for one day. The seedling was then grown in the greenhouse. Observations were made, and collections were taken after 8 DPI (due to the bad condition of the plants).

Method 4: Co-cultivation with micro-wounding

The apical shoot of the seedling was pierced with a sterile needle every 3-5 mm in three vertical lines. The shoot was then immediately co-cultivated with the Infiltration buffer for 30 minutes. The seedling was rinsed with sterile water, and infected parts were covered for one day. Afterwards, the seedling was transferred to the greenhouse. Observations were made, and collections were taken after 14 DPI.

4.3.4 Metabolomic extraction

The collected, frozen apical shoot (140-430 mg) was ground and resuspended with 1-1.5 ml of metabolomic extraction buffer. The supernatant was collected (13 000 RPM, 10 minutes, 4°C), and absorbance was measured at 535 nm, which is the absorbance maximum of betanine, the most abundant betalain (Shakir & Vincenzi, 2024; Salazar-Gonzáles et al. 2023). The spectrophotometer was calibrated with the buffer, and absorption at 535 nm was validated with beetroot extract, which was prepared from beetroot (purchased in Hemköp) in the same manner as the spruce samples. The absorbance was then converted to concentration according to Beer-Lambert Law: $A=\varepsilon \cdot c \cdot l$. In our case, A is the absorbance at 535 nm, ε is the extinction coefficient of betanin (60,000 L/mol·cm) (Calva-Estrada, 2022), and l is the path length through the cuvette (1 cm). The concentration in mol/L was converted to mg/kg to account for the weight differences between samples and data was analysed with two-way ANOVA (Mass ratio (mg/kg) ~ Method * Sample type).

4.3.5 PCRs of *DAL12* and *DAL13* and subsequent BP cloning

In the first round of PCR, the volume of 3 µl of cDNA synthesised from male cones' mRNA was mixed with other reaction components in the same manner as in section 4.1.4. Because BP cloning depends on compatible attB and attP sequences (Figure 4), primers used for this PCR were gene-specific with a partial attB overhang (11-12 bp) (Table 6 in appendix). The settings used for the PCR run are described in the appendix Table 4. PCR product of expected size (*DAL12* - 742 bp; *DAL13* - 661 bp) was purified from gel and used for the second PCR. The PCR mix was again prepared according to the same protocol, but full-length attB primers were used (Table 6 in appendix). The specific parameters of the run are detailed in the appendix (Table 4). The PCR products were then purified from the gel and used for the BP reaction (Figure 4).

At least 15 ng of the att-B-flanked product in 1-6 µl of elution buffer were combined with 1 µl of pDONR™/Zeo (150 ng/µl) and with 1-6 µl of TE buffer (pH 8, Thermo Fisher Scientific, Waltham, MA, USA) to the final volume of 8 µl (PCR product : vector = 1 : 10). The reaction was supplemented with 2 µl of BP clonase and mixed. The sample was incubated for 1 hr at 25°C, supplemented with 1 µl of protease K, and incubated for 10 min at 37°C. The reaction was cooled down on the ice, and TOP10 cells were transformed and placed on selection, like in section 3.1.4. In this case, however, the selective antibiotic was Zeocin™ (25 µg/ml, Thermo Fisher Scientific, Waltham, MA, USA), and Low-salt LB medium was used as a substrate (regular LB deactivates Zeocin™). Colony PCR from isolated colonies was performed with gene-specific primers, and plasmids were isolated and sent for sequencing.

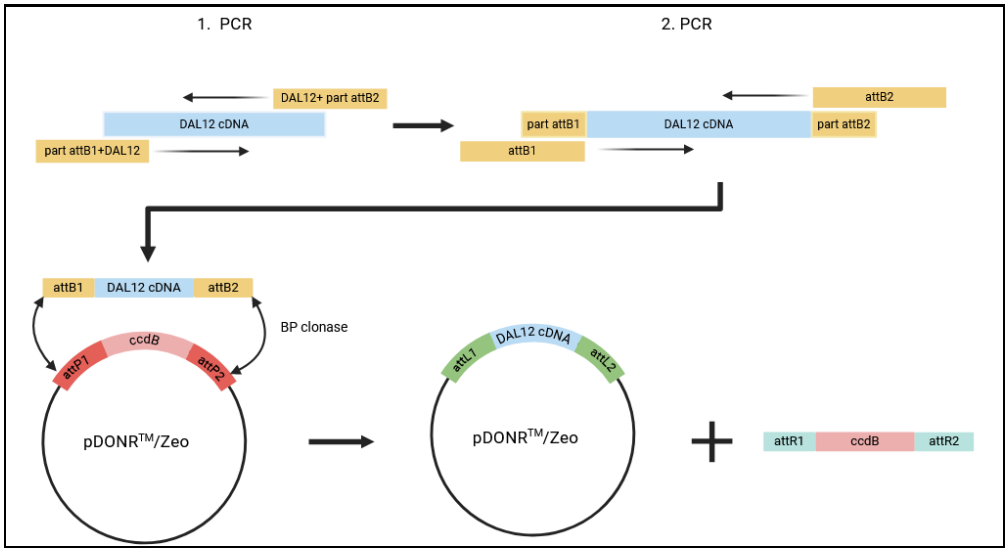


Figure 4: **PCR quantification of the *DAL12* gene with att-B-flanked primers followed by BP reaction.** Designed in BioRender.com and adapted from Gateway™ manual.

4.3.6 Quantitative PCR (qPCR) of *DAL12* and *DAL13*

The volume of 2 µl of diluted cDNA (50 ng/µl) synthesised from male cones' mRNA was added in a well on a 96-plate along with 5 µl SYBR™ Green PCR Master Mix (2x), 0.2 µl of *DAL12/13* qPCR primers (0.5 µM), and 2.8 µl of water. Reactions with constitutively expressed housekeeping genes (*H2A*, *actin*, *polyubiquitin*) were prepared in the same manner. Each reaction was prepared in 3 technical replicates. qPCR was then run according to the settings described in the appendix (Table 4). After the run, the quality of the collected data was determined based on the consistency of expression among the technical replicates of housekeeping genes and data was interpreted.

5. Results

5.1 Preparation of yeast libraries

To determine if the selected transcription factors (Table 1) bind to the promoters of *PaSPL1* from different genotypes and activate transcription of the *PaSPL1* gene, we decided to conduct a Y1H assay. This assay will help elucidate whether these potential interactions are present. The first step towards this goal was creating TF-containing yeast libraries, prepared through the following process.

The group members collected the male, female, and vegetative buds on various dates (August 2016, April 2024, and June 2023) and at different times of the day (morning, afternoon, or unspecific). We isolated RNA from these samples, prepared cDNA and performed PCR amplification of the TF-encoding sequences of interest.

We successfully obtained PCR products for most of the TF-encoding sequences. However, we did encounter a minor challenge where many of the TF-encoding sequences were amplified along with several other products. This has allowed us to refine our processes for the next steps. The spruce transcriptome has lots of potential for additional description and it is still being annotated. Additionally, we do not have any knowledge regarding alternative splicing of the examined genes. Therefore, in some instances, we could not determine which product should be isolated if the sizes were very similar or none of the products was within ± 100 bp from the expected position. This problem was overcome by performing nested PCR. We created another set of primers that bind to the untranslated regions (UTRs) that were used for the first round of PCR, and then we used the gene-specific primers (forward primer with CACC overhang) for the second round. The products that had similar sizes during both rounds and were close to the expected size were then considered the most probable TF-encoding sequences and were isolated from the gel. The concentrations of gel-purified PCR products were then measured on Nanodrop

The following procedure, D-TOPO™ cloning, was another critical step. Although we optimised the manufacturer's (Thermo Fisher Scientific) protocol, the number of colonies was usually deficient, as was the transformation efficiency, so we had to typically repeat this step several times before a positive colony was successfully obtained. The plasmid was then isolated from overnight culture and sent for sequencing (Obtained sequences supplemented in appendix). Some sequences corresponded exactly to the reference sequences obtained from Plantgenie (Sundell et al., 2015, https://plantgenie.org/) (Figure 5A), while some differed because not all allelic variants and copies of the genes are described at the moment (Figure 5B). Sequences that only partially matched the reference (Figure 5C) were further analysed using NCBI BLAST to identify similarities with other

sequenced conifer genomes and to confirm homology to the gene of interest. However, for our experiment, it was only necessary to inspect that there are no stop codons, frameshifts, or mutations in the DNA binding sites. Other differences (SNPs, amino acid substitutions) should be negligible for this type of experiment. Vectors that passed the inspection were subsequently utilised in the LR reaction.

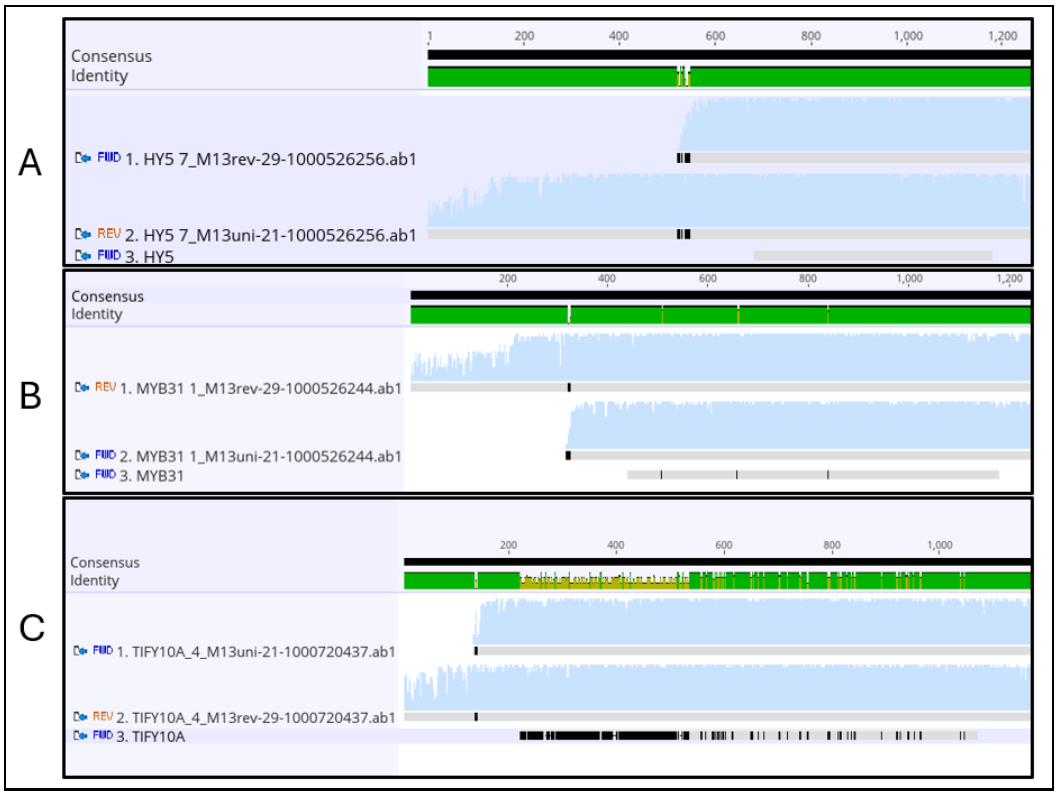


Figure 5: **Examples of alignment of sequencing results to reference sequence.** The first two rows of each example are sequencing results obtained with vector-specific M13 primers, and the third row is the reference sequence from Plantgenie. A) Sequence alignment of *PaHY5-like-1* (*HY5*) where the sequences match exactly (only green consensus identity with the reference) B) Sequence alignment of *PaMYB31-like-1* (*MYB31*) with SNPs that do not change the protein sequence (three orange stripes in the consensus identity with the reference) C) Sequence alignment of *PaTIFY10A-like-1* (*TIFY10A*), which matches the known sequence only partially (Many orange stripes and segments in the consensus identity with the reference). Blasting confirmed homology with a gene from *Picea sitchensis*. Allignments assembled in Geneious Prime® 2023.0.4.

In the LR reaction step we combined the TF-containing pENTR vectors with the pDEST-AD-2u destination vectors using LR Clonase™ II Enzyme mix. In this case, we followed the manufacturer's protocol precisely, and transformation typically resulted in at least 1 positive colony. Plasmids from positive colonies were isolated and sequenced to confirm the presence of the TF genes in the pDEST-AD-2u vector (= expression clone) (Figure 6). Once confirmed, we transformed the expression clone was inserted in the Yα1867 yeast cells.

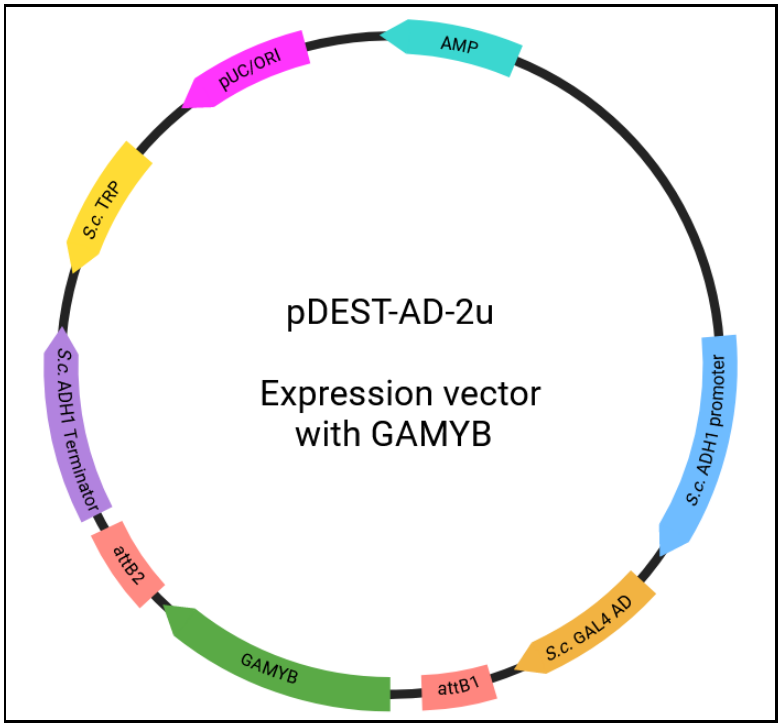


Figure 6: **An example of the expression vector with *PaGAMYB-like-1* (*GAMYB*).** The vector also contains *Saccharomyces cerevisiae alcohol dehydrogenase 1* (*S.c. ADH1*) promotor and terminator, *S.c. GAL4* activation domain, *S.c. N-C5'phosphoribosyl-anthranilate* that is needed for Tryptophan biosynthesis (S.c. *TRP*), *pUC origin of replication* (*pUC/ORI*), and *ampicillin resistance* (*AMP*). Designed in BioRender.com.

Initially, we tried to transform the yeast cells according to a protocol published by Gaudinier et al. (2017), which largely inspired the experimental setup of our Y1H assay. Unfortunately, using this protocol did not result in a successful transformation and due to the large number of steps and variables in this protocol, it would have been difficult to optimise. Fortunately, the Frozen-EZ Yeast Transformation II™ kit provided a more streamlined transformation protocol, successfully producing transformants in all instances. Given that the selection process is deficiency-based, all resulting colonies were expected to be positive (Figure 7). This expectation was further validated through colony PCR (Figure 7). Positive yeast libraries were stored for further experiments.

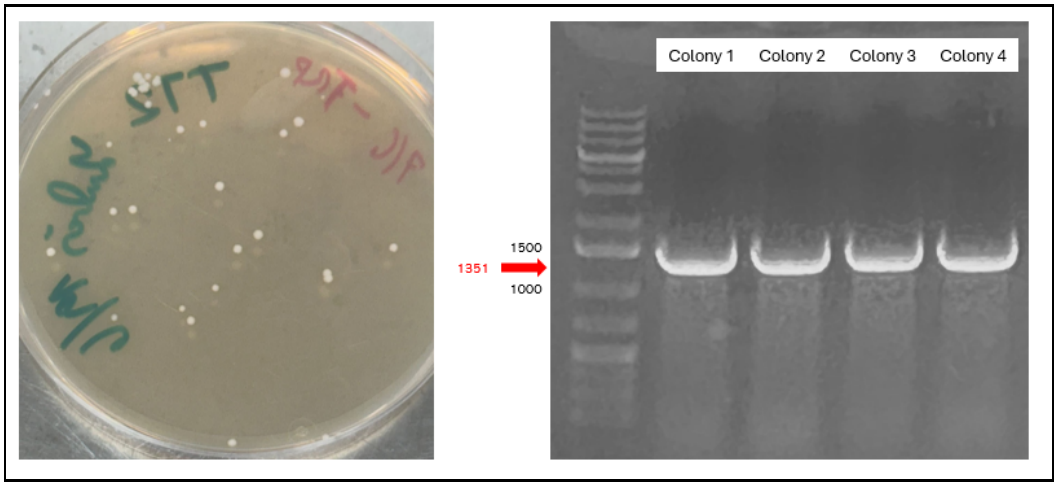


Figure 7: **On the left: Transformed Yα1867 yeast colonies containing expression vector with *PaTT2-like-1*, growing on -TRP selection. On the right: Colony PCR of four of the depicted *PaTT2-like-1* colonies with a band at the expected position 1351 bp.**

Not all 17 TFs of interest reached the final stage "in Yeast" within the given time frame. Table 2 describes the current status of each of the TFs.

***Table 2*: The current status of each of the examined TFs.** Stage "in Yeast" means that the TF is ready for the Y1H experiment. Stage "pDEST" means that colonies tested positive in colony PCR but were not sequenced yet. Stage "in TOPO or in pDEST" means that colonies need to be screened for positive transformants. Stage "PCR" means that the product was obtained but D-TOPO™ reaction was not successful yet. Stage "Not obtained in PCR" means that the TF was not amplified from available cDNA. **PaSPL1* was obtained already ligated in the entry vector which was prepared by Nathan Zivi. F = female, V = vegetative.

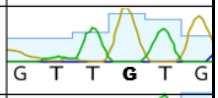
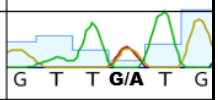
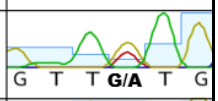
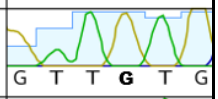
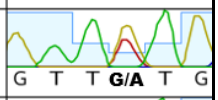
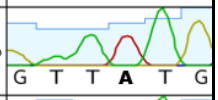
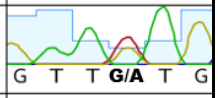
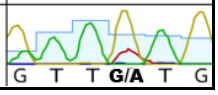
ID (Plantgenie)	TF	Obtained from tissue(s)	Stage
MA_8147g0020	*PaMYB31-like-1*	August F and V	in Yeast
MA_55123g0010	*PaGAMYB-like-1*	August F and V	in Yeast
MA_10208000g0010	*PaHY5-like-1*	August F and V	in Yeast
MA_14452g0010	*DAL10*	August F and V	in Yeast
MA_97319g0010	*PaAP2-like-1*	August F and V	in Yeast
MA_2193g0020	*PaTT2-like-1*	703 V	in Yeast
MA_16120g0010	*DAL19*	703 V	in TOPO or pDEST
AF064080.1	*PaSPL1*	*	in TOPO or pDEST
X80902.1	*DAL1*	703 V	in pDEST
MA_9455802g0010	*PaTIFY10A-like-1*	703 V	in TOPO or pDEST
MA_10433497g0010	*PaTIFY10B-like-1*	August F and V	in pDEST
AY701762.1	*PaNLY*	703 V	PCR
AY701763.1	*PaLFY*	703 V	PCR

MA_41006g0010	*PaR2R3-like-1*	703 V	PCR
MA_11267g0020	*PaLHY-like-1*	-	Not obtained in PCR
MA_115536g0010	*PaCCA1*	-	Not obtained in PCR
OU384166.1	*PaAP2 ERF-like-1*	-	Not obtained in PCR

5.2 Calli genotyping and transformation

Before we could evaluate the transformability of the spruce calli with RUBY vectors, we had to identify which alleles of *PaSPL1* these calli have. We isolated DNA of 11 spruce calli cell lines was, and the *PaSPL1* gene was PCR amplified. Then we sent the unpurified PCR product for sequencing, and we determined zygosity determined based on the SNP (nucleotine 1421, A = *acrocona*, that differs between *acrocona* and WT (Table 3). We were not able to acquire DNA of 3 out of the 11 tested genotypes in sufficient quality, so they were not genotyped. The goal was to identify at least 1 WT homozygote, 1 *acrocona* homozygote, and 1 heterozygote for the transformation experiments.

Table 3: **The PaSPl1 zygosity of the examined genotypes (WT = wild-type, Acro = *acrocona*).**

Genotype	*PaSPL1*	Nucleotide 1421 ⬇
15:H7	WT/WT	
15:H12	WT/Acro	
15:H11	WT/Acro	
15:H1	WT/WT	
16:H9:7	WT/Acro	
16 H3:11	Acro/Acro	
16:H3:10	WT/Acro	
16:H8:8	WT/Acro	

The calli of four genotypes that were healthy and actively growing (15:H7, 15:H11, 15:H12, 16:H9:7) were used for the transformation test with 35S:RUBY and UBQ:RUBY. The agro-control calli were transformed with GV3101 containing pTok47 without the RUBY vectors. We followed the protocols thoroughly, and

tested both liquid plant culture media and solid plant media methods. At 12 days post-infection (DPI), there was no noticeable difference between agro-control and RUBY colours. At 31 DPI, the calli were again carefully inspected, and four had small red spots (Figure 8 depicts one of them), showing that the transformation was inefficient but successful. It is also possible that the transformants require more time to show the phenotype.

To determine which approach might be the most suitable, we also had to evaluate how the growth of different genotypes is affected by the choice of the transformation method and the vector.

Figure 9 demonstrates that the "solid" method is significantly more effective than the "liquid" method in fostering actively growing calli. Specifically, the "solid" process results in 25-100% of actively growing calli, while the "liquid" method predominantly results in no actively growing calli, with a maximum of only 50%. Results do not suggest that the choice of vector affects the growth on solid media; however, both RUBY vectors had no growing calli after the “liquid” treatment, whereas the control did. It is worth noting that in this case, the growth was inferior, and it will likely not progress further.

Additionally, not all genotypes were responding in the same manner. 15:H7 was tested with all six combinations (35S or UBQ or Control with liquid or solid), and 100% calli grew abundantly after solid media treatment, and none grew after the liquid treatment. In the case of 16:H9:7 (4 combinations), the growth was very limited in all cases independently of treatment. Therefore, some genotypes (for example, 16:H9:7) may require a different approach. The differences in active growth could also be a direct result of the initial vitality of the “parent” callus when the experiment was started.

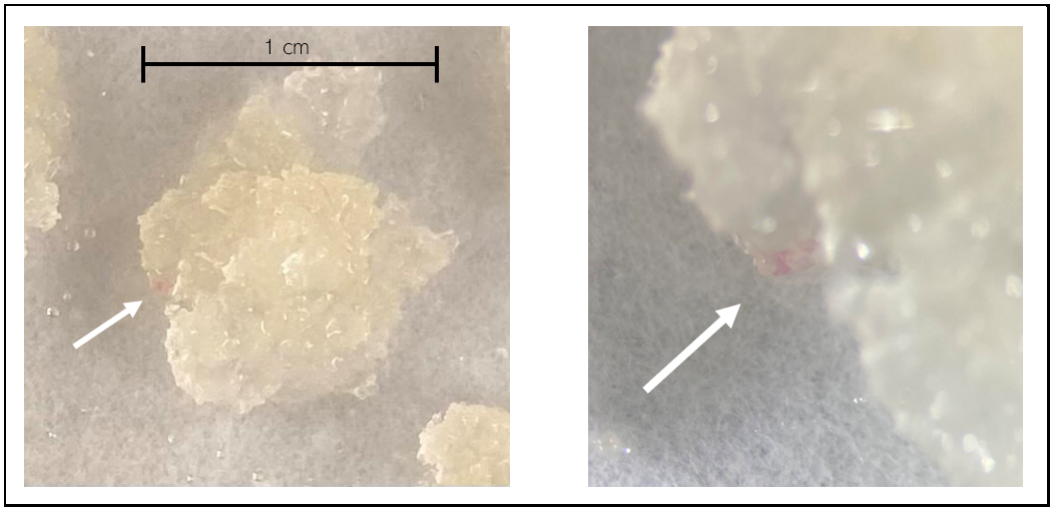


Figure 8: **A callus (genotype 15:H11) transformed with 35S:RUBY showing expression of betalains at 31 DPI.**

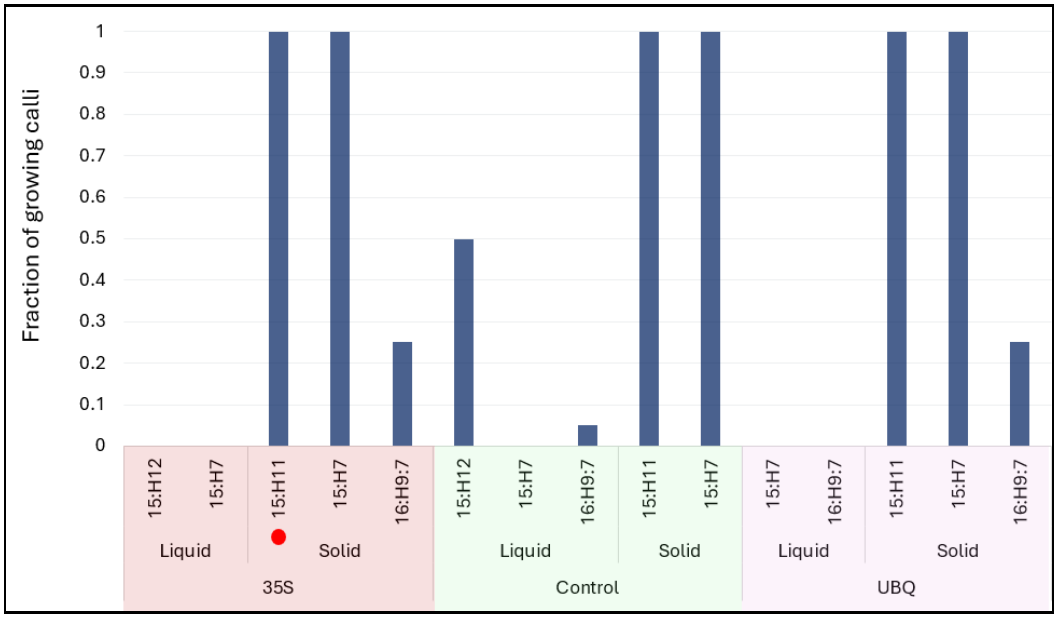


Figure 9: **The effect of the transformation method and vector choice on the healthy growth of calli of selected genotypes.** The red dot marks the treatment that resulted in a positively transformed 15:H11 calli.

5.3 *DAL12* and *DAL13*

Before we can study the function of the putative B-class genes *DAL12* and *DAL13* by introducing them into *acrocona* calli, we must take the initial step which involves their amplification from cDNA and subsequent cloning.

The cDNA synthesised from RNA from male cones (samples from 18/8/23) was used for PCR amplification of both genes with partial-attB overhangs. Products of the correct size were purified from gel and used as substrates for the second round of PCR with complete attB primers. Both were again gel purified and used for BP reaction with pDONR/Zeo (overview in Figure 4).

Both BP reactions yielded colonies; however, only *DAL12* colonies tested positive. The plasmid was isolated and verified by sequencing and is now ready to be recombined into a destination vector of choice.

As *DAL13* was difficult to re-amplify (the PCR yielded product only once) from the August samples, we tried to amplify it from the male cones collected in April. The amplification was successful, indicating that *DAL13* may have been expressed more at that point (Figure 10).

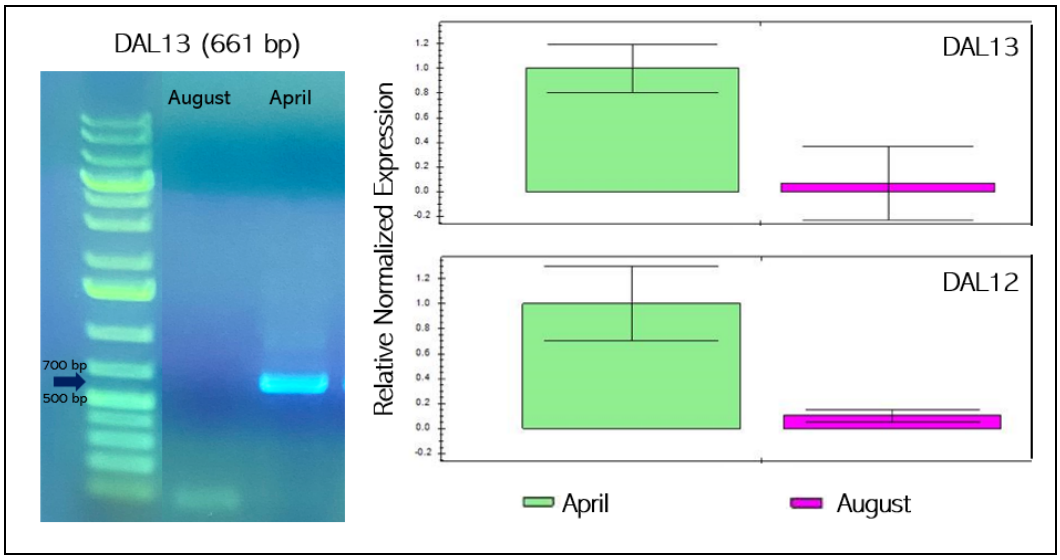


Figure 10: **On the left - Comparison of *DAL13* PCR products in the samples from August and April. The dark blue arrow indicates the expected position (661 bp). On the right - Relative normalized expression of *DAL12* and *DAL13* in the April and August samples.**

To provide additional insight into the expression patterns of *DAL12* and *DAL13*, we conducted a qPCR with both August and April samples. The data showed (Figure 10) that both *DAL12* and *DAL13* were not expressed at all or minimally expressed in the August sample and relatively much more expressed in the April sample. This finding was unexpected, as previous study of Sundström et al. (1999) suggested otherwise.

5.4 Tests of *in-planta* transient expression in apical shoot of spruce seedlings

As transient expression of *DAL12* and *DAL13* genes directly in spruce plants might be another way of studying their function, we tested several methods derived from validated transient transformation protocols.

The four selected methods, sonication followed by infection (SI), infection followed by sonication (IS), vacuum infiltration, and wounding, were tested on three biological replicates per RUBY construct and three agro-controls (only GV3101 with pTok47). The experiments adhered strictly to the designed protocols except for vacuum infiltration, where, due to technical difficulties, some of the infiltration suspensions were acclimating up to 6 hrs instead of the recommended 3 hrs. After the infiltration, the trees were observed daily for signs of successful transformation. However, no red colour was observed. At 13-14 DPI, SI, IS, and wounded plants were photographed (Figure 11), and apical shoots were collected and frozen in liquid nitrogen. The plants that have undergone vacuum infiltration were heavily affected by the procedure and had to be collected at 8 DPI (Figure 11). Some of them started drying, so samples were not collected.

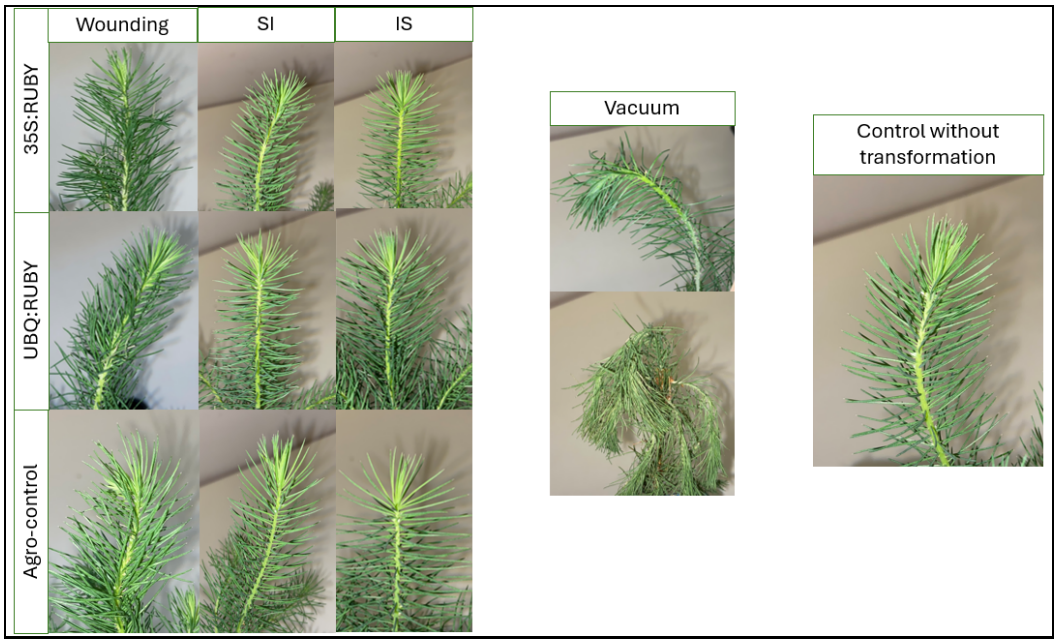


Figure 11: **Apical shoots of spruce seedlings agro-transformed with different methods.** No betalain expression can be observed in any of the samples. The middle pictures show the possible detrimentary effects of vacuum infiltration (35S:RUBY on the top and UBQ:RUBY on the bottom).

The collected, frozen tissue was ground, and 140-430 mg was used to extract betalains. The tissue was resuspended in the metabolic extraction buffer, centrifuged, and absorbance at 535 nm was measured. The absorbance data was normalized and expressed as mg of substance absorbing at 535 per kg of tissue (Figure 12, data in Table 7). The two-way ANOVA test showed a significant difference between the different methods (p-value = 1.52E-05) but not between the different types of samples (p-value = 0.158). The interaction between methods and samples also was not significant (p-value = 0.265). From these results, it can be inferred that the RUBY genes were expressed in very low amounts, possibly below the sensitivity of the detection method, or not expressed at all. For context, the beetroot standard exhibited a betanin mass ratio of 135.62 mg/kg, while the average betanin mass ratio of all RUBY-transformed samples was only 1.34 mg/kg, similar to the Agro-control average of 1.21 mg/kg.

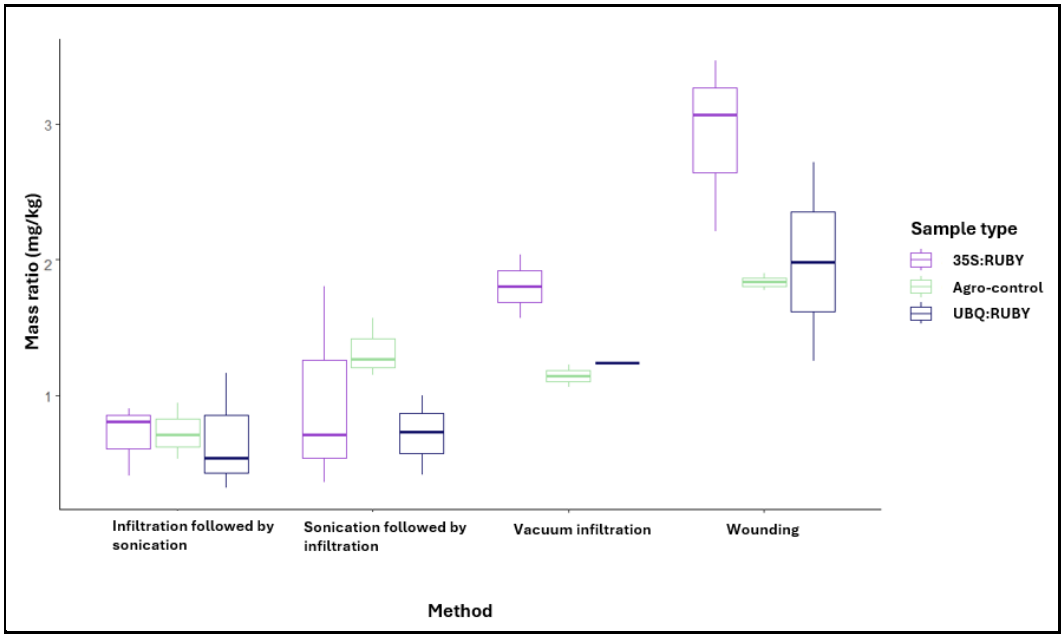


Figure 12: **Mass ratio (mg/kg) of substances absorbing at 535 nm per method and sample type**.

6. Discussion

6.1 Preparation of yeast libraries

The main challenge that the Norway spruce breeders face is the long time (20-25 years) it takes for the trees to transition into the reproductive stage (Lindgren et al., 1977). The natural spruce variant *acrocona* displays an early cone-setting phenotype (Flachowsky et al., 2009; Uddenberg et al., 2013). Uncovering the genetic mechanisms responsible for this phenotype thus interested the breeders and researchers. Akhter et al. (2022) discovered that *acrocona* contains SNP in the *PaSPL1* gene, which affects the binding of regulatory miRNAs and results in higher levels of *PaSPL1* mRNA. *PaSPL1* was, therefore, linked to the acrocona phenotype, making it highly important to understand not only the characteristics of the gene but also its regulatory framework. To better understand how the expression of *PaSPL1* is regulated, our group aims to conduct a Y1H experiment with potential transcription factors (TFs) that may bind to the promoter of this gene. One of the goals of this thesis was to prepare yeast libraries containing these potential TFs, and this section describes the procedures and optimisations undertaken to achieve this goal.

Creating TF-containing yeast libraries for the Y1H experiment required many optimisations; however, it resulted in the successful preparation of 6 TF-containing yeast libraries and 5 TF-encoding sequences in the entry or destination clone, which will be advanced to the yeast library stage in the coming weeks.

Some TF-encoding sequences could only be obtained from cDNA synthesised from a specific tissue type collected at a particular time of the year. For example, the cDNA synthesised from June 2023 vegetative tissue samples, particularly from the 703 genotype, was suitable for amplifying TF-encoding sequences that were difficult to obtain from the samples from August 2016 (For instance, *PaTT2-like-1*, *PaTIFY10A-like-1*). This variation could result from differential expression of these genes through time or across genotypes. Nevertheless, it could also be caused by the degradation of RNA throughout the years of storage in the -80°C freezer or by the insufficient quality of the cDNA. The latter seems, however, unlikely as the quality of the cDNA was evaluated by semi-quantitative PCR with constitutively expressed polyubiquitin.

Even after trying all available cDNAs, some genes simply couldn't be amplified. For example, we never obtained *PaLHY-like-1* and *PaCCA1* (*Picea abies CIRCADIAN CLOCK ASSOCIATED 1*). These two genes, similar to their Arabidopsis homologs, likely play a role in the circadian rhythm of spruce (Karlgren et al., 2013). Thus, their expression levels could largely differ throughout the day, and the peak could be very close to the dark-to-light transition. The August

2016 and April 2024 samples were collected at either a non-specific time or in the afternoon, and to test if time could play a role, we collected samples (already growing vegetative shoots) at 9:30 in the morning (June 2024), but the genes were still not quantified. It is possible that samples collected even earlier, right after sunrise, may contain more mRNA of these genes, which can be tested in further experiments. *PaAP2 ERF-like-1* is another gene that was not successfully amplified, not even with nested PCR. The reason for this remains unclear, and we could use qPCR to determine if it is due to low expression levels or if there might be some other reason (bad primer design, incorrectly annotated gene sequence in Plantgenie, etc.).

The genes successfully obtained from PCR continued to the D-TOPO™ reaction step, which also required several adjustments. We had to increase the amount of PCR product added to the reaction tenfold and allow the reaction to run longer (2-20 hours), as following the manufacturer's instructions did not result in a successful transformation. There are several possible explanations for why these alterations were needed. In almost all cases, we purified the PCR product from the gel with the Zymoclean Gel DNA Recovery Kit, and the eluate typically had a quite prominent 230 nm peak. This could indicate the presence of compounds that may interfere with the D-TOPO™ reaction and inhibit the ligation. Selecting an alternative extraction kit (GeneJET Gel Extraction Kit, ThermoFisher Scientific) and corresponding elution buffer (10 mM Tris-HCl, pH 8.5) did not yield different results. The D-TOPO™ reaction process can thus be optimized further to increase reaction efficiency. We could, for example, try another elution buffer or even elute the PCR product with only sterile water. An optimal approach is to use the PCR product directly without purification. However, we mostly tried to avoid that, as there were usually several bands in the gel, and the present primer dimers and other products could also be ligated. The subsequent steps, *E. coli* transformation and LR reaction followed by transformation, worked sufficiently and typically yielded at least 1-2 positive colonies. Therefore, there is likely not a problem with the TOP10 competent cells. Once the final expression vector was isolated from *E. coli,* it could be introduced into yeast cells to create the yeast libraries.

The yeast transformation protocol also required refinement. After carrying out unsuccessful transformations following the protocol by Gaudinier et al. (2017), we decided to use the much simpler protocol offered by the Frozen-EZ Yeast Transformation II™ kit. This protocol resulted in high transformation efficiency, and its simplicity enables easy replicability of the experiments. It is not advisable to optimise the Gaudinier et al. (2017) protocol further as it includes several variable steps and is much more time-consuming. The TF-containing yeast libraries were stored and are now ready for the Y1H experiment (see section 8.).

6.2 Calli transformation with RUBY vectors

The second goal of this project was to validate a calli transformation protocol and evaluate the transformability of embryonic calli of wild-type and *acrocona* lines with RUBY vectors. Transformable lines will later be used for transformation experiments with *DAL13* and *DAL12* to determine their putative B-class function in male cone development (Sundstöm et al., 1999).

The *PaSPL1* zygosity of most of the available spruce calli genotypes was determined, and calli with good growth (15:H7, 15:H11, 15:H12, 16:H9:7) were transformed with control *Agrobacterium*, *Agrobacterium* with 35S: RUBY, and *Agrobacterium* with UBQ: RUBY.

For 2 to 3 weeks post-infection, no apparent differences in the phenotypes of the control and RUBY calli were detected. At 31 DPI, the first calli transformed with 35S: RUBY were identified. This delay in detection may be attributed to the initially weak signal, which became visible only after a sufficient number of betalain-expressing cells accumulated. Alternatively, our expectation of a more robust transformation might have led us to overlook the small transformed spots. Another possible explanation is the inherent complexity of the betalain biosynthesis pathway, which involves a triple-enzyme mechanism (He et al., 2020) and may result in a slower expression rate requiring time to produce an observable amount of betalains. This is due to the necessity of converting several intermediate metabolites at a sufficient rate. To test this hypothesis, we could supplement the calli or the growth medium with exogenous tyrosine, the precursor substrate, to expedite betalain production. In study of Fadzliana et al. (2017) similar approach was tested on calli of pitaya, which has betalain pathway naturally, and they disovered that 20 mg/L of tyrosine supplementation results in 1.7-fold increase in betalain content.

Besides the slow expression, the natural availability of tyrosine could also selectively disadvantage the transformed cells (TCs) when the RUBY vector is present without positive selection. Theoretically, TCs and non-transformed cells (NTCs) possess equivalent tyrosine levels. However, TCs convert a portion of tyrosine into betalains, potentially giving NTCs a competitive edge in protein biosynthesis and cell proliferation.

Ford et al. (2023) demonstrated that tyrosine is among the less abundant amino acids. Across the 72 plant species analyzed in their study, which focused on fruits and vegetables, tyrosine constituted only approximately 2% of the overall amino acid content. Suppose this low percentage is conserved among plant species, as no similar data is available for gymnosperms. We can deduce that any decrease in tyrosine availability can potentially impair protein biosynthesis and related processes, thus giving the NTCs with more available tyrosine an advantage.

As previously mentioned, the currently employed RUBY vectors lack components that would enable the discrimination of non-transformed cells (NTCs). Zhao et al. (2024) provided a comprehensive overview of transformation experiments conducted on gymnosperm species, highlighting that the *Bialaphos resistance* gene (*Bar*), which confers resistance to the herbicide phosphinothricin, is among the most frequently used selection markers. Several reviewed studies also employed kanamycin resistance for selection purposes. Furthermore, a recent study by Wang et al. (2024) on the transformation of peach calli using RUBY genes incorporated hygromycin resistance to select positive transformants. To improve our selection process, at least one of these resistance genes, under an appropriate promoter, could be isolated from a commercially available vector through restriction digestion and subsequently ligated into the RUBY vectors we are currently using. Additionally, combining resistance-based selection with tyrosine supplementation could potentially facilitate the rapid and efficient identification of positive transformants.

In comparing the liquid and solid methods for plant infection regarding the healthy growth of calli, it is evident that the solid method yields healthier and more vigorously growing calli. The reduced growth rate observed in calli subjected to the liquid method is likely due to the rapid changes in conditions and/or limited oxygen availability in the suspension culture. Consequently, we should prefer the solid method for subsequent experiments.

At 31 DPI, the betalain production was detected only in 4 calli of 15:H11 and transformed with 35S: RUBY. Therefore, this genotype is confirmed as "transformable" and can be used in further transformation experiments. Nonetheless, as the number of identified betalain-expressing transformants was very low, it would be premature to state that 35S: RUBY is more suitable for the transformability evaluation than UBQ: RUBY. All growing 35S: RUBY and UBQ: RUBY calli were sectioned and subcultured at 35 DPI. Some sections seemingly displayed a slight red colour, which will be observed further.

6.3 Cloning and qPCR of *DAL12* and *DAL13*

As stated, the heterozygous and homozygous *acrocona* calli, evaluated as transformable, will be transformed with the *DAL12* and *DAL13* genes to determine their role as putative B-class genes. The third goal of this thesis was to clone these genes into entry vectors, which will later be recombined into the T-DNA region of destination vectors used for agro-transformation.

DAL12 was successfully quantified and ligated into the pDONR^{TM}/Zeo vector and can be used for further steps. On the contrary, *DAL13* was difficult to obtain from the cDNA synthesised from male buds collected in August 2016. When the PCR quantification was repeated with cDNA synthesised from April 2024 male

buds, a strong band was present at the expected position (not confirmed by sequencing yet). This indicated that there might be different expression patterns of *DAL13* between the two samples, which contradicts previous findings. The expression of *DAL13* was measured to be comparatively higher in the early autumn period when the microsporophylls are differentiated than during other times of the year (Sundström et al., 1999). Therefore, the gene should be present more abundantly in the August sample.

To better understand, we ran qPCR with both *DAL12* and *DAL13* samples. The results showed that both genes were relatively strongly expressed in the April samples and very little or not at all in the August samples. A potential explanation is that the buds collected in 2016 were incorrectly identified as male but were female or vegetative instead. However, the *DAL12* gene was obtained from this cDNA. So, it seems likely that the buds were a collection of vegetative/female buds with at least one male bud. In Figure 13 we can observe that especially male and vegetative buds are quite similar in size and shape, although vegetative buds are more "pointy". The depicted buds are from the April collection. At this point, the buds are larger and more developed than in August (development is taking place from May to May of the next year) (Carlsbecker et al., 2013), so it is easy to imagine that the vegetative bud was incorrectly categorised as male during the August collection. It is also possible that the error happened later and that we simply used a wrong sample for the cDNA synthesis.

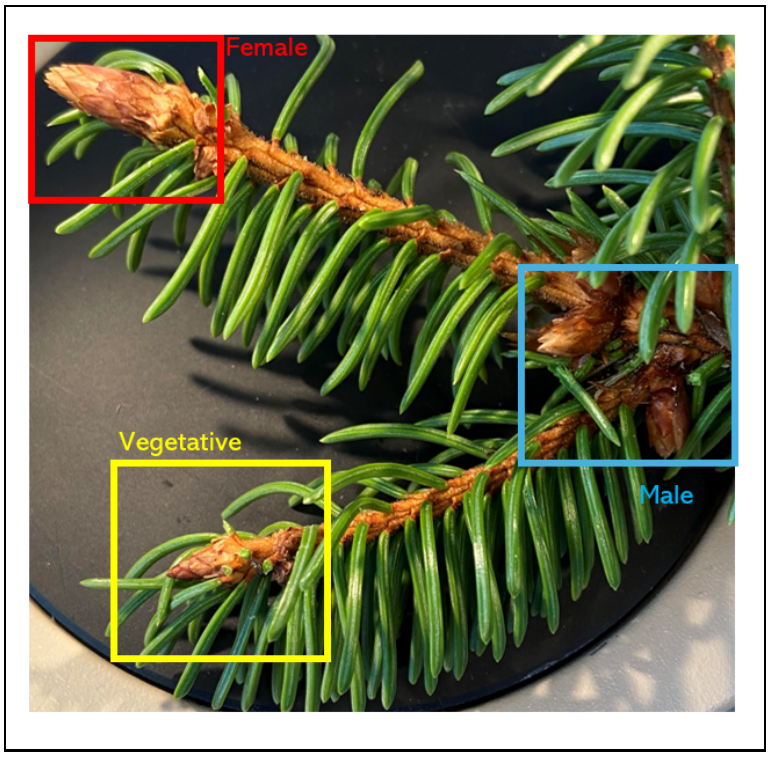


Figure 13: **Female, male, and vegetative buds on a branch collected in April 2024.**

6.4 Interpretation of results of *in-planta* transient expression tests in the apical shoot of spruce seedlings

An alternative to the stable transformation of *acrocona* calli with *DAL12* and *DAL13* genes followed by regeneration into a tree could be a transient transformation of bud-forming tissue in already grown trees. Since no protocol currently exists for this type of transformation, this study's fourth and final task was to test four different transformation methods and evaluate their potential for future experiments.

To assess the effectiveness of these methods, a two-way ANOVA of spectroscopic data of extracted metabolites was conducted. The analysis suggested that none of the transformations with RUBY vectors resulted in the expression of betalains significantly different from the control samples. Still, there was a significant difference between the four methods. The very low p-value (1.52E-05) is likely attributed mainly to the slight difference in the processing of wounded and the other samples. The wounded samples were resuspended in 1 ml of extraction buffer, and 0.9-1 ml was then transferred to the cuvette. This proved challenging due to contamination from the pellet, leading to the resuspension of the other samples in 1.5 ml of buffer, from which 1 ml was transferred. This process likely resulted in a more heterogeneous suspension, particularly when 1 ml was taken from the wounded samples. Additionally, the samples from vacuum-infiltrated seedlings exhibited different behaviour, as they did not sediment as effectively as the SI and IS samples. Consequently, the absorbance readings may have been slightly elevated due to the increased presence of tissue debris in the extract.

The calli transformation experiments suggest that detecting betalains in the tissue may require an extended period. Allowing a few additional days might be advantageous for future transient expression experiments before collecting the tissue. If the transformation efficiency is notably low, spectroscopy may need to be more sensitive to detect differences in betalain levels. Thus, employing a more sensitive detection method could be necessary. To this end, we preserved half of the ground tissue for qPCR analysis. Should the transformation be successful, we can expect to detect RUBY transcripts in the samples.

Additionally, there remains a possibility that the plants were not transformed at all. If qPCR results confirm this, we must devise and evaluate a new approach. An initial step could involve integrating previously tested methods. For instance, combining wounding with infiltration followed by sonication (IS) or sonication followed by infiltration (SI) might yield better results, as incisions on hypocotyls and needles have been shown to enhance transformation efficiency in *in vivo* studies (Liu et al., 2020). The current settings for vacuum infiltration have proven too

harsh, often damaging the plants. Therefore, future attempts should omit this method or apply milder settings to avoid detrimental effects.

7. Conclusions

Throughout this thesis, our primary objectives were to prepare yeast libraries with 17 potential transcription factors (TFs) for a yeast one-hybrid (Y1H) experiment, transform selected calli using RUBY vectors, generate pDONR™ vectors with *DAL12* and *DAL13*, and evaluate methods for in planta transient transformation of spruce seedlings. Significant progress has been made in all the following areas:

- We successfully prepared yeast libraries with 6 TFs; most remaining TFs are now in entry/destination vectors and ready for further processing.
- The transformation of several calli with the RUBY vector was successful, leading to the validation of the transformation protocol and the formulation of recommendations for its improvement.
- *DAL12* was successfully inserted into the pDONR™/Zeo vector, and *DAL13* was prepared for the BP reaction.
- The *in-planta* transient transformation methods were tested, and analysis of metabolic extracts did not indicate successful transformation. Nevertheless, the final evaluation will be made with qPCR.

Overall, this work lays a solid foundation for future Y1H experiments and provides valuable insights into optimising transformation protocols and developing efficient strategies for the genetic engineering of spruce calli and trees.

8. Future perspectives

Moving forward from the achievements outlined in this thesis, several key directions for future research and development are evident.

In the upcoming weeks, the TF-containing yeast libraries created as a part of this project will be mated with yeast libraries that contain the promoter of *PaSPL1* isolated from different genotypes (Figure 1, steps 14b-15). The offspring will be then grown and placed on selection to identify successfully mated diploid offspring (Figure 1, step 16). Subsequently, X-gal assay and 3AT assay will be used to identify positive interactions (Figure 1, step 17). In the X-gal assay, colonies that will be blue will indicate an interaction between the TF and the promoter. Similarly, in the 3AT assay, the actively growing colonies will suggest interaction (Gaudinier et al., 2017). The Y1H will thus help us elucidate how the regulatory framework of *PaSPL1* expression operates.

However, as this experiment will only provide us with knowledge regarding the interactions *in vivo*, we have to consider the following steps we may need to take to confirm this interaction. One option could be an electrophoretic mobility shift assay (EMSA), which would provide us with information about the DNA-protein interaction *in vitro*. We may also consider performing chromatin immunoprecipitation sequencing (ChIP seq) to verify that the interaction occurs *in planta*. This experiment would, however, require the development of TF-specific antibodies, which may be too costly. We could also consider performing a dual-luciferase assay to explore the interaction between transcription factors (TFs) and promoters transiently expressed in tobacco. This assay has been previously validated using genes from the related species *Picea crassifolia* (Feng et al., 2024). We can adopt the established protocol where Renilla luciferase (REN), derived from *Renilla reniformis*, is constitutively expressed under the *35S* promoter. The expression of REN then serves as a normalization control for the signal detected from firefly luciferase (LUC), which is under the control of the promoter of interest (Feng et al., 2024).

Regarding the stable calli transformation with *DAL12* and *DAL13*, the next steps should be focused on designing a suitable destination vector. As the positive transformants will not be visible, like in the case of RUBY vectors, it will be necessary to add a gene for antibiotic/herbicide selection, such as hygromycin. A suitable promoter for the *DAL12/13* genes will also be required. Using a robust tissue-nonspecific promoter, such as *CaM35S*, may result in unwanted phenotypes due to the activity of *DAL12/13* in tissues where they normally do not occur. Thus, It would be reasonable to select a promoter that is not so strong or, optimally, even specific to the tissue in which we want *DAL12/13* to be expressed. Furthermore, *DAL11* should also be cloned into a vector and used for the same experiments as it is another potential B-class candidate gene. We can also think about utilising the

2A peptides for linking the genes and co-expressing them under a single promoter. This way we can create a series of 7 constructs:

- *promoter:DAL12*
- *promoter:DAL13*
- *promoter:DAL11*
- *promoter:DAL12:2A:DAL13*
- *promoter:DAL12:2A:DAL11*
- *promoter:DAL11:2A:DAL13*
- *promoter:DAL12:2A:DAL13:2A:DAL11*

These contructs can then be used for transformation of calli, and we can compare the phenotypes of the regenerated trees. This could allow us to get a better insight into the specific/redundant roles of these genes.

A transient transformation could be another way of introducing and expressing the *DAL12/13* genes to a specific tissue at a specific time. As most methods tested in this study, except vacuum infiltration, did not negatively affect the viability of transformed tissue, combining several methods or performing them more invasively could be advisable. Designing a working method for transient transformation would be beneficial for investigating the roles of *DAL12/13* and other genes that are active in specific tissue at specific times. This approach could significantly shorten the time it takes to observe phenotypical changes associated with such genes.

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Software:

Geneious Prime® 2023.0.4 (https://www.geneious.com/)

10. Popular science summary

If you decide to pursue a career as a Norway spruce breeder, you will probably be shocked to find out that you will be able to breed 1 or maximally 2 generations of spruce during your entire career. That is the same amount of wheat generations that you can breed per 1/3 of a year. Needless to say, it is not an optimal speed in a world that faces rapid climate change.

However, genetic diversity is a gift that keeps on giving, and already in the 19th century, a scientist discovered a natural variant of spruce called *acrocona*, which has some very interesting traits. One trait that particularly captured the interest of researchers in the past decades is its ability to produce female cones very early, especially if related trees are bred together.

A group of scientists from the Swedish Agricultural University conducted a series of molecular biology experiments on the *acrocona* tree and discovered that one gene, in particular, seems to play a very important role in the female cone-setting process. This gene, called *PaSPL1*, became a hot candidate for breeding efforts and researchers want to thoroughly characterise this gene, but they also want to know how it is affected by other genes and bring some light into the undiscovered molecular networks in spruce.

That is why this thesis is focused on the preparation of an experiment that will reveal if some genes that are known for having regulatory functions during the transcription of DNA to RNA, also regulate the transcription of *PaSPL1* gene. The experiment that will later be conducted to uncover this is called yeast 1-hybrid. It is named that way because two types of yeast, one with a gene that may regulate *PaSPL1* and one with the regulatory region that is positioned on the genome in front of *PaSPL1*, are mated (”hybridised”). If the gene interacts with the regulatory region in the offspring yeast cells, they will change colour thanks to a special reporter gene. This experiment will then tell us where our research and breeding efforts should aim next.

As you may have noticed, there is a small problem with the early cone-setting phenotype - it only results in female cones. But we may just have a solution for this problem as well. two genes, *DAL12* and *DAL13*, were already 25 years ago hypothesised to have essential roles in the formation of male cones. This role can be finally decided by introducing them into the *acrocona* trees. That can possibly be done by utilizing state-of-the-art genetic engineering methods and introducing

these genes permanently in either small clusters of *acrocona* cells (“calli”) that can be later grown into adult trees, or by introducing them transiently into tissues of grown trees or seedlings. This thesis lays the groundwork for these large-scale experiments, offering valuable insights into transformation processes and preparing the *DAL12* and *DAL13* genes for future research.

11. Acknowledgements and Dedication

I want to thank my supervisors Dr. Jens Sundström and Dr. Laxmi Mishra for their guidance and an exemplary supervision.

I also want to thank the members of the lab and of the department for creating friendly, yet very academically stimulating environment.

I can not also forget to thank my boyfriend Adonisek, my family, and friends for their love and support.

This thesis is dedicated to my grandfather, Jan Borský, who loved forest and its trees and whom I miss dearly.

12. Appendix

12.1 Tables

Table 4: **Thermal cycler settings used for different PCR protocols.**

Step	1.	2.	3.	4.	5.	6.	7.	8.	9.
Repeats	1x	10x			25x (30x yeast)			1x	1x
PCR of TF (Phusion)	98°C 30 sec	98°C 10 sec	54-57°C 30 sec	72°C 40 - 120 sec	98°C 10 sec	58-61°C 30 sec	72°C 40 - 120 sec	72°C 5 min	4°C hold
E. coli colony PCR (Taq)	95°C 3 min	95°C 30 sec	54-57°C 30 sec	72°C 60 - 180 sec	95°C 30 sec	58-61°C 30 sec	72°C 60 - 180 sec	72°C 5 min	4°C hold
Yeast colony PCR (Phire)	98°C 5 min	98°C 5 sec	54-57°C 5 sec	72°C 40 - 80 sec	98°C 5 sec	58-61°C 5 sec	72°C 40 - 80 sec	72°C 1 min	4°C hold
Calli genotyping (Phusion)	98°C 30 sec	98°C 10 sec	61°C 30 sec	72°C 30 sec	98°C 10 sec	61°C 30 sec	72°C 30 sec	72°C 5 min	4°C hold
DAL12 and DAL13 partial attB	98°C 30 sec	98°C 10 sec	58-60°C 30 sec	72°C 30 sec	98°C 10 sec	58-60°C 30 sec	72°C 30 sec	72°C 5 min	4°C hold
DAL12 and DAL13 full attB	98°C 30 sec	98°C 10 sec	58-60°C 30 sec	72°C 30 sec	98°C 10 sec	58-60°C 30 sec	72°C 30 sec	72°C 5 min	4°C hold
Step	1.	2.	3.	4.	5.	6.	7.		
Repeats	1x	40x		1x	1x	1x	1x		

DAL12 and DAL13 qPCR	95°C 3 min	95°C 10 sec	60°C 30 sec	65°C 0.05 min	95°C 5 sec	65°C 0.05 min	95°C 10 sec

Table 5: **Primer sequences used in this study**; "-" means that only TOPO primers were used. *IDs from Plantgenie, **IDs from GenBank, ***IDs from European Nucleotide Archive.

ID	Gene name abbreviation used in this study	Gene name used in this study	UTR fwd	UTR rvs	TOPO fwd	TOPO rvs	Expected final size (bp) + 4bp (CACC)	Origin of primers
MA_8147g0020*	*PaR2R3-like-1*	*Picea abies R2R3-MYB-like-1*	AGAGTTGTGCC TGTCGAATCA	TGTGTGCAA ATTAAACAA CACTGC	CACCATGGTCA TCACTAGCCAA	TGTGTGCAAATTA AACAACACTGC	1315	This study
MA_55123g0010*	*PaMYB31-like-1*	*Picea abies MYB31-like-1*	-	-	CACCATGGGTC GCTCTCCTT	CTAATCCCAACAA CCAGAGCAG	742	This study
MA_10208000g0010*	*PaGAMY-like-1*	*Picea abies GIBBERELIC ACID-RESPONSIVE MYB-like-1*	-	-	CACCATGAGTT CAGAGACGAGT	TCAAGGAAGATC GGCGATTTGTG	1813	This study
MA_14452g0010*	*PaTT2-like-1*	*Picea abies TRANSPARENT TESTA 2-like-1*	CCCTGCTCTCT ACCTTGTGC	ATATGCCCT GCAACGCTT CA	CACCATGATAA GCGAGAAGTAT C	ATATGCCCTGCAA CGCTTCA	1351	This study
MA_97319g0010*	*PaAP2 ERF-like-1*	*Picea abies APETALA 2 ETHYLENE RESPONSIVE FACTOR-like-1*	CTTTATATTCC AGCTGTGATAG AT	GCTTCTTGA CATCTCAGC TC	CACCATGACGC TATGTTGCAGC AT	GAGTTAATAAGTA AATAAGTTCAAA GAC	745	This study
MA_2193g0020*	*PaAP2-like-1*	*Picea abies APETALA 2-like-1*	ACAACAAACCC ACGTCCAAC	GCTTCAGAA GAAGGGCAT TTC	CACCATGACTC CGGGACGT	TCACTTCGTCTCT CTTGATAAGC	1846	This study
MA_16120g0010*	*DAL19*	*DEFFICIENT AGAMOUS LIKE 19*	TGGATCTAAAC TTAAGCTACTG GGTG	CCAAGCCCA TAACCGTTT ACCA	CACCATGGTGA CGGGCAAGACG CA	AGAGCAAACACG TACACAACCA	247	This study
AF064080.1**	*DAL10*	*DEFFICIENT AGAMOUS LIKE 10*	-	-	CACCATGGGGC GGGGGAA	TCATTTGATGTTA ACAGAACCATATT CC	813	This study
X80902.1**	*DAL1*	*DEFFICIENT AGAMOUS LIKE 1*	GCTGAAGCAGA AGAGGGATC	GGGTTTGTTT GCCCAATCA ACT	CACCATGGGGC GGGGTCGAG	ATCCTTAAATATC AAACCCACCACCC TTG	797	This study
MA_9455802g0010*	*PaTlFY10A-like-1*	*Picea abies TIFY10A-like-1*	CATGTGTGAGC CATAACCGA	AGCATCCAC CGACATTTT GA	CACCATGTGGT TGAGGGCACGT	TCATGGACAGCTC AACGACAGTATG	844	This study
MA_10433497g0010*	*PaTlFY10B-like-1*	*Picea abies TIFY1B-like-1*	-	-	CACCCTGGAAA TGGATAAGGAT AG	TTAGTTTTGCGGA GGGGGAGC	856	This study
AY701762.1**	*PaNLY*	*Picea abies NEEDLY*	-	-	CACCATGACGG AGATGGG	CTATGGACACTCT TTGCTTCT	1002	This study
AY701763.1**	*PaLFY*	*Picea abies LEAFY*	-	-	CACCATGCAGA GGGGTGGAG	TTATAGATGGGAC TGTTTGCTTTTCTC	1161	This study
MA_41006g0010*	*PaHY5-like-1*	*Picea abies ELONGATED HYPOCOTYL 5-like-1*	-	-	CACCATGCAAG CAATAGCTCC	CTACCCTTCTGCT TCAACAAATCC	507	This study
MA_11267g0020*	*PaLHY-like-1*	*Picea abies LATE ELONGATED HYPOCOTYL-like-1*	-	-	CACCATGGCTT CTTTTCCGGC	TCAGGGCTCGATT TGTGCCATCA	3013	This study

MA_115536g0010*	*PaCCA1*	*Picea abies CIRCADIAN CLOCK ASSOCIATED 1*	-	-	CACCATGAAAATGTCTCTGCCGT	TTACATGGAACAACGCTCGTAC	2200	This study
ID	Gene		-	-	Genotyping fwd	Genotyping rvs	Size	Origin
OU384166.1***	*PaSPL1*	*Picea abies SQUAMOSA-PROMOTER BINDING PROTEIN-like-1*	-	-	CAGAGCTCCAAGGCTTCCTC	GTCCTTGCAGAAAGCCATGC	502	Akhter et al., 2022

***Table 6: DAL12* and *DAL13* primers**. Nucleotides highlighted in blue are the parts of attB overhangs that are are shared between the primers used in the first PCR and the second PCR.

GenBank ID	Gene	PCR gene specific + partial attB1 fwd	PCR gene specific + partial attB2 rvs	PCR full att B1 fwd	PCR full att B2 rvs	qPCR fwd	qPCR rvs	Expected size (bp)
AF158542.1	*DAL12-2*	AAAAAGCAGGCTATGATGAGAATGGGGAGGGG	GAAAGCTGGGTAATCTCCATCCTAGCTTTTC	GGGGACAAGTTTGTACAAAAAAGCAGGCT	GGGGACCACTTTGTACAAGAAAGCTGGGT	GATGGCTGTGTTGTCAAGGG	TCCAAATCTCCTCCCGTCAGA	717
AF158543.1	*DAL13-1*	AAAAAGCAGGCTATGGGGAGGGGAAAGA	GAAAGCTGGGTGCCTTCAGCAGTTAATATCC			ACCAAGCGACAGAACAAGGA	TGTCCGTGATGCTGGTCAAC	661

12.2 CDS sequences based on our sequencing results

>AF064080.1
DAL10 (813 bp)

ATGGGGCGGGGGAAGATAGAGCTGAAGAAGATAGAGAGCACAAGCA
ACAGGCAGGTGACGTTCTCGAAGCGGCGGATGGGGTTGCTGAAAAAG
GCACAGGAGCTTTCCGTCTTATGCGATGCCGAGGTGGGCGTCATCATC
TTCTCTAATACCGGCAGACTCTACGACTTCTCCAGCTCCAGTATGGAG
AAGATGATTGAAACATACTATCGATTTCTTGAAAAAAATAACCATGGC
CAACAAGCGCACCTACAGATACCATCCAATCAAGACCTGGGAAGACT
GATGCAAGAGCTACAGGCAATTGAGAGTATGTACAAAAAGTCAATAG
GCGAGGAGTTGTCTTCCTTATCCATCAGTGACTTGAAGCACCTGGAGC
ACCAGCTCGAAGTAGGAATCAGTCGAATAAGAGGAAGAAAGAGCGAA
CTTGTTGAGGAGCAGATAGCTAATCTTCAAAGCAGGGAGCGTGATTTA
CTAGAAGAGAACAATGTCCTACAACAGCTGCTGTCAGAAGCTCGGAG
CTCCACTGAGATGGTAGCATCAAGGTTTGTAAACAATAATCTTGCAGC
TAATATACTGCCTGCACCCATAACGTCTACCAACCCCTATCAACAAAA
CTTTGGGCAGACATCACAAGTTTGTATAGGAAACTCTTCGTCATCCAA
AAATATCGAACAGAGCTCGTCAGGGCCACAGTCAACCGCCAAGGAGC
GTCCTTGTCCAGATCAGCATAGTTACCCAACTCAATCCATCAATCTACT

ACTAGATGGATGGGATATAATGGAGGAATATGGTTCTGTTAACATCAA
ATAA

>MA_9455802g0010
PaTIFY10A-like-1 (812 bp)

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>MA_41006g0010
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> AF158542.1
DAL12-2 (717 bp)

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12.3 Calculations of betalain concentration

Table 7: Complete list of collected data and calculations used for graphs in section 5.4.

Sample	Number	Method	Weight	A	uM	c (mg/ml)	c(mg/ml) till 1ml	Mass ratio (mg/kg)
35S	1	Wounding	220	0.053	0.883333	0.000486		2.208333333
35S	2	Wounding	230	0.077	1.283333	0.000706		3.06884058
35S	3	Wounding	230	0.087	1.45	0.000798		3.467391304
UBQ	1	Wounding	220	0.03	0.5	0.000275		1.25
UBQ	3	Wounding	270	0.08	1.333333	0.000733		2.716049383
Control	2	Wounding	300	0.058	0.966667	0.000532		1.772222222
Control	3	Wounding	140	0.029	0.483333	0.000266		1.898809524
35S	1	IS	270	0.008	0.133333	7.33E-05	0.00011	0.407407407
35S	2	IS	320	0.021	0.35	0.000193	0.00028875	0.90234375
35S	3	IS	340	0.02	0.333333	0.000183	0.000275	0.808823529
UBQ	1	IS	300	0.007	0.116667	6.42E-05	0.00009625	0.320833333
UBQ	*2	IS	280	0.011	0.183333	0.000101	0.00015125	0.540178571
UBQ	3	IS	390	0.033	0.55	0.000303	0.00045375	1.163461538
Control	1	IS	270	0.014	0.233333	0.000128	0.0001925	0.712962963
Control	2	IS	380	0.026	0.433333	0.000238	0.0003575	0.940789474
Control	3	IS	260	0.01	0.166667	9.17E-05	0.0001375	0.528846154
35S	1	SI	230	0.006	0.1	0.000055	0.0000825	0.358695652
35S	2	SI	290	0.015	0.25	0.000138	0.00020625	0.711206897
35S	3	SI	290	0.038	0.633333	0.000348	0.0005225	1.801724138
UBQ	*2	SI	200	0.006	0.1	0.000055	0.0000825	0.4125
UBQ	1	SI	220	0.016	0.266667	0.000147	0.00022	1
UBQ	3	SI	150	0.008	0.133333	7.33E-05	0.00011	0.733333333
Control	1	SI	350	0.04	0.666667	0.000367	0.00055	1.571428571
Control	2	SI	370	0.031	0.516667	0.000284	0.00042625	1.152027027
Control	3	SI	370	0.034	0.566667	0.000312	0.0004675	1.263513514
35S	1	Vacuum	430	0.049	0.816667	0.000449	0.00067375	1.566860465
35S	2	Vacuum	270	0.04	0.666667	0.000367	0.00055	2.037037037
UBQ	1	Vacuum	200	0.018	0.3	0.000165	0.0002475	1.2375
Control	1	Vacuum	350	0.027	0.45	0.000248	0.00037125	1.060714286

Control	3	Vacuum	180	0.016	0.266667	0.000147	0.00022	1.222222222
								1.294535207
	*Samples UBQ 2 may have been mixed but that does not affect the final result							

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