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EXZELLENT.

Ectomycorrhiza Development:
Investigation of Selected Ectomycorrhiza
Induced Poplar Genes

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“However difficult life may seem, there is always something you can do, and succeed at. It matters that you don’t just give up”

- Stephen Hawking (1942-2018)

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For my beloved father and in memory of my mother

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Zusammenfassung

Mutualistische Interaktionen wie Ektomykorrhiza (ECM) sind wichtig für die Funktion von Waldökosystemen. Hier bilden zwei Königreiche, Pflanzen und Pilze, eine Symbiose und tauschen Nährstoffe und Kohlenhydrate aus. Ein Engpass in der Ektomykorrhiza-Forschung ist der Zeitbedarf für die transgene Pflanzengeneration. Eine alternative Strategie ist die Bildung sogenannter Kompositpflanzen, bei denen transgene Wurzeln aus nicht-transgenen Sprossen gebildet werden. Ein *Agrobacterium rhizogenes*-vermitteltes Wurzeltransformationsprotokoll wurde in dieser Arbeit mit axenischen *Populus tremula* × *tremuloides* und *P. tremula* × *alba* Stecklingen entwickelt. Von den vier verschiedenen *A. rhizogenes*-Stämmen wurde K599 als am besten geeignet befunden. Wurzeln von zusammengesetzten Pappeln waren in der Lage, Ektomykorrhiza zu bilden, wenn sie mit *Amanita muscaria* inokuliert wurden.

Mit Hilfe der quantitativen Echtzeit-PCR wurde eine vergleichende Analyse der Transkriptions-Level für ausgewählte Gene in mykorrhizierten und nicht-mykorrhizierten Pappel-Feinwurzeln durchgeführt. Insgesamt wurden 50 Ektomykorrhiza-induzierte Gene auf Basis einer genomweiten Microarray-Analyse ausgewählt (Nehls, unveröffentlicht). Da das Array-Oligomer-Design auf dem *Populus trichocarpa*-Genom basiert, die Hybridisierung aber mit *P. tremula* × *tremuloides* cDNA durchgeführt wurde, ist eine Kreuzhybridisierung möglich, die zu Fehlinterpretationen führen könnte. Als erster Schritt wurden *P. tremula*/*P. tremuloides* Datensätze auf die besten Homologe untersucht. Nach dem Primer-Design und ersten qRT-PCR-Tests blieben 14 Kandidatengene übrig. Schließlich wurde eine Expressionsanalyse mit mehreren unabhängigen Chargen von Pappel-Feinwurzeln und Mykorrhizen für sechs Gene durchgeführt.

Zwei Gene, ein Transkriptionsfaktor (TF- Potri.008G071100) der AP2/ERF-Superfamilie und eine potentielle Glykosyltransferase (GT- Potri.007G095000) wiesen die höchste Genexpressionsdifferenz auf und wurden für die weitere Analyse ausgewählt. Eine Fusion mit super yellow fluorescent Protein zeigte eine wahrscheinliche subzelluläre Lokalisation im Kern und Zytoplasma für Potri.007G095000 und den Kern für Potri.008G071100. Nach ihrer Etablierung wurden Kompositpappeln für die Promotoranalyse der ausgewählten Gene verwendet. Dazu wurden etwa 3 kb Promotorfragmente aus genomischer DNA von *P. tremula* × *tremuloides* amplifiziert,

sukzessiv vom 5'-Ende gekürzt und die resultierenden Fragmente vor der kodierenden cDNA eines peroxisomal lokalisierten yellow fluorescent Protein geklont. Die längsten Promotor-Reporter-Konstruktionen wurden für die Erzeugung von Kompositpappeln verwendet. Für den TF und die GT wurde keine zuverlässige ECM-induzierte Expression gefunden, was auf die Notwendigkeit eines längeren Promotorfragments hinweist. Da das beste Arabidopsis-TF-Homolog bekanntermaßen selbstregulierend ist, wurde die Identifizierung potenzieller *cis*-Elemente in der Promotorregion geplant. Daher wurde die Proteinüberexpression in *E. coli* eingeleitet. Alle Versuche, ein nativ gefaltetes Protein in löslicher Form zu erhalten, waren jedoch erfolglos.

Summary

Mutualistic interaction such as ectomycorrhiza (ECM) is important for forest ecosystem function. Here two kingdoms, plant and fungi, form a symbiosis and exchanges nutrients and carbohydrates. A bottle neck in ectomycorrhizal research is the time demand for transgenic plant generation. Formation of so-called composite plants, where transgenic roots are formed on non-transgenic shoots, is an alternative strategy. An *Agrobacterium rhizogenes*-mediated root transformation protocol was developed in this work using axenic *Populus tremula* × *tremuloides* and *P. tremula* × *alba* cuttings. Out of four different *A. rhizogenes* strains, K599 was found to be the most suitable one. Roots of composite poplars were able to form ectomycorrhiza when inoculated with *Amanita muscaria*.

By using real time quantitative PCR a comparative analysis of transcript levels was done for selected genes in mycorrhized and non-mycorrhized poplar fine roots. A total of 50 ectomycorrhiza-induced genes were chosen based on a genome wide microarray analysis (Nehls, unpublished). As the array oligomers were designed based on *Populus trichocarpa* genome but the array hybridization was performed using *P. tremula* × *tremuloides* cDNA, cross hybridization leading to misinterpretation is feasible. Therefore, the first step was to screen *P. tremula*/*P. tremuloides* datasets for the best matching homologs. After primer design and first qRT-PCR tests 14 candidate genes remained. Finally, expression analysis with several independent batches of poplar fine roots and mycorrhizas were obtained for six genes.

Two genes, a transcription factor (TF- *Potri.008G071100*) belonging to the AP2/ERF superfamily and a potential glycosyltransferase (GT- *Potri.007G095000*) revealing the highest gene expression difference, were selected for further analysis. Fusion with super yellow fluorescent protein revealed a probable subcellular localization of *Potri.007G095000* in nucleus and cytoplasm and *Potri.008G071100* in nucleus. Following their establishment, composite poplars were used for promoter analysis of the selected genes. For this purpose, around 3 kb promoter fragments were amplified from genomic DNA of *P. tremula* × *tremuloides*, successively shortened from the 5' end and the resulting fragments were cloned in front of the coding sequence of a peroxisomal located yellow fluorescent protein. The longest promoter-reporter constructs were used for generation of composite poplars. No reliable ECM induced expression was found for

the TF and the GT indicating the need for longer promoter fragments. Furthermore, as the best *Arabidopsis* TF homolog is known to be auto-regulated, the identification of potential *cis*-elements in the promoter region was planned. Therefore, protein overexpression in *E. coli* was initiated. However, all attempts of receiving a natively folded protein in soluble form were unsuccessful.

1. Introduction

1.1 Mycorrhiza: union of two kingdoms

In 1885 pathologist A.B. Frank first coined the term mycorrhizae, which means fungal root (from the Greek 'mycos', meaning fungus and 'rhiza', meaning root). He described the relationship as the union of two different organisms, plant and fungus forming a single morphological organ where they nourishes each other by exchanging carbohydrate for inorganic nutrients and water from soil (Rakovan 2012). Mycorrhizal structures can be found on every continent around the world across different climate zones and are known to be traced back as far as 460 million years in time (Sadava 2008).

This unique relationship is not only beneficial for the partners themselves but also has high functional significance in numerous ecosystems (Peter et al. 2001). The association not only increases nutrient and water supply for plants but also provides some protection from parasitic fungi and nematodes (Quoreshi et al., 2003). Mycorrhizal fungi provide a critical linkage between roots and soil resources and have the capacity to utilize organic N and breakdown phenolic compounds (Smith and Read 1997). They are also known to play an important role in carbon sequestration into forest soil (Quoreshi et al., 2003). Plants provide carbohydrates to roots and mycorrhizae, as a result mycorrhizae acts as a carbon sink and play a crucial role in below ground carbon allocation (Ryziewicz and Andersen 1994). An essential factor for forest ecosystem prosperity in regions with nitrogen poor soil is believed to be the plant-fungal symbiosis (Smith and Read 2008).

1.1.1 Types of Mycorrhiza: in and out

Depending on whether the fungal hyphae penetrate the primary/secondary plant cell wall, mycorrhiza is mainly divided into two groups, endomycorrhiza and ectomycorrhiza. However, endomycorrhiza can be subdivided into ectoendomycorrhiza, arbutoid, monotropoid, ericoid or orchid mycorrhiza (Quoreshi et al. 2003).

Arbuscular mycorrhiza is thought to be the most ancient and widespread type of symbiosis that is formed by 70-90 % of terrestrial plants (Parniske 2008). The fungal hyphae penetrate the cell wall of individual root cells and grow straight through the cells forming a tree like (arbuscular) structures within the cells, but always remains outside

the plasma membrane of the infected plant cell (Smith and Read 2008). These structures act as the primary nutrient exchange site between the symbionts (Sadava 2008).

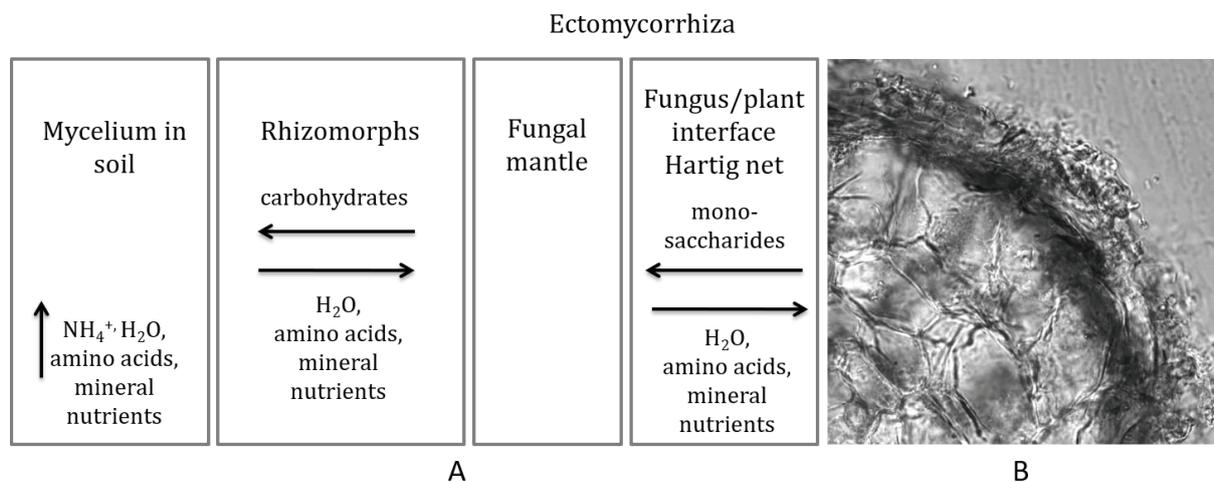


Figure 1: Schematic diagram of an ectomycorrhizal fungal colony without the fruiting bodies (adapted from Nehls 2008).

Soil living fungal hyphae (A) are responsible for water and nutrients uptake for fungal nutrition, which are often distributed in the fungal rhizomorphs. Ectomycorrhiza (B) is composed of a fungal mantle and a hyphal network that grows within the apoplast of root cortex, known as Hartig net, where exchange of nutrient and metabolite between the partners occur.

Ectomycorrhiza on the other hand, is predominant in trees of boreal and many temperate forests (Parniske 2008). In contrast to arbuscular mycorrhiza, the ECM fungus grows outside and between the root cells, where the fungal and plant cell walls are in direct contact and form the interface between them (Balestrini and Bonfante 2014). The fungi covers the root and establishes the so called Hartig net by growing exclusively within the middle lamella and wrapping around individual root cell walls (Nylund 1980). The Hartig net provides a large surface area of contact between the partners and allows efficient metabolites transfer (Marino 2008; Nehls 2008). An extensive web of fungal hyphae extends from the infected root into the rhizosphere and takes up around 25 % of the soil volume near the root (Sadava 2008). The mycorrhiza thus acts as a sponge which not only increases the surface area for nutrient absorption but also holds water efficiently, which enhances the host plants drought tolerance (Selosse et al. 2006; Sadava 2008). The fungal mantle acts as an intermediate storage for a) nutrients delivered by soil-growing hyphae originally intended for Hartig net and b) carbohydrates taken up by hyphae in the Hartig net, designated to be transported towards the soil growing mycelium (Jordy et al. 1998; Nehls 2008). It also protects the

root physically from pathogens (Schechter and Bruns 2012) and heavy metals (Colpaert et al. 2011).

1.1.2 Symbiotic root system signals in ectomycorrhiza formation: I halt, you go there

Ectomycorrhizal fungal mycelia are known to comprise up to 30 % of microbial biomass and 80 % of the total fungal biomass in boreal forest soils (Högberg and Högberg 2002; Wallander 2006). They are thus supposed to be key elements of forest ecosystem processes such as nutrient cycling and carbon entry (Read et al. 2004; Anderson and Cairney 2007). Although ECM mycelial biomass differs with soil depth and host tree species, it correlates with the distribution of plant roots surrounded by the respective soil profile (Wallander et al. 2004; Nehls 2008). Therefore, the entire forest can be considered as one biological entity, below ground connected by a 'wood-wide web' of ectomycorrhizal fungi – a term that was coined by Simard et al. (Simard et al. 1997).

Colony formation

The general strategies of ECM fungal root colonization involve generative spore germination. If compatible plant partners are recognized, hyphal growth of soil-growing hyphae is directed towards the emerging fine roots (Martin et al. 2001), binds to the root surface, penetration of the root cell apoplast and branching and ingress of hyphae occur (Barker and Tagu 2000). Similar to the chemical cross talk between plant and bacteria in nodule symbiosis, host and fungal partner in an ECM symbiosis exchange several rhizospheric signals such as phytohormones or phenol compounds. Molecular communication is followed by morphological changes both in fungal hyphae and roots (Barker and Tagu 2000; Wiemken and Boller 2002). Young root tips are accessible for fungal infection, which indicates the ability of fungus to penetrate only certain roots probably depending on the physiologic or structural state (pectin-cellulose ratio) (Duddridge and Read 1984) and gradient of unknown morphogens (phytohormones like auxin and cytokinin), altering plant morphology and defence mechanism (Barker and Tagu 2000).

After the initial colonization, the fungus secretes cell wall degrading enzymes in order to allow its way into the rhizodermal tissue (Smith and Read 2008) and root hairs usually formed by root rhizodermal cells, are suppressed by ectomycorrhiza formation (Nehls

2008). In order to maximize the substrate exchange, the intimate symbiosis is expected to have regulatory effects on both organisms profoundly at molecular level, such as altering the metabolism, enzyme activities and essential gene expression such as cell growth, membrane formation etc. (Nehls et al. 2001; Wiemken and Boller 2002). In this thesis, ectomycorrhizal interaction between *Populus sp.* and the Basidiomycotic fungus *Amanita muscaria* was studied.

1.2 Model organisms in ectomycorrhizal research: together we are strong- the ally

Forests cover 30 % of earth's terrestrial surface harbouring substantial biodiversity and providing humanity enormous benefits with clean air, water, timber, fibre and fuels (Tuskan et al. 2006). Plant's ability to form symbiotic relationship with different soil fungi also allows them to absorb water and nutrients more efficiently in drought or nutrient limited conditions (Smith and Read 2008).

1.2.1 *Populus* species: an ECM plant model

Poplar is a member of the clade 'Eurosoid I' in the updated Angiosperm Phylogeny Group (APG) system (APG, 2003) and is also a member of the family Salicaceae in the order Malpighiales. *Arabidopsis thaliana*, the widely used model organism in plant science, by contrast is a member of the clade 'Eurosoid II' and is placed in family Brassicaceae in the order Brassicales (Cronk 2005). *Populus sp.* has been successfully used as a model plant for functional analysis of *Arabidopsis* genes (Jansson and Douglas 2007). Although *Arabidopsis* with its small physical size, rapid generation time, straight forward genetics and small genome is an obvious model species, it lacks other different reproductive strategies or other essential traits such as wood formation and seasonality of growth (Jansson and Douglas 2007). Furthermore, beneficial association with mycorrhizal organisms are also not found for *Arabidopsis*. In contrast *Populus* is a host for ectomycorrhizal and arbuscular mycorrhizal fungi (Jansson and Douglas 2007). With the available genome sequence of *Populus* and ectomycorrhizal fungi such as *Laccaria bicolor* or *A. muscaria* interesting investigations of the molecular, physiological and environmental basis for such interactions are possible (Lammers et al. 2004).

Populus trichocarpa known as black cottonwood or western balsam poplar, native to western North America is the first tree species to be fully sequenced. This marks a

milestone in eco-devo as *P. trichocarpa* is the first ecologically important plant species having numerous congeners around the northern hemisphere with significantly different morphological and ecological characteristics (Brunner et al. 2004). The poplar genome consists of some 45,555 genes spread over 19 chromosomes ($2n = 38$) comprising 500 million base pairs (Tuskan et al. 2006). The poplar genome sequence is an approximately 6 × depth adding to a long list of important research attributes such as facile transformation, vegetative propagation, rapid growth, modest genome size and extensive expressed sequence tags (Brunner et al. 2004).

The ability of poplar species to form ectomycorrhiza in a closed petri dish system (Hampp et al. 1996) and formation of transgenes (Fillatti et al. 1987) is of high importance in the ectomycorrhizal research. However, it is difficult to cultivate and form stable transformation with *P. trichocarpa* under sterile laboratory conditions (Neb, 2017).

Populus tremula is a widely distributed species in northern Europe, often growing in mixed stands with pine, spruce and birch. The hybrid poplars of European aspen *P. tremula* L. with North American trembling aspen ***P. tremuloides*** Michx. and white poplar *P. alba* L. produced by artificial hybridization have shown superior growth characteristics. Such interspecific hybrids grow faster than the progenies of intraspecific crosses (Heimbürger 1936; Einspahr and Benson 1964; Melchior and Seitz 1966; Heimbürger 1968; Zsuffa 1975; Yu et al. 2001). The genetic diversity in aspen can be manipulated through selective breeding, interspecific hybridization and cloning (Li 1995). Due to the properties of its wood quality hybrid species are favourable for paper industry. Hybrid aspen, along with many other *Populus* species is also competent both for micro-propagation and genetic transformation (Klopfenstein et al. 1997; Häggman et al. 2003). Hybrid poplar plants ***Populus tremula x tremuloides*** and ***Populus tremula x alba*** being relatively easier to handle under laboratory sterile conditions are used in this present thesis.

1.2.2 *Amanita muscaria*: an ECM fungal model

Amanita muscaria is commonly known as fly agaric belonging to the family Amanitaceae of the phylum Basidiomycota (Nehls et al. 2007). It is native to the temperate and boreal zone and widely spread in the coniferous forests in the north of America, Asia and

Europe. The round white stem of the fruiting body has a length of approximately 8-20 cm and a diameter of about 1.5-3 cm standing on the tuberous base. The characteristic red cap is covered with small white to yellowish pyramid shaped warts that can grow up to a diameter of 15 cm. Mushroom spore dispersal is known to be a two phase process – active ejection of spores from the gill surface by surface tension catapults, followed by passive dispersal by wind and animals (Dressaire et al. 2016). After settling on appropriate substrate, hyphae grow from the spore and form a highly branched mycelium, representing the base for new fruiting body. *Amanita* is known to naturally build mycorrhiza with a variety of trees such as spruces, birches or eucalyptus. Under laboratory conditions, *Amanita muscaria* is able to form mycorrhiza with a greater host spectrum and is known to be relatively easy to handle, thus it has been established to be a good model organism for mycorrhizal symbiosis study (Klothe 2009). The recent genome project of *A. muscaria* even strengthens the relevance of using it as a model organism for research (Kohler et al. 2015).

1.3 Plant transformation: I change you briefly or forever

Transformation was first discovered by F. Griffith in the late 1920s, when he reported the successful conversion of pneumococcal cells from a harmless form to disease-causing type (Griffith 1928; McCarty and Avery 1946). The term ‘transformation’ describes the insertion of foreign molecules into bacteria, fungi or plant cells. DNA being a highly charged macromolecule, difficult to manipulate and not being able to diffuse through the protecting hydrophobic cell membrane, remained a major problem (Rivera et al. 2012). Over the years, continuous research and molecular advancement brought solution to gene manipulation.

1.3.1 History, advancement and importance of plant transformation

With the achievement of producing the first recombinant DNA at the beginning of 1970s (Meselson and Yuan 1968; Smith and Welcox 1970) making DNA manipulation easier, plant transformation was first developed for tobacco in 1984 (De Block et al. 1984; Horsch et al. 1984). With the help of *Agrobacterium tumefaciens*, the causative agents of crown gall disease was first demonstrated to be harnessed by researchers in order to introduce defined DNA fragments into plant cells (Newell 2000). Since then we have emerged from the period of plant research dominated by the necessity to develop

genetic transformation methods into the era of transformation application as an essential tool in plant biology and cultivar improvement (Birch 1997). In 1994 the Food and Drug Administration (FDA) approved tomato as the first transgenic crop for human consumption in USA (Herrera-Estrella et al. 2004; Schlegel 2007). Meanwhile, the success of introducing foreign gene into plant has been extended to over 120 plant species belonging at least to 35 different families including most major economic crops, vegetables, fruit, medicinal and pasture plants (Birch 1997).

Advancements in the production of novel recombinant proteins (Van Der Krol et al. 1988) in transformed plants opened a new horizon for the pharmaceutical industries (Canter et al. 2005; Meyers et al. 2010) due to its lower production costs, rapid scalability, absence of human pathogens and capability to fold and assemble complex proteins with high precision. Furthermore, the ability to introduce and express (or inactivate) specific genes in plants allows direct testing of hypotheses in plant physiology that have been difficult to resolve using other biochemical approaches (Coruzzi and Puigdomènech 2013). Therefore, molecular genetic analysis of cellular events controlling sexual reproduction and plant-microbe interaction (Newbigin et al. 1995; Staskawicz et al. 1995), became feasible.

1.3.2 Transient and stable plant transformation

Transfer of foreign DNA into plant cells can lead to transient expression, which lasts only for few days, but it is flexible and gene expression is not afflicted by positional effects (Fischer et al. 1999). Therefore this approach is a useful niche in areas such as development of metabolic studies (Van Montagu 1998). Stable plant transformation on the other hand is often a time consuming process that involves tissue culture techniques to regenerate plants from treated cells or tissue explants (Hansen and Wright 1999). As a result, stable transformation allows the integration of the introduced DNA into the host cell genome and therefore it can be passed on to the succeeding generations (Newell 2000).

1.3.3 Transformation methods – I alter you sincerely

The essential requirements to produce transgenic plants are a) an efficient method for DNA introduction into cells/tissues b) a screening procedure to select transgenic plants and c) efficient plant regeneration (Birch 1997).

Commonly either indirect or direct transformation techniques are used (Rao et al. 2009). Vacuum infiltration (Bechtold et al. 1993; Bechtold and Pelletier 1998; Tjokrokusumo et al. 2000; Charity et al. 2002; Gao and Peng 2004; Acereto-Escoffié et al. 2005; Joh et al. 2005; Canche-Moo et al. 2006; Tague and Mantis 2006; Subramanyam et al. 2011), bombardment with DNA coated microprojectiles (Klein et al. 1987; Hartman et al. 1994; Rajasekaran et al. 1996; Christou and Pelica 2000; McCabe et al. 2000; SL et al. 2005; Ceasar and Ignacimuthu 2009) and electroporation or PEG treatment of protoplast (Krens et al. 1982; Neumann et al. 1982; Fromm et al. 1985; De la Pena et al. 1987; Toriyama et al. 1988; Zhang and Wu 1988; Georges et al. 1990; Spörlein and Koop 1991; Vasil 2005) are the most popular strategies of direct transformation (Birch 1997; Newell 2000).

Indirect transformation uses the capability of bacteria to transfer genes into higher plants (Broothaerts et al. 2005). Most commonly used microorganisms are two soil born bacteria *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Chilton et al. 1977; Ow et al. 1986; Zupan et al. 1997; Patnaik and Khurana 2001; Broothaerts et al. 2005; Christensen and Müller 2009; Dhar et al. 2011; Manimaran et al. 2011). Since the first gene introduction using *Agrobacterium* (Herrera-Estrella et al. 1983; Bevan 1984), this strategy has further been optimized by plasmid development (Rivera et al. 2012), and strain optimization (Nadolska-Orczyk et al. 2000; Gelvin 2009).

1.3.4 Selection and visualization strategies for stable transformation

After the introduction and integration of exogenous DNA into the plant genome, it is important to select the transformed cells expressing the integrated foreign DNA (Newell 2000). For transformation systems generating substantial numbers of non-chimeric primary transformants, useful strategies are a) genes conferring resistance to selective chemical agents (Wilmsink and Dons 1993) and b) genes with specific phenotype allowing visual or physical screening (Bowen 1993; Bower et al. 1996). Later identification of the transformed plants is also possible with desired genes by PCR (Christou et al. 1992; Kim and Minamikawa 1996). The problems of selection strategies are cross-protection of untransformed tissue by transformed tissue, hence generating non-uniform hybrid plants (Park et al. 1998). Scorable markers or reporter genes (e.g. codes for enzymes having activities on substrates which are not usually present in the plant host) have been utilized as indicators of successful transformation since the early

days of plant transformation (Newell 2000). Selectable marker genes can be divided into several categories depending on their positive or negative and conditional or non-conditional selection, approximately fifty such selectable marker genes are known (Miki and McHugh 2004).

Today, the use of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* as reporter gene allows sophisticated visualization of transgenic cells without the need of any substrate supply to the plant (Sheen et al. 1995).

1.4 Agrobacterium: the natural plant genetic engineer

1.4.1 Agrobacterium genus and host range

The genus *Agrobacterium* is gram-negative bacteria mostly known to be plant pathogen that induces atypical growth of plant tissues due to unique inter-kingdom gene transfer (Chilton et al. 1977). The transferred genes cause uncontrolled proliferation of plant cells synthesising nutritive compounds that are metabolized by the bacteria (Escobar and Dandekar 2003). The genus *Agrobacterium* is divided into a number of species based on their disease symptomology and host range. *A. radiobacter* does not induce any plant disease (Hildebrand 1940; Conn 1942), *A. tumefaciens*, *A. rhizogenes* and *A. rubi* cause crown gall, hairy root and cane gall diseases respectively (Chandra 2012). *Agrobacterium vitis* is known to cause galls on grape and other few plant species (Ophel and Kerr 1990; Miranda et al. 1992). With the discovery of plasmids (pTi) conferring the 'oncongenicity' to an *Agrobacterium* strain, the basis of pathogenicity and application to a plant transformation tool, became visible (Zaenen et al. 1974; Drummond et al. 1977).

Depending on the type of tumorigenic plasmid, a specific disease property develops. For example, plant infection with *A. tumefaciens* C58 containing the nopaline-type Ti - plasmid causes crown gall teratomas. Curing this plasmid results in a non-pathogenic strain, while introduction of a Ri (root inducing) plasmid results in a rhizogenic growth (White and Nester 1980; Lam et al. 1984). *Agrobacteria* are able to transfer DNA to a remarkably broad range of plant groups, which includes dicot and monocot angiosperms (De Cleene and De Ley 1976; Anderson and Moore 1979; Porter and Flores 1991; van Wordragen and Dons 1992) and gymnosperms (Loopstra et al. 1990; Morris and Morris 1990; Stomp et al. 1990; McAfee et al. 1993; Yibrah et al. 1996; Levee et al. 1999; Wenck et al. 1999). The host range is however not limited to plants, as Ascomycotic and

Basidiomycotic fungi (Bundock et al. 1995; Bundock and Hooykaas 1996; Piers et al. 1996) (De Groot et al. 1998; Abuodeh et al. 2000) and even human cells can be transformed (Kunik et al. 2001).

1.4.2 Transformation and integration of T-DNA from *Agrobacterium* to plant genome

1.4.2.1 Recognition of suitable plant host

Activity of *Agrobacterium* virulence (*vir*) proteins is essential for the generation of single stranded T-strand molecules (ssT-strand) and its subsequent transport to the plant cells. VirA and VirG proteins function as members of a two-component sensory-signal transduction genetic regulatory system. Along with the monosaccharide transporter ChvE and in the presence of relevant phenolic and sugar molecules VirA, a periplasmic antenna that senses the presence of plant phenolic compounds induced upon wounding (Stachel and Zambryski 1986; Albright et al. 1989; Winans 1991; Jin et al. 1993; Turk et al. 1994; Lee et al. 1995; Doty et al. 1996), autophosphorylates and subsequently transphosphorylates the VirG protein (Jin et al. 1990; Jin et al. 1990), which upon phosphorylation becomes active and helps in the activation or increase of the level of *vir* genes transcription, most probably by interacting with *vir*-box sequences, a component of *vir* gene promoters (Das et al. 1986; Das and Pazour 1989; Pazour and Das 1990)

1.4.2.2 Attachment of *Agrobacteria* to host cells

DNA and protein transfer from *A. tumefaciens* to plant cell strongly suggests an intimate association between pathogen bacteria and the host plant cells. Quantitative estimation revealed two types of *Agrobacterium*-plant cells interaction: a) a non-specific, non-saturable, aggregation-like interaction, which can readily be removed by buffered salt solution wash and b) a specific, saturable interaction (200–1000 bacteria per plant cell), which is impervious to such washing (Neff and Binns 1985; Gurlitz et al. 1987). The specific attachment of *A. tumefaciens* to host cells is independent of the Ti-plasmid (Douglas et al. 1982; Neff and Binns 1985), which rules out the T-pilus as a causal agent for a stable attachment. Three chromosomally encoded bacterial genes, *chvA*, *chvB*, and *pscA* (*exoC*) involved in the synthesis and/or localization of periplasmic β 1-2 glucan are necessary for attachment. Their role in the process is however not well understood (Douglas et al. 1985; Cangelosi et al. 1987; Thomashow et al. 1987; Zorreguieta et al.

1988; Cangelosi et al. 1989). Furthermore, a series of genes were identified as attachment deficient (*att* genes) in the genome (Matthysse 1987; Matthysse et al. 2000). Their role in the attachment process however been questioned due to their location on a 542 kb plasmid pAtC58 (Goodner et al. 2001; Wood et al. 2001), not required for virulence (Hynes et al. 1985; Nair et al. 2003). *Arabidopsis thaliana* mutants, recalcitrant to *Agrobacterium* transformation (*rat* mutants) were isolated (Zhu et al. 2003), where *Agrobacterium* can no longer bind efficiently to some of these mutants probably due to surface alteration in these plants (Nam et al. 1999). One of the best characterized such mutant is with an insertion in the promoter region of an arabinogalactan protein, likely to be found in the cell wall which probably results bacteria to bind poorly to the root cells (Nam et al. 1999; Zhu et al. 2003).

1.4.2.3 T-DNA: Key to the *Agrobacterium*- mediated transformation

The essential molecular basis of *Agrobacterium*- mediated genetic transformation is the T-DNA region of tumour-inducing (Ti) or rhizogenic (Ri) plasmids (Gelvin 2003). While Ti plasmids are known to be between 200 kbp to 800 kbp in size (De Vos et al. 1981; Hood et al. 1984; Jouanin 1984; Knauf et al. 1984; Komari et al. 1986; Gérard et al. 1992; Fortin et al. 1993; Otten et al. 1996; Goodner et al. 2001), the piece of transferred DNA is only approximately of 10-30 kbp in size (Lemmers et al. 1980; Zambryski et al. 1980; Barker et al. 1983; Byrne et al. 1983; Suzuki et al. 2000) representing less than 10 % of the Ti plasmid. Ti-regions can be present in single or multiple copies on such transfer plasmids (Barker et al. 1983; Suzuki et al. 2000), for example nopaline Ti plasmid, mannopine and cucumopine Ri plasmid contain only a single T-DNA, whereas in octopine (pTi) and agropine (pRi) types two regions (TL-DNA and TR-DNA) have been identified (Trovato and Linhares 1999).

A 15 kb of non-transferred DNA separates two T-DNA; the right T-DNA (TR) contains genes homologous to the T-DNA from Ti plasmids (Huffman et al. 1984; Jouanin 1984). T-DNA processing from the large Ti plasmid and its subsequent export from *Agrobacterium* to the plant cell is controlled by the virulence (*vir*) genes carried by the Ti plasmid (Garfinkel and Nester 1980; Klee et al. 1983; Hooykaas et al. 1984; Lundquist et al. 1984; Horsch et al. 1986; Stachel and Zambryski 1986). Upon integration into the plant cell genome the T-DNA expresses enzymes that direct production of opines, synthesised and excreted by the transformed cells and consumed by *Agrobacterium* as a

nutrient source (Dessaux et al. 1992). In addition, T-DNA also encodes several other enzymes responsible for auxin biosynthesis (Inzé et al. 1984) and isopentyl transferase that converts AMP to cytokinin isopentyl adenosine (Akiyoshi et al. 1984).

T-regions are defined by 25 bp long T-DNA border sequences that are highly homologous (Yadav et al. 1982; Jouanin et al. 1989) and flank the T-region in a directly repeated orientation (Peralta and Ream 1985; Rubin 1986) (Wang et al. 1984; Van Haaren et al. 1988; Veluthambi et al. 1988). Generally, the T-DNA borders delimit the T-DNA as these are the sequences specific for endonuclease VirD1/2 targets for processing of T-DNA from the Ti-plasmid. Polarity among the T-DNA borders is well established, right borders initially appeared to be more important compared to the left one (Wang et al. 1984; Hepburn and White 1985; Jen and Chilton 1986; Wang et al. 1987; Sen et al. 1989). Border sequences not only serve as the target for VirD1/2 endonucleases but also as the attachment site for VirD2 protein that nicks the border (Vogel and Das 1992) resulting in the single stranded T-DNA, also known as T-strand (Herrera-Estrella et al. 1988; Ward and Barnes 1988; Young and Nester 1988; Dürrenberger et al. 1989; Howard et al. 1989; Filichkin and Gelvin 1993), which is then transferred to the plant cell (Tinland et al. 1994; Yusibov et al. 1994). Therefore, attachment of VirD2 protein to the right border establishes the polarity and importance of right borders.

1.4.2.4 T-DNA and virulence protein transfer into plant cell nucleus

Together with the VirD4 protein, 11 VirB proteins, which either form the membrane channel or serve as ATPases providing energy for channel assembly and export processes, make up a type IV secretion system required for transfer of the T-DNA and various other Vir proteins, such as VirE2 and VirF (Christie 1997; Vergunst et al. 2000). VirD4 serves as a linker promoting the interaction between the processed T-DNA/VirD2 complex and the VirB-encoded secretion apparatus (Hamilton et al. 2000), other proteins (VirB2, VirB5, and possibly VirB7) form the T-pilus (Jones et al. 1996; Lai and Kado 1998; Eisenbrandt et al. 1999; Schmidt-Eisenlohr et al. 1999; Lai and Kado 2000; Sagulenko et al. 2001) and VirB2 is known as the major pilin protein (Jones et al. 1996; Lai and Kado 1998; Eisenbrandt et al. 1999; Lai and Kado 2000). The VirD2 and VirE2 proteins play crucial and perhaps complementary roles in *Agrobacterium*-mediated transformation by constituting with the T-strand (Howard and Citovsky 1990). Function of the pilus in T-DNA transfer is not well understood, it may serve as a conduit for T-

DNA-Vir protein transfer or act as a hook to bring the bacterium in close proximity to the host cell helping the molecular transfer (Gelvin 2003). Induction of *vir* genes is reported to be maximal approximately at 25 to 27°C (Turk et al. 1991; Jin et al. 1993; Al-Kaff and Covey 1996), however pilus of some *Agrobacterium* strains is most stable at lower temperatures around 18 to 20°C (Fullner and Nester 1996; Lai et al. 2000; Baron et al. 2001).

Attached to the 5' end of the T-strand, VirD2 serves as a pilot protein to guide the T-strand through the type IV export apparatus and once inside the plant cell it may function in additional stages of transformation process (Gelvin 2003). VirD2 contains nuclear localization signal (NLS) sequences that helps to direct it and the attached T-DNA to the plant nucleus (Gelvin 2010). It is reported that NLS of VirD2 protein can direct reporter proteins and *in vitro*-assembled T-complexes to the nuclei not only of plant but also animal and yeast cells (Herrera-Estrella et al. 1990; Howard et al. 1992; Tinland et al. 1992; Koukolikova-Nicola and Hohn 1993; Koukolikova-Nicola et al. 1993; Citovsky et al. 1994; Guralnick et al. 1996; Mysore et al. 1998; Ziemienowicz et al. 1999; Tzfira and Citovsky 2001; Ziemienowicz et al. 2001). VirE2, a non-sequence-specific single-stranded DNA binding protein (Gietl et al. 1987; Christie et al. 1988; Citovsky et al. 1988; Citovsky et al. 1989; Sen et al. 1989), that protects T-strands from nucleolytic degradation occurring both in the plant cytoplasm and perhaps in the nucleus (Rossi et al. 1993; Young et al. 2001), when bound to single-stranded DNA, can alter the DNA from a random-coil conformation to a shape resembling a coiled telephone cord (Citovsky et al. 1997), which due to its elongated shape may help direct the T-strand through the nuclear pore. In addition, VirE2 contains NLS sequences that direct fused reporter proteins to plant nuclei (Citovsky et al. 1994; Cluster et al. 1996; Zupan et al. 1996; Tzfira and Citovsky 2001). According to the most popular current model accepted by research community, T-strand is coated with hundreds of VirE2 in the plant cells and the two bipartite NLS sequences targets T-strand to the nucleus, even without the VirD2 NLS sequence (Gelvin 1998). It has also been reported that T-strand along with the associated Vir proteins interacts with plant proteins such as *Arabidopsis* importin- α in order to form super-T-complexes (Gelvin 2010).

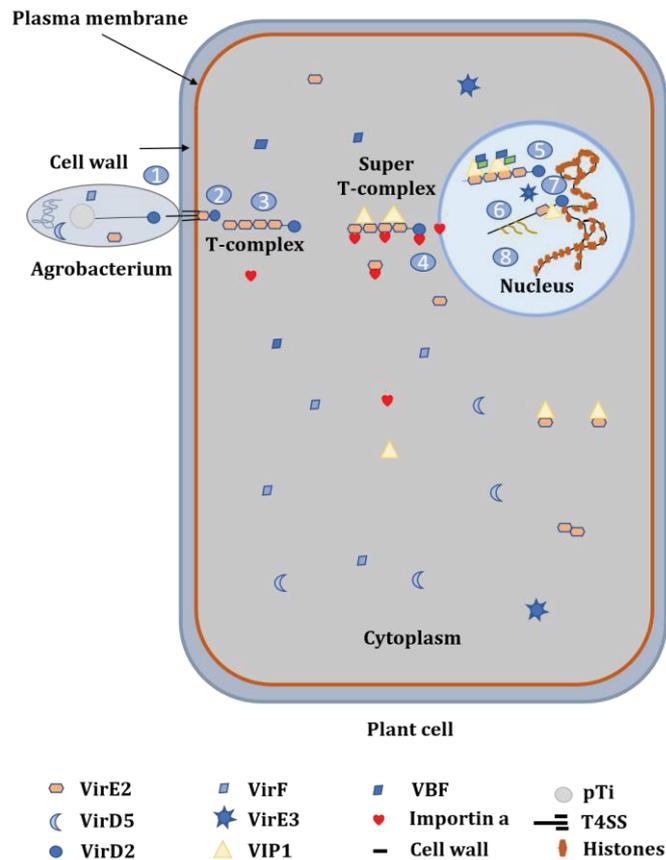


Figure 2 : Schematic representation of *Agrobacterium*-mediated plant transformation (adapted from Gelvin 2010).

Several important virulence effectors and plant proteins are noted. Circled numbers represent the following steps: 1. attachment of *Agrobacterium* to the plant cell; 2. transfer of virulence effector proteins and T-strands through the plant plasma membrane into the host cell; 3. formation of T-complex and super-T-complex and subsequent cytoplasmic trafficking; 4. nuclear targeting; 5. targeting of the super-T-complex to chromatin; 6. protein removal from the super-T-complex prior to T-DNA integration; 7. integration of T-DNA into the plant genome; and 8. transgene expression. T4SS - type IV secretion system. The figure is not drawn to scale.

1.4.2.5 T-DNA integration into the plant genome

Once inside the nucleus, T-strands are targeted towards plant chromatin prior to integration (not required for transient transformation). Several plant proteins in coordination with virulence effectors may facilitate this step. CAK2Ms, a kinase has been identified that phosphorylates VirD2 and the large subunit of RNA polymerase II, which recruits the TATA box binding protein and initiate the transcription (Bako et al. 2003). VirD2 can also associate with TATA box binding protein, which may guide T-strands to transcriptionally active regions of chromatin for integration (Bako et al. 2003). Surveys of several T-DNA tagged libraries in *Arabidopsis* and rice indicated that T-DNA preferentially integrates into promoter regions of genes and transcriptionally active

chromatin (Brunaud et al. 2002; Szabados et al. 2002; Schneeberger et al. 2005; Li et al. 2006). Several reports suggest the role of histones in targeting T-DNA to chromatin integration sites where VIP1 protein was shown to interact with various histones (Li et al. 2005; Loyter et al. 2005). C-terminal region of VIP1 is important for such interaction and T-DNA integration (Li et al. 2005). VIP1 can also direct *in vitro* interaction of T-DNA complexes consisting single stranded DNA, VIP1 and VirE2 with nucleosome monomer (Lacroix et al. 2008), acting as a molecular bridge to guide T-strands to plant chromatin for subsequent integration.

T-DNA integration mechanism is controversial and two major models have been proposed (Tzfira et al. 2004): a) The strand-invasion model, which suggests that VirD2 T-strands search for micro homology between T-DNA and plant DNA and induce locally melting the homologous regions within target DNA. Furthermore, VirD2 causes a nick in one strand of the plant DNA where the T-strand is ligated to, during mitosis replication the complementary strand of T-DNA is synthesized and incorporation of the T-DNA double strand copy into the plant genome is finalized (Tinland 1995); b) Double strand break repair and integration model based on a non-homologous-end-joining (NHEJ) process hypothesizes the replication of single stranded T-strand in the plant nucleus to a double stranded form which subsequently integrates into double stranded breaks in plant genome. This model explains the presence of inverted repeat copies of T-DNA, frequently found in transgenic plants and the observation that various T-DNAs introduced into the same plant cell often link together upon integration (Neve et al. 1997; Krizkova and Hroudá 1998; De Buck et al. 1999). The double strand break repair and integration model was also proved in studies where preferential T-DNA integration could be targeted by introducing deliberate double stranded breaks into plant DNA, (Salomon and Puchta 1998; Chilton and Que 2003; Tzfira et al. 2003).

1.4.3 *Agrobacterium rhizogenes* – biology and applications

The term 'hairy root' was first mentioned by Stewart et al. in 1900 (Stewart et al. 1900). *A. rhizogenes* recognizes phenolic compounds resulting from plant wounding, moves to the wound site and integrates genetic material into the plant genome. Infection by *A. rhizogenes* causes hairy-root-disease which is characterized by plagiotropic root growth, high degree of lateral root formation, profusion of root hairs. However, in contrast to undifferentiated callus formation by *A. tumefaciens* a highly differentiated

and functional root organ is maintained (Tepfer 1984; Balandrin et al. 1985; Charlwood and Charlwood 1991; Flores et al. 1999). In 1930, Riker et al., described and named the hairy root causing organism as *Phytomonas rhizogenes*, which later was renamed to *Agrobacterium rhizogenes* (Riker et al. 1930). An important feature of such *Agrobacterium*-induced roots is their unique ability to grow *in vitro* even in the absence of exogenous plant growth regulators (Rao and Ravishankar 2002), which made hairy roots a widely used research tool for secondary metabolite production, study of gene function and root biology.

1.4.3.1 Infection diversity and biology of *A. rhizogenes*

In nature *A. rhizogenes* appears to be restricted to a limited plant host species such as apples, cucumber, tomato and melon (Smith and Townsend 1907; Riker et al. 1930; Davioud et al. 1988; Isogai et al. 1990; Weller et al. 2000; Weller et al. 2004; Weller et al. 2006). However, under laboratory condition more than 450 different plant species, varying from dicotyledonous and monocotyledonous plant families (Cleene and Leu 1981; Tepfer 1989) to some gymnosperms (Diouf et al. 1995; Yibrah et al. 1996) are reported to be susceptible to *A. rhizogenes* infection (Tepfer 1984; Porter and Flores 1991). *A. rhizogenes* is able to infect and genetically transform most plant tissues and organs including the hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root, and tuber, with the resulting production of hairy roots (Mugnier 1988; Han et al. 1993; Drewes and Van Staden 1995; Giri et al. 2001; Królicka et al. 2001; Azlan et al. 2002).

A. rhizogenes strains are characterized by the presence of a large root inducing (Ri) plasmid and classified as either biotype 1 or 2 (Veena and Taylor 2007). According to the comparative genome mapping and DNA/DNA hybridization experiments, the *A. rhizogenes* isolates have been shown to possess a highly conserved “core” DNA region, essential for hairy root formation (Filetici et al. 1987). *A. rhizogenes* strains can be classified into different sub-groups based on the type of opines they produced. Commonly known *A. rhizogenes* strains identified to date include agropine-type strains (Ri plasmids pRiA4, pRi1855, pRiHRI, pRi15834, and pRiLBA9402), mannopine-type strains (Ri plasmid, pRi8196), cucumopine-type strains (Ri plasmid, pRi2659), and mikimopine-type (Ri plasmid pRi1724) (Petit et al. 1983; Filetici et al. 1987; Davioud et al. 1988; Isogai et al. 1988; Suzuki et al. 2001). Among various strains of *A. rhizogenes*,

K47, K599, and HRI are hyper-virulent types capable of infecting a wide variety of different plant species (Veena and Taylor 2007). *A. rhizogenes* strains also differ from each other by their ability to induce root growth only on the apical surface of carrot root discs or root proliferation both upon inoculation of apical and basal surfaces (Cardarelli et al. 1985; Ryder et al. 1985).

1.4.3.2 Molecular basis of hairy root formation

In terms of composition as well as structure, the Ri plasmid of *A. rhizogenes* and Ti plasmid of *A. tumefaciens* are broadly similar. However, minor differences do exist (Zhu and Welander 1998; Moriguchi et al. 2001), for example, unlike Ri plasmids Ti plasmids contain so-called overdrive sequences, the 24-bp conserved sequence located adjacent to the right border on Ti plasmids (Shurvinton and Ream 1991) that increases efficiency of tumour formation by enhancing T-DNA border processing (Peralta and Ream 1985; Peralta et al. 1986; Van Haaren et al. 1987; Toro et al. 1988). Ri plasmids on the other hand possess 8-bp repeated sequences, known as T-DNA transfer stimulator sequences (TSS) that enhance efficiency of T-DNA transfer to the plant genome (Hansen et al. 1992; Moriguchi et al. 2001). The absence of *virE1* and *virE2* genes, known to be critical for pathogenesis of *A. tumefaciens* (Christie et al. 1988; Citovsky et al. 1992; Ward and Zambryski 2001; Duckely and Hohn 2003) that are absent from *A. rhizogenes* genome and Ri plasmid DNA (Moriguchi et al. 2001; Hodges et al. 2004) are thought to be functionally satisfied by the *GALLS* gene (Hodges et al. 2004).

The mechanism of root induction and proliferation is the least understood aspect of hairy-root disease. All *A. rhizogenes* strains contain a specific region in their T-DNA located on the Ri plasmid, which carries genes involved in root initiation and development (*rol*-genes), related to opine biosynthesis and of unknown function (Slightom et al. 1986; Hansen et al. 1994). In the early 1990s, it was reported that a combination of *rolA*, *rolB*, and *rolC* loci was sufficient for producing the hairy-root phenotype, depending on various plant species and tissue type (Christey 2001). However, the effect of T-DNA genes of *A. rhizogenes* in plant phenotypes and their interference in various hormone pathways clearly indicate that further genes may also play an important role for hairy-root induction (Porter and Flores 1991). For some subtypes, genes involved in auxin biosynthesis (*aux1* or *iaaM* and *aux2* or *iaaH*) along with further genes of unknown function may be carried by a second TDNA (Veena and

Taylor 2007). Although both TL-DNA and TR-DNA are transferred and integrated independently into the host plant genome, transfer of TL-DNA is known to be crucial for the induction of hairy root syndrome (Phelep et al. 1991; Nilsson and Olsson 1997; Sevón and Oksman-Caldentey 2002).

1.4.3.3 Composite plant system

In 1989, Hansen et al. developed a novel tool called Composite plants that consists of a wild type shoot from which transgenic roots are induced (Hansen et al. 1989). This method was later further developed by Torregrosa and Bouquet (Torregrosa and Bouquet 1997). The first *ex vitro* composite plants were generated by inoculating wild type shoots with *A. rhizogenes* under non-sterile conditions (Collier et al. 2005; Taylor et al. 2006). Composite plants were successfully generated for over 20 different species meanwhile (Estrada-Navarrete et al. 2006; Estrada-Navarrete et al. 2007; Kereszt et al. 2007).

This method is simple, cost effective and requires minimal infrastructure. Moreover, it significantly reduces the time required for generation of transgenics from several months to few weeks compared to traditional transgenic plant production using *A. tumefaciens*. Additional T-DNA containing binary plasmid present in the bacteria can also be efficiently transferred and around 20-60 % of the produced roots inoculated with wild type *A. rhizogenes* contain both hairy-root-inducing and binary T-DNA (Collier et al. 2005). Reporter gene constructs are helpful to visualize roots expressing T-DNA of interest and can also be used for promoter analysis studies and subcellular protein localization. Hairy roots were successfully used to determine the expression pattern of various promoters fused with visual marker genes such as GUS or green fluorescent protein GFP (Isayenkov et al. 2005; Estrada-Navarrete et al. 2006; Nontachaiyapoom et al. 2007; Suttipanta et al. 2007) and to examine tissue-specific and targeted subcellular localization of desired protein to various organelles (Suzuki et al. 1993; Moreno-Valenzuela et al. 2003; Marjamaa et al. 2006). Several reports have shown that RNAi silencing is functional (Kumagai and Kouchi 2003) and moves systemically (Limpens et al. 2004) in hairy roots of composite plants produced *in vitro*. Moreover, composite plant system plays a crucial role in the plant-biotic interaction research, where biotic interactions or gene functions can be tested without the need to produce fully transformed plants. *A. rhizogenes* induced *in vitro* hairy root cultures have been used to

study interactions between roots and nematodes (Kifle et al. 1999; Narayanan et al. 1999), rhizobia (Diaz et al. 1989; Quandt et al. 1993) and mycorrhizal fungi (Mugnier 1997). Hairy roots of composite plants, for example *Lotus corniculatus* (Hansen et al. 1989), *Vicia hirsuta* (Quandt et al. 1993), *Medicago truncatula* (Boisson-Dernier et al. 2001), *Sesbania rostrata* (Van de Velde et al. 2003), *Trifolium repens* (Diaz et al. 1989) and *Coffea arabica* (Alpizar et al. 2006) have been used for gene expression studies during nitrogen fixation and mycorrhizal symbiotic associations.

1.4.4 Binary vector system

The Ti and Ri plasmids are large and present only in low copy number in *Agrobacterium* and are unable to replicate in *E. coli* (Lee and Gelvin 2008). With the discovery that *vir* genes and T-DNA can be located on separate replicons in the same *Agrobacterium*, binary vector system was developed (de Framond et al. 1983; Hoekema et al. 1983; Lee and Gelvin 2008). Following are the essential elements of a binary vector – a) origin of replication (*ori*) that allows maintenance both in *E. coli* and *Agrobacterium*, b) left and right border sequences important for T-DNA transfer, c) a suitable bacterial selection marker, d) multiple cloning site (MCS) with endonuclease recognition site for easy T-DNA manipulation and e) a plant marker for selection of transgenic cells (Lee and Gelvin 2008).

pPLV11 is a pGreen based modified vector (Hellens et al. 2000; De Rybel et al. 2011) with ligation independent cloning (LIC) site for expression analysis and protein localization studies (De Rybel et al. 2011). It contains a pColE1-Ori and a pSa-Ori for replication in *E.coli* and *Agrobacterium* respectively, a kanamycin resistance cassette for bacterial selection (Hellens et al. 2000) and a tdTomato fluorescent protein for protein localization. Due to its relatively smaller size and efficient *ori*, these vectors are easy to manipulate (Hellens et al. 2000). In this work modified versions of pPLV11 were used: a) pPLV11-SharinaI harboring a constitutively expressed super yellow fluorescent protein (sYFP) for subcellular localization and b) pPLV11-SharinaII contains a peroxisomal targeted sYFP coding region for promoter analysis (Nehls, unpublished).

pCAMBIA is a series of medium size (vector backbone around 6 kb) vectors with ColE1 and pVS1 origin of replication for *E. coli* and *Agrobacterium* maintenance. These vectors contain all required genes for replication encoded on their vector backbone; therefore, no helper plasmid is required.

1.5 Agroinfiltration – for transient protein expression system

Transient expression systems are useful due to their fastness, flexibility and their independence from chromosomal positional effects (Fischer et al. 1999). *A. tumefaciens* is commonly used for such transient expressions (Fischer et al. 1999). When infiltrated into plant leaves as a liquid culture, it mediates transfer of the genes from the T-DNA region of bacterial Ti-plasmid into the plant cells. Most of the plant cells in and around the infiltrated region express the transgene (Kapila et al. 1997). Although this system unlike viral vectors does not lead to systemic expression of the desired foreign gene, it can be used with long (>2 kb) genes, which are in general unstable in viral vectors (Porta et al. 1996). A major advantage of Agroinfiltration is that several transgenics can be delivered into the same cells (Kapila et al. 1997) by mixing different transgene *Agrobacterium* cultures prior to infiltration (Voinnet et al. 2003). Therefore, long, multimeric proteins, such as antibodies can be expressed and assembled *in vivo* in plant cells (Vaquero et al. 1999).

N. benthamiana is the most easy to use plant for high throughput protein localization studies in nuclei, endomembrane and other structures within cells (Goodin et al. 2005; Goodin et al. 2007). Moreover, continued improvements in expression vectors, auto-fluorescent proteins, and imaging technologies (Goodin et al. 2007) make *N. benthamiana* a significant research model for localizing plant proteomes (Pepperkok and Ellenberg 2006; Simpson and Pepperkok 2006; Laketa et al. 2007). In agroinfiltrated tissues, the expression yield of heterologous proteins can dramatically decline by post-transcriptional gene silencing (PTGS) activation in host plant (Circelli et al. 2010). Use of plant virus suppressors of gene silencing such as P19 was proven to reduce this response greatly and increases the yield of recombinant proteins in agroinfiltrated leaves by several folds (Johansen and Carrington 2001; Voinnet et al. 2003; Circelli et al. 2010).

1.6 Ligation independent cloning (LIC): I tie the knot myself

Ligase independent cloning does not require any DNA ligase or restriction enzyme for cloning purposes, instead this method relies on the 3' → 5' exonuclease activity of T4 DNA polymerase in order to generate long cohesive ends on both sides of vector and DNA fragments desired for cloning (Aslanidis and de Jong 1990).

In this technique, the purified PCR product and linearized vector backbone are incubated with T4 DNA polymerase that comprises both 3' → 5' exonuclease activity and 5' → 3' polymerase activity (Cha and Alberts 1989; Young et al. 1992). In presence of a single nucleotide, it helps to remove the 3' terminal nucleotides and produces highly specific single stranded overhangs at both ends of the insert (Bonsor et al. 2006). For example, when vector backbone and insert are incubated with only dCTP and dGTP in absence of other dNTPs respectively, the exonuclease activity of T4 DNA polymerase is active as long as it does not encounter any Cytosine or Guanine, thus removes the 3' terminal ends by hydrolysis (Reddy et al. 1992). Encountering with Cytosine and Guanine present in the mixture helps switch to the polymerase activity of the T4 DNA polymerase (Reddy et al. 1992).

The resulted single stranded overhang sequences of the insert is complementary to the overhangs of the linearized LIC engineered vector whose two overhangs are not complementary to each other to ensure directional cloning (Bonsor et al. 2006). This is an added advantage over blunt end cloning strategy. The T4 DNA polymerase treated insert and vector anneals in a ten minute reaction and the mix can directly be used for transformation into cloning or expression bacterial strains (Bonsor et al. 2006). The complementarity of the overhangs guarantees the vector and insert remain annealed during the transformation and ligation later occurs by the DNA repair enzymes inside the host cells (Bonsor et al. 2006).

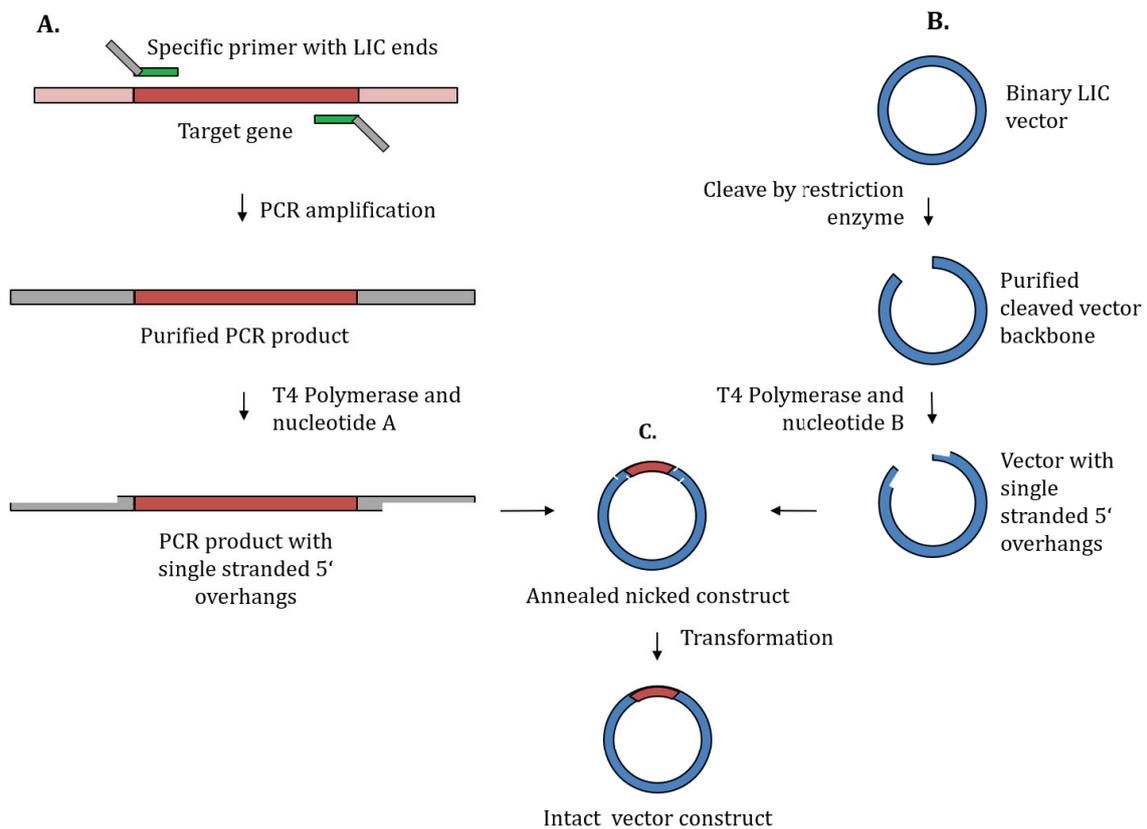


Figure 3 : Ligation independent cloning

Illustration of ligation independent cloning (LIC): A. Target gene is PCR amplified using gene specific primers with LIC ends attached to their 5' termini. The PCR product is purified and incubated with T4 DNA polymerase and a single deoxyribonucleotide (A = dATP, dTTP, dCTP or dGTP) to generate a LIC insert with single stranded 5' overhangs. B. vector is cleaved at the LIC site using a specific restriction endonuclease (blunt end cleave by *HpaI* is used in this thesis). The linearized vector is purified and incubated with T4 DNA polymerase and a single deoxyribonucleotide complementary to that used for the PCR product-LIC insert preparation, to generate single stranded 5' termini overhang complementary to that of the insert. C. purified vector and insert are mixed and annealed at room temperature. The nicks are repaired when transformed into *E. coli* cloning strain.

1.7 Regulation of gene expression in eukaryotic cells

Gene expression in multicellular organism requires complex regulation of activities in many different cell types (Ahluwalia 2009). Eukaryotic promoters are composed of a core promoter and distal regulatory regions. The core promoter contains a docking site for the so-called preinitiation complex (PIC) that often contain an AT-rich site (TATA box) found ~30 bp upstream from the transcription start. Such AT-rich sequence motive is, however, missing in many plant promoters; other regions such as CAAT and GC boxes increases the RNA polymerase binding activity (Adams 2008). Further upstream activation site (UAS) elements are found around 80-1800 nucleotide away from the initiation site (Graw 2010). Unlike UAS the presence of silencer or enhancers in the

vicinity of the gene is less specified and can be as far as 50 kb away from the starting codon and can be found inside or outside of the coding part of the gene (Graw 2010). Eukaryotic RNA polymerases require a set of additional proteins called the general transcription factors for efficient initiation.

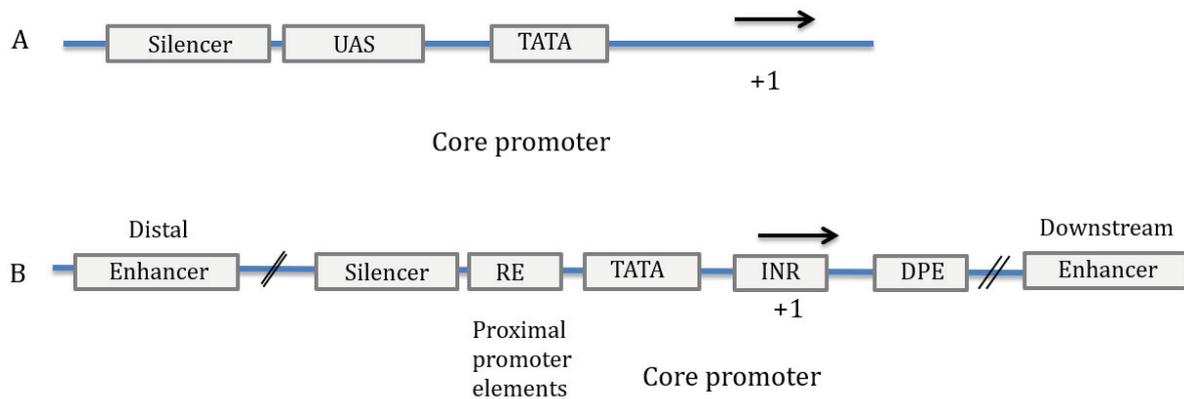


Figure 4 : Eukaryotic promoter

Structures of a simple eukaryotic promoter (A) and an extensively diversified metazoan regulatory module (B) (adapted from Levine and Tjian 2003).

(A) Shown is the promoter region of a simple eukaryotic cell, with the core promoter (the TATA box), the upstream activating site (UAS) and a silencer, responsible for downregulating the expression of a gene. The “+1” indicates the start of transcription in the direction of the arrow.

(B) The regulatory module of a metazoan cell presents, the initiator element (INR), downstream promoter elements (DPE) and downstream enhancers. Upstream regulating elements like a distal enhancer can be over 1000bp away from the transcription initiation site (“+1”).

1.8 Plant transcription factors: I define you

The interaction of transcription factors of basic transcription machinery to *cis* elements localized in a given distance to the core promoter is crucial for the regulation of cell activities (Guilfoyle 1997). Study by mutational and functional analysis demonstrated that typical plant transcription factors consist of a DNA-binding region, an oligomerization site, a transcription regulation domain and a nuclear localization signal (NLS), some may lack either a transcription regulation domain (Goff et al. 1992) or a specific DNA binding region (Vetten and Ferl 1995; Hill et al. 1996; Washburn et al. 1997). Moreover, identification of conserved RNA-binding motifs in the carnation ethylene responsive element-binding protein-1 (Maxson and Woodson 1996), a G-protein b-subunit-like motif in COP1 (constitutive photomorphogenic 1) (Deng et al. 1992), or a putative membrane-spanning region in plastid envelope DNA-binding

(PEND) (Sato et al. 1998) proteins also demonstrate novel functional domains in plant transcription factors.

Transcription factors belonging to the same family generally have distinct actions due to the differences in their regulation domains and the regions of the proteins that tend to diverge from one another (Yanagisawa and Sheen 1998). Regulation domains, and hence transcription factors, function as either repressors or activators, depending on whether they inhibit or stimulate the transcription of target genes. Repression of gene expression probably occurs via direct interaction with RNA- polymerase interaction complex or with further *cis*-acting factors (Liu et al. 1999).

Compared to proteins from other organisms, plant transcription factors do contain single or multiple Nuclear localization signals (NLSs) (Boulikas 1994; Varagona and Raikhel 1994; Dehesh et al. 1995; Klinge et al. 1996; Meisel and Lam 1996; Michael et al. 1996; Lyck et al. 1997), which probably be functionally independent (Varagona and Raikhel 1994; Dehesh et al. 1995; Meisel and Lam 1996) and either clustered (Klinge et al. 1996) or dispersed within the protein.

1.8.1 Regulation of transcription factor genes

Plant transcription factor genes may either be expressed constitutively or in organ-limited (Waldmüller and Link 1995; Meissner and Michael 1997; Kater et al. 1998), stimulus-responsive (Aguan et al. 1993; Kusano et al. 1995; Mikami et al. 1995; Vetten and Ferl 1995; Nakagawa et al. 1996; Gubler et al. 1997; Wang et al. 1997), development-dependent (Vollbrecht et al. 1991; Chern et al. 1996; Li et al. 1996; Yamamoto and Minamikawa 1997) and cell-cycle-specific manners (Minami et al. 1993). Members of a gene family are not necessarily responsive to the same stimulus; for instance some genes of the bZIP family are regulated by light (Weisshaar et al. 1991; Schindler et al. 1993), while others respond to abscisic acid, auxin and salicylic acid (Niu and Guiltinan 1994; Hong et al. 1995; Miao and Lam 1995; Nakagawa et al. 1996). Plant transcription factor genes also respond to multiple environmental signals.

Coincidental accumulation of mRNA from plant transcription factor genes and their targets occurs in response to several stimuli, and remain consistent with a role in activation; it has been shown the proliferation of many regulatory gene mRNAs precedes the expression of their effector genes (Vetten and Ferl 1995; Nakagawa et al. 1996;

Gubler et al. 1997; Wang et al. 1997). In contrast, quantitative changes in transcripts levels from other regulatory and target genes are inversely correlated, which indicates the products of the former probably be transcriptional repressors (Weisshaar et al. 1991; Waldmüller and Link 1995). Members of the same plant transcription factor multigene family might also be expressed differently, and the distinct kinetics implies differences in the regulatory capacity of the related transcription factors.

Quantitative variations in transcription factor mRNA by suppression and over-expression experiments, shown to bring substantial changes in plants (Kater et al. 1998). Therefore, accurate regulation of transcription factor genes by their *cis*- and *trans*-acting elements is potentially essential (Urao et al. 1996). Regulatory and regulated genes are sometimes influenced by the same transcription factors because of common *cis*-acting elements (Izawa et al. 1994; Sakamoto et al. 1996). *cis*-acting elements of some transcription factor genes are affected by their own products (Izawa et al. 1994; Mikami et al. 1995; Sakamoto et al. 1996). Alternative splicing has been reported for mRNAs originating from several plant transcription factor genes (Grotewold et al. 1991; Dietrich et al. 1997; Magaraggia et al. 1997).

1.9 AP2/ERF gene family: large and crucial

Various transcription factors and *cis*-acting elements in stress-responsive promoters function together for plant adaptation to environmental cues. It is estimated that *Arabidopsis* and rice genomes encode about 1300–1600 transcription factors (Chen and Zhu 2004). The APETALA2/ethylene-responsive element binding protein (AP2/ERF) superfamily is one of the largest groups of plant specific transcription factors. They play crucial roles in plant growth, development and response to diverse abiotic and biotic stresses such as extreme temperature (freezing damage, and high temperature stress), drought, high salinity and pathogen infection (Dubouzet et al. 2003; Gutterson and Reuber 2004; Kume et al. 2005; Sakuma et al. 2006; Yamaguchi-Shinozaki and Shinozaki 2006; Lim et al. 2007; Sharoni et al. 2010). Their role in various hormones-related signal transduction pathway including abscisic acid (ABA), ethylene, cytokinin and jasmonate has also been reported (Ohme-Takagi and Shinshi 1995; Shen et al. 2003; Pauwels and Goossens 2008; Rashotte and Goertzen 2010; Hu et al. 2013).

AP2/ERF genes share a highly conserved AP2 DNA binding domain with 50 to 70 amino acid residues consisting of a three-stranded anti-parallel β -sheets and an α -helix (Allen et al. 1998). This AP2 domain was first discovered in the homeotic gene *APETALA2* (*AP2*) while studying *Arabidopsis* flower and seed development (Jofuku et al. 1994). Two main classification methods have been proposed for the plant AP2/ERF superfamily depending on sequence similarities and the number of AP2 domains (Li et al. 2017). Sakuma and colleagues classified the AP2/ERF superfamily into five families: *APETALA* (*AP2*), related to *ABA* Insensitive3 (*ABI3*)/ *Viviparous1* (*RAV*), dehydration responsive element-binding (*DREB*), ethylene responsive factors (*ERF*), and soloist (Sakuma et al. 2002) (Sakuma et al. 2006). The *ERF* family is also known as the *EREBP* (ethylene-responsive element binding proteins) family (Nakano et al. 2006). The *AP2* family, essential for plant development contains two AP2 domains (Okamuro et al. 1997; Shigyo and Ito 2004), the *RAV* family functioning as negative regulators in plant development and important for mediating abiotic and biotic stress responses, contains one AP2 domain and one B3 domain (Li et al. 2015), while the *DREB* (dehydration-responsive element-binding protein), *ERF* (ethylene response factor), and *Soloist* families each contain a single AP2 domain. According to sequence similarity of the single AP2 domain, *DREB* family genes and *ERF* family genes are further classified into the groups A1-6 and B1-6 respectively (Sakuma et al. 2002). On the other hand proteins containing AP2/ERF predicted amino acid sequences are classified into three major families: *AP2*, *ERF* (including both *DREBs* and *ERFs*), and *RAV* (Nakano et al. 2006). In *Arabidopsis* and rice the *ERF* family was sub-divided into 12 and 15 groups respectively based on the structure and similarity of the AP2 domain (Nakano et al. 2006).

AP2/ERF superfamily genes and transcripts have been identified and characterized in many plants and were extensively studied in the context of plant stress tolerance (Xu et al. 2011; Mizoi et al. 2012). *DREB* family genes have been reported to respond to drought, desiccation, osmotic stress, salt, low and elevated temperature and are supposed as the candidate genes for improving plant stress tolerance in crop plants (Lata and Prasad 2011). The genome-wide identification of the *AP2/ERF* gene family has been accomplished in many plant species (Czechowski et al. 2005) (Zhuang et al. 2008; Licausi et al. 2010; Xu et al. 2013) (Lei et al. 2016) (Sun et al. 2014) (Song et al. 2013) (Shu et al. 2016) (Lakhwani et al. 2016) including poplar (Zhuang et al. 2008).

1.9.1 Poplar *AP2/ERF* and *DREB* gene families

Nanjo et al (2004) identified 13 candidate clones containing the AP2/ERF domain from the poplar expressed sequence tags (ESTs) (Nanjo et al. 2004) and a total of 200 AP2/ERF family members were identified in the *Populus trichocarpa* (Zhuang et al. 2008). This superfamily included 5 putative *RAV* family genes, encoding one AP2/ERF domain together with one B3 domain, 26 genes were predicted to encode protein containing two AP2/ERF domains classified as AP2 family and 168 predicted genes encoding proteins consisting a single AP2/ERF domain which have been further divided into two families based on their amino acid sequences. 77 genes were identified as possibly encoding DREB family and 91 genes were predicted to encode ERF family members. A single Soloist gene that includes an AP2/ERF-like domain was identified, whose homology appeared quite low comparing to other AP2/ERF genes (Zhuang et al. 2008). The *AP2/ERF* and *DREB* families of *P. trichocarpa* were 1.4-fold larger than those of *A. thaliana* (Zhuang et al. 2008). *Populus* has generally more protein-coding genes than *Arabidopsis*, ranging on average from 1.4 to 1.6 putative *Populus* homologs for each *Arabidopsis* gene (Tuskan et al. 2006). Like in *Arabidopsis* and rice, Poplar *AP2/ERF* genes are distributed among all the linkage groups similarly.

As AP2/ERF, members of the dehydration responsive element-binding (*DREBs*) protein family are known to play an important role in regulating plant growth and the response to external environmental stresses. The DREB subfamily belongs to the APETALA2/ethylene-responsive element-binding protein (AP2/EREBP) family in the plant kingdom. The *DREB* subfamily recognizes the dehydration-responsive element (DRE) with a core motif of A/GCCGAC (Liu et al. 1998), or the *cis*-acting element AGCCGCC, known as GCC box (Sakuma et al. 2002). A combination of genetic and molecular approaches have been used to characterize a series of *DREB* family regulatory genes involved in various pathways, including genes related to cold, drought, high salinity, heavy metals, and abscisic acid (ABA) (Peng et al. 2013).

In 2013, Chen et al. have identified 75 *DREB* genes in the *Populus trichocarpa* genome (Chen et al. 2013), based on the alignment of the AP2/ERF coding regions and the *Arabidopsis DREB* subfamily into six groups, A1, A2, A3, A4, A5, and A6, containing 6, 17, 2, 26, 15, and 9 members respectively (Chen et al. 2013). *DREBs* share high homology and contain a conserved WLG motif (β -sheet 3 containing WLG element -W-

Tryptophan, L - Leucine , G - Glycine) in the AP2/ERF domain of *Populus* (Chen et al. 2013; Li et al. 2017). They all contain alanine at position 319 in a β -sheet (Chen et al. 2013). Furthermore, position 14 is a valine and 19 is glutamic acid expected to play an important role in the recognition of DNA binding sites in the DRE and GCC box *cis*-elements. *P. trichocarpa* DREB Group A-1 proteins share remarkable similarities with known C-repeat binding factors (CBF) /DREB proteins of *Arabidopsis* and carry critical amino acids needed for binding to the C-repeat (CRT) elements in the target genes (Chen et al. 2013).

1.10 Genes selected in this study

PtrDREB1 (**Potri.008G071100.1**) located on chromosome 8, is a dehydration responsive element-binding (DREBs) transcription factor of the DREB Group A-3 of *P. trichocarpa* (Chen et al. 2013). Specific details of the DREB subfamily have already been introduced above. Based on program of ExPASy (the SIB Bioinformatics Resource Portal) tool theoretical pI of the protein was calculated to be 8.34 and online analysis using SOSUI tool (Hirokawa et al., 1998) predicted a soluble protein without transmembrane domains. The next homolog of *PtrDREB1*, is *PtrDREB2*, a second member of the DREB subfamily group A3. In contrast to poplar, *Arabidopsis* has only one member *ABI4* (Abscisic Acid Insensitive4) in the same subfamily (Chen et al. 2013).

Arabidopsis *ABI4* is involved in phytohormone (abscisic acid) response regulation. The plant hormone abscisic acid (ABA) regulates developmental and environmental aspects like seed development, including synthesis of storage proteins and lipids (Finkelstein and Somerville 1989; Rock and Quatrano 1995) or acquisition of desiccation tolerance and dormancy (Black 1983; Karssen et al. 1983; Koornneef et al. 1989). Moreover, vegetative responses to ABA include induction of stomatal closure and tolerance of salt, drought, and cold stresses (Leung and Giraudat 1998). Many ABA-regulated genes and an array of corresponding transcriptional regulators have been identified (Busk and Pagès 1998), and *Abscisic acid insensitive* (*abi*) mutants revealed several phenotypes for few of them (Koornneef et al. 1984; Finkelstein 1994). *ABI1* and *ABI2* encode for phosphatases (Leung et al. 1994; Leung et al. 1997) while *ABI3*, *ABI4* and *ABI5* encode for transcription factors (Giraudat et al. 1992; Finkelstein and Lynch 2000).

Although mutations in *ABI3*, *ABI4*, and *ABI5* have the maximum impact on gene expression during seed maturation, all three genes shown to be expressed only to a limited degree in vegetative tissues (Finkelstein et al., 1998; Rohde et al., 1999; Finkelstein and Lynch, 2000). Reports have also indicated some cross-regulation of expression among *ABI3*, *ABI4*, and *ABI5* that suggests their function in a combinatorial network, rather than a regulatory hierarchy to control seed development and ABA response (Söderman et al. 2000). *ABI4* was reported to be involved in glucose signalling (Arenas-Huertero et al. 2000; Rook et al. 2001; Arroyo et al. 2003; Price et al. 2003), several sugar signalling and its response pathways (Huijser et al. 2000; Laby et al. 2000; Bossi et al. 2009), ABA signalling (Finkelstein 1994; Arenas-Huertero et al. 2000; Huijser et al. 2000; Laby et al. 2000; Söderman et al. 2000) and lipid mobilization in the embryo and germinating seeds (To et al. 2002; Penfield et al. 2006). Furthermore a function in chloroplast functioning and retrograde signalling was observed in seedlings (Koussevitzky et al. 2007; Muñoz-Bertomeu et al. 2010) as well. Other studies using older plants, showed that *ABI4* is involved in responses to plant pathogens and tolerance to β -amino-butyric acid (BABA)-induced water stress (Jakab et al. 2005; Ton et al. 2005; Adie et al. 2007; Kaliff et al. 2007), sugar insensitivity in fully grown leaves (Oswald et al. 2001), chloroplast photosynthesis (Oosten et al. 1997; Yabuta et al. 2007), mitochondrial retrograde signalling (Giraud et al. 2009) and nitrate modulated root branching (Signora et al. 2001), modulation of hormonal control of lateral root formation (Shkolnik-Inbar and Bar-Zvi 2010). It has also been shown that *ABI4* acts as a major modulator of root development and various other functions (Shkolnik-Inbar et al. 2013).

Potri.007G095000, located on chromosome 7 is predicted to encode a uridine diphosphate (UDP) glycosyltransferases (UGTs) (Nehls, unpublished). *Potri.007G095000* corresponds to *AT1G22340* (Nomenclature: *UGT85A*, also referred as *ATUGT85A7* in literature) of *Arabidopsis thaliana* (Nehls, unpublished). The *Arabidopsis* ortholog is predicted to be located in intracellular membrane-bounded organelles or the nucleus of cotyledons, lateral root primordia, root tips, vascular leaf primordia (The *Arabidopsis* Information Resource (TAIR) - www.arabidopsis.org). Despite their long-established role in animal metabolism, UGT activities in plants have received relatively little attention (Vogt and Jones 2000; Ross et al. 2001). Plant UGTs have a diverse substrate such as flavonoids, terpenes, auxin, cytokinin, salicylic acid, and sterols and

postulated to play key roles in plant development, metabolism, and defence (Bowles 2002; Dixon 2004; Bowles et al. 2006). Although they also comprise important targets for agronomic and medicinal applications (Dixon and Ferreira 2002), to date however, information about their expression, function, substrate, substrate specificity, and biological effects in plants is limited to only few individual UGTs (Woo et al. 1999; Jones et al. 2003; Quiel and Bender 2003; Woo et al. 2003; Woo et al. 2004; Woo et al. 2007). Glycosyltransferases catalyse the transfer of activated sugar to a wide range of acceptor molecules with exposed hydroxyl groups (Ross et al. 2001), thereby creating a glycosidic bond. These enzymes are found in most living organisms but are particularly important in plants. Here photosynthesis products are converted into oligo or polysaccharides for carbohydrate storage or cell-wall polysaccharides and glycoproteins (Keegstra and Raikhel 2001). Glycosyltransferases have been classified into different families on the basis of sequence similarity, catalytic specificity and the existence of consensus sequences (Campbell et al. 1997; Mackenzie et al. 1997; Kapitonov and Yu 1999).

Glycosylation is an essential metabolic pathway with diverse roles in cellular processes (Jaeken and Matthijs 2001). Generally in plants, glycosylation of phytochemicals by the addition of glucose or other sugars results in enhanced water solubility and lower chemical reactivity, allowing long-term storage in vacuoles or cell walls (Dixon 2004). Reversible conjugation of hormones such as auxin and cytokinin may also be crucial in 'homeostasis' for the regulation of physiologically active hormone levels (Szerszen et al. 1994).

A very large glycosyltransferase superfamily, family 1 contains 107 putative UGT genes and 10 UGT pseudogenes (Li et al. 2001). The family 1 GTs are classified based on the presence of a 44-50 amino acid C-terminal consensus sequence and are predicted to transfer nucleotide-diphosphate-activated sugars to a diverse array of low-molecular weight 'secondary' metabolites (Paquette et al. 2003; Gachon et al. 2005; Bowles et al. 2006). This consensus sequence is known to represent the nucleotide sugar binding site and is termed as the Plant Secondary Product Glycosyltransferase (PSPG) consensus (Gachon et al. 2005), with 120 putative PSGTs identified in *Arabidopsis* (Bowles et al. 2006). Family 1 GTs of *Arabidopsis* are classified into 14 groups (A-N) based on their substrate specificity (Li et al. 2001; Ross et al. 2001). *Potri.007G095000* and *ATUGT85A7* are the only members of subgroup of group G (Woo et al. 2007).

1.11 Heterologous protein expression

Obtaining specific proteins from their natural host cells is challenging. Therefore, recombinant protein production frequently is the sole applicable procedure (Sørensen and Mortensen 2005). Expression is generally induced from a plasmid harboured by a genetically compatible system. Essential genetic elements in a recombinant expression system are a) origin of replication (*ori*) of expression plasmid, b) an antibiotic resistance marker, c) transcriptional promoters, d) translation initiation regions (TIRs) and e) transcriptional and translational terminators (Sørensen and Mortensen 2005). Most plasmids used for recombinant protein expression have the ColE1 or p15A replicon and these are stably replicated under selective conditions (Summers 1998). Such expression plasmids require a strong transcriptional promoter to control high-level gene expression and the most common inducer is the sugar molecule isopropyl-beta-D-thiogalactopyranoside (IPTG) (Hannig and Makrides 1998). By far the T7 based pET expression system is mostly used in recombinant protein over expression. In the early 1990s Studier et al. first described the pET expression system that over the years has been developed for several expression applications (Studier et al. 1990; Dubendorf and Studier 1991) and now more than 40 different pET plasmids are available commercially (Sørensen and Mortensen 2005).

The most commonly used system for rapid production is *Escherichia coli* due to its well characterized genetics and rapid growth in inexpensive culture media. However, heterologous proteins expressed as insoluble aggregates of folding intermediates known as inclusion bodies is often a major problem for *E. coli* system (Ghosh et al. 2004; Esposito and Chatterjee 2006). Over the years although considerable progress has been made to produce protein in biologically active soluble form (Lilie et al. 1998), optimal expression conditions differ greatly from protein to protein (Ghosh et al. 2004). In this work pET28a was used as an expression vector.

1.12 Aim of the thesis

To be able to produce fast poplar transgenics, a protocol to produce composite poplar was developed in this thesis in collaboration with D. Neb (group Nehls). It allows induction of transgenic root within a short period of time capable in mycorrhiza formation. For this purpose, four different *Agrobacterium rhizogenes* strains were

tested for their ability to induce transgenic roots from poplar cuttings and transgenic root growth properties were studied. Transformation efficiency of binary vector T-DNA transmission was also investigated by expression of a reporter gene construct.

Another aim of this thesis was the comparative analysis of transcript levels of preselected poplar genes in mycorrhized and non-mycorrhized poplar fine roots. A total of 50 genes were chosen for this work based on a whole genome array analysis (Nehls, unpublished), out of which few revealed a reproducible strongly induced expression in the mycorrhized roots based on qRT-PCR analysis, which provided an independent experimental confirmation of the microarray analysis done by Nehls. Based on the comparable expression pattern found both in microarray and qRT-PCR, two genes were chosen for further detailed investigation a) a transcription factor belonging to the AP2/ERF superfamily TFs, one of the largest group of plant specific TFs important for plant growth, development and response to various biotic and abiotic stresses and b) a glycosyltransferase necessary for synthesis of important cellular molecules such as cell-wall polysaccharides.

A homology analysis of promoter regions of the ectomycorrhiza-induced AP2/ERF transcription factor was performed to identify the putative conserved DNA binding domains of the poplar gene and was also compared to that of the other genus. Subsequently, composite poplar plants were used for initial promoter analysis. For this purpose, the respective promoter fragments were amplified, truncated and cloned in a binary vector such a way that the promoter of interest controls the transcription of a peroxisomal localized super yellow fluorescent protein (sYFPPTS1). Fragments of various promoter lengths were then examined in composite poplars for any mycorrhiza induced difference in sYFPPTS1 expression.

In addition, the subcellular localization of the gene products of both mycorrhiza induced genes coding for a transcription factor and a glycosyltransferase were investigated. For this purpose, C-terminal fusion constructs with a sYFP were generated for the coding sequences (CDS) of both genes. The constructs were then transformed into *Nicotiana benthamiana* by Agrobacterium infiltration and the subcellular localization of the fusion protein was elucidated by confocal laser scanning microscopy (cLSM) in leaf cells.

2. Materials and Methods

2.1 Bioinformatics and *in silico* analysis

2.1.1 *In silico* identification of *P. trichocarpa* homologs

A set of *P. trichocarpa* based 60 bp oligonucleotide sequences, previously used for a DNA microarray analysis (Nehls et al. unpublished) were used for the investigation. These oligonucleotides were used to identify the best fitted *P. tremula x tremuloides* homologs in the *in silico* homology search. The most likely homolog of the other two species *P. tremula* and *P. tremuloides* were considered for those genes where a best matched homolog of the hybrid poplar could not be found. The NCBI BLAST program suite embedded in the Geneious software package (version 6.1.7, Biomatters Ltd., New Zealand) was used for the search and identification. Available genome, coding sequence and transcript databases of *Populus trichocarpa* and genome databases of *Populus tremula*, *Populus tremuloides*, *Populus tremula x tremuloides* were utilized for this *in silico* analysis (table 1).

Table 1: Sources and versions of different poplar databases used in this thesis.

Listed are the databases used in the present study. At the time of analysis only genome databases were available for *P. tremula*, *P. tremuloides* and *P. tremula x tremuloides*.

Organism	Source	Sequence	Version
<i>Populus trichocarpa</i>	Phytozome v9.1, http://www.phytozome.com	Coding region	V3.0210
		Genome	V3.0210
		Transcripts	V3.0210
<i>Populus tremula</i>	UPSC draft genome http://popgenie.org	Genome	V0001
<i>Populus tremuloides</i>	UPSC draft genome http://popgenie.org	Genome	V0001
<i>Populus tremula x tremuloides</i>	UPSC draft genome http://popgenie.org	Genome	V0001

The Megablast program within the Geneious software package was used to align the 60 bp oligonucleotide sequences to the available databases. The short oligomers were only used to identify the potential homologs, however for confirmation of the probable *P. tremula x tremuloides* homologs, a longer conserved sequence i.e. the coding region (CDS) of the respective *P. trichocarpa* gene was used.

The next step was to align the homologs from the *P. tremula*, *P. tremuloides* and *P. tremula x tremuloides* databases with the entire *P. trichocarpa* coding region (CDS) using the program “Geneious alignment”. Standard parameters were selected for the alignment (figure 5) along with a ‘Cost Matrix’ of 65% similarity, gap open penalty of 12, gap extension penalty of 3 and “Global alignment with free end gaps”. Some homologs were found in the 3’ to 5’ direction i.e. on the complementary strand; the software subsequently reversed and complemented the sequence to present a consensus sequence in the 5’ to 3’ direction. In such cases the sequence was automatically marked as “REV” (i.e. reversed) for the ease of identification while in general the 5’-3’ sequences are marked as “FWD” representing “forward”. The best matching *P. tremula*, *P. tremuloides* and *P. tremula x tremuloides* homologs were selected for further analysis.

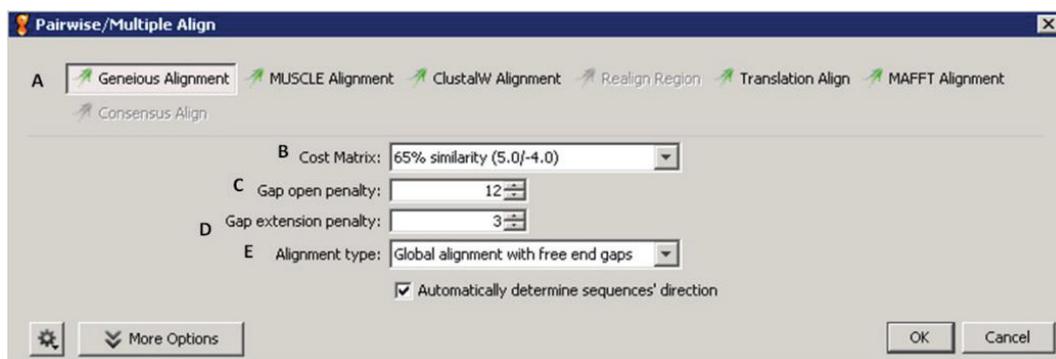


Figure 5: Parameters used for aligning a homolog found in the *P. tremula x tremuloides* genome database with the coding region (CDS) of respective *P. trichocarpa* gene.

Shown are the used alignment conditions: Geneious Alignment (A), Cost Matrix at 65% similarity (B), Gap open penalty of 12 (C), Gap extension penalty of 3 (D) and Global alignment with free end gaps as the alignment type (E).

2.1.2 Primer designing for real time qPCR

The best homolog sequences selected from *P. tremula*, *P. tremuloides* and *P. tremula x tremuloides* were used as template for primer designing. The integrated Primer 3 programme of the Geneious software package was used for the purpose (Untergasser et al. 2012).

Following strategies were chosen for maximum specificity in qPCR

1. **Desired amplicon length:** Shorter fragment gives higher PCR efficiencies in SYBR® green I based qPCR (Spandidos et al. 2010). The longer the amplicons, the more SYBR® green I are incorporated resulting in an increased signal

magnitude which in turn raises the background fluorescence. In this experiment, a region between 110 bp to 200 bp was selected for amplification.

2. **Area of specificity:** In general coding region (CDS) is highly conserved between different species. Choosing a less conserved region outside the CDS in 3' untranslated region (3' UTR) increases the specificity for a particular species. A maximum of 50 bp downstream from the 3' end of the coding sequence (3'-UTR region) were included for the qPCR amplification.

Keeping in mind the above-mentioned strategy, following guidelines were considered for the primer design (figure 6):

1. The length of the qPCR amplicon should be between 110 bp and 200 bp
2. The reverse primer should be outside the coding region, i.e. in the 3' UTR region.
3. The 3' end of the reverse primer should not be more than 50 bp from the 3' end of the coding sequence (CDS).

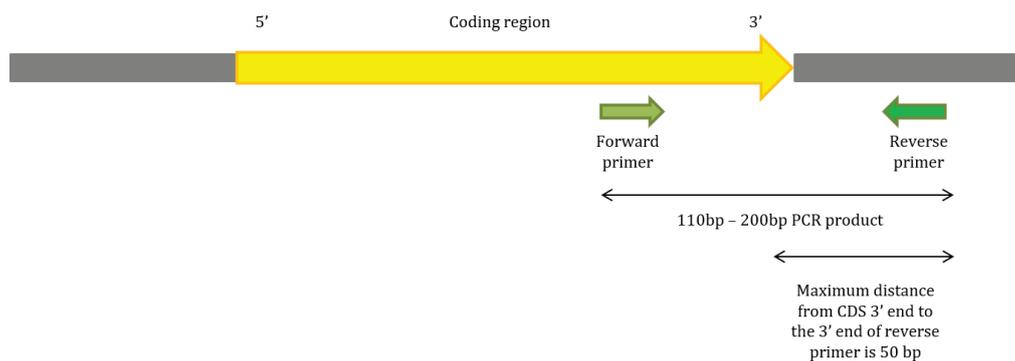


Figure 6: Strategies for qPCR primer designing.

110 bp and 200 bp were chosen as an optimal targeted PCR product. The reverse primers were placed downstream from the coding region, but not more than 50 bp away from the stop codon.

Other selected parameters were – a) primer length of 18-27 bp with an optimum of 20 bp, b) Optimum GC (Guanine-cytosine) content was 50 % with a range between 20 %-80 % and c) the melting temperature (T_m) of primer pairs was 57 °C – 63 °C with an optimum of 60 °C. For a primer pair maximum of 2 °C T_m difference was selected. A total of three alternative primer pairs were designed per gene. The primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany) and were dissolved in 5 mM Tris- HCl, pH 7.5 to a final concentration of 100 μ M for real time qPCR analysis.

2.2 Organisms and plasmids

2.2.1 Organisms

2.2.1.1 Bacteria

Escherichia coli

For cloning purposes *Escherichia coli* TOP 10 F- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*lacZ* Δ M15 Δ *lacX74 recA1 araD139 Δ (*araleu*)7697 *galU galK rpsL* (StrR) *endA1 nupG* (Invitrogen, Groningen, The Netherlands) was used. BL21 DE3 *fhuA2*[*lon*]*ompTgal*(λ DE3)[*dcm*] Δ *hsdS* λ DE3= λ *SbamH*Io Δ *EcoRI*Bint::(*lacI*::*PlacUV5*::*T7gene1*) *i21* Δ *nin5* (Merck Chemicals GmbH, Darmstadt, Germany) and Rosetta-gami F- *ompT hsdS_B*(*r_B* *m_B*) *gal dcm* (DE3) pRARE (Cam^R) (Merck Chemicals GmbH, Darmstadt, Germany) were utilised for protein over expression.*

Agrobacterium sp.

Agrobacterium tumefaciens C58C1 (Goodner *et al.*, 2001; Wood *et al.*, 2001) and GV3101 (kindly provided by Prof. Dr. Rita Groß-Hardt - Holsters *et al.*, 1980) were used to transform tobacco leaves for subcellular localisation. The following three *Agrobacterium rhizogenes* strains were analyzed for their capability to induce poplar composite plants: 1724, a mikimopine strain (Shiomi *et al.*, 1987); 2659, a cucumopine strain (Daimon *et al.*, 1990); 8196, a manopine strain (Hansen *et al.*, 1991) and 15834, an agropine strain (Mankin *et al.*, 2007). The *A. rhizogenes* strain K599 was further used for *in planta* promoter analysis.

2.2.1.2 Fungus

Poplar plants were ectomycorrhized using pre-grown *Amanita muscaria* HB2010 (collected and kindly provided by Annette Hintelmann, working group Nehls).

2.2.1.3 Plant

Populus sp.

RNA from poplar species *Populus tremula* L. \times *tremuloides* Michx., T89 (Tuominen *et al.*, 1995) was mainly used for all the molecular biology experiments (first strand c-DNA synthesis, genomic DNA isolation etc.) and the same poplar species was also further

used for *in planta* analysis along with *Populus tremula* × *alba* (No. 7171-B4, Institut de la Recherche Agronomique, INRA).

Nicotiana benthamiana

For subcellular localization of proteins, tobacco plants *Nicotiana benthamiana* (thanks to Prof. Dr. Hänsch, TU- Braunschweig, Germany) were used.

2.2.2 Plasmid

2.2.2.1 *Escherichia coli* transformation and expression vectors

Cloning vector pJet1.2/blunt

The cloning vector pJet1.2/blunt (GenBank/EMBL Accession number EF694056) was obtained from Thermo Fisher Scientific GmbH (Darmstadt, Germany).

Protein over expression vector – pET-28a

The protein expression vector pET28a (Novagen) was kindly provided by Dr. Frank Dietz (Biochemistry, University of Bremen). Target gene was cloned into the vector such that its expression is under the control of a T7 promoter and a Lac operator which can be induced upon IPTG addition. 3' Strep tag II is present enabling specific purification of the recombinant protein. The pET28a backbone contains a kanamycin resistance cassette and ColE1 pBR322 origin of replication.

2.2.2.2 Plant transformation vectors

Vectors for sub-cellular localisation of protein

pCXUN-sYFP is a pCAMBIA based modified vector (Nehls, unpublished), containing a maize ubiquitin promoter, ligase independent cloning (LIC) site and super yellow fluorescent protein (sYFP) in the T-DNA was used to determine the subcellular localisation of selected genes. The pCAMBIA backbone has a pBR322 origin of replication for maintaining high copy number of plasmid in *E. coli* and pVS1 replicon for high stability in *Agrobacterium*. The vector map is illustrated in appendix figure 39.

pPLV11 – Sharina I, a pGreen based modified vector (Nehls, unpublished) was used for the sub-cellular localisation in tobacco. The T-DNA contains a constitutive promoter, followed by a ligase independent cloning site and super yellow fluorescent protein

(sYFP). The pGreen backbone contains origin of replication both for *E. coli* and *Agrobacterium sp.* (ColE1 and pSa respectively), making DNA manipulation easier.

Vector for promoter analysis

pPLV11 – Sharina II, a pGreen based modified vector (Nehls, unpublished) was used for *in planta* promoter study. The T-DNA contains a peroxisomal targeted super yellow fluorescent protein (sYFP) which can be driven by the promoter of interest inserted into the multiple cloning site via either by ligase independent or conventional cloning. The pPLV11 (Genbank accession number JF909464) backbone contains ColE1 and pSa origin of replication for promoting high copy number of plasmids in *E. coli* and *Agrobacterium sp.* respectively. A 1056 bp long shutter fragment was inserted into the MCS, making DNA manipulation easier.

2.3 Culture of organisms

All cultures were maintained and propagated under sterile conditions. Different incubators were used for incubation of solid and liquid cultures (with shaker). Media and solutions were either autoclaved at 121 °C for 20 min under 15 psi or filter sterilised using 0.2 µm pore sized (Cellulose acetate membrane) filters (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), if heat sensitive. In case of light sensitivity (for e.g. Rifampicin), medium was stored and maintained in dark. All the used glass wares were also either autoclaved or dry heat sterilised at 180 °C for 4 hours.

2.3.1 Bacteria

E. coli was grown in LB medium at 37 °C (Lysogeny broth: 10 g/l peptone, 5 g/l yeast extract, 5 g/l NaCl), supplemented with respective antibiotics (ampicillin 100 mg/l, kanamycin 50mg/l and streptomycin 100 mg/l) and 18 g/l agar (if required).

All *Agrobacterium* strains were cultivated at 28 °C in CPY medium (5 g/l peptone, 5 g/l sucrose, 1 g/l yeast extract, 0.5 g/l MgSO₄ × 7H₂O and 15 g/l agar; pH 5.3 – 6.0). The medium was supplemented with antibiotics when required (50 mg/l kanamycin, 2.5 mg/l tetracycline, 40 mg/l gentamycin and 100 mg/l rifampicin).

2.3.2 Fungus

Stocks of *Amanita muscaria* were maintained in modified Melin Norkrans medium (MMN) (Marx, 1969; Kottke *et al.*, 1987) containing 50-100 mM glucose. For sub-

culturing and further propagation 3×3 mm excised agar blocks from edge of a growing colony was placed in the middle of a fresh MMN plate and were incubated at 18 °C for several months.

2.3.3 *Populus sp.* cultivation

P. tremula × alba and *P. tremula × tremuloides* were cultivated in MS6 (Murashige and Skoog medium) (Murashige & Skoog, 1962) and all steps were carried out in sterile condition under the laminar air flow. 150 ml sterile MS6 agar medium was poured into 0.5 l sterile glass jars. Tweezers and scalpels were sterilized by keeping them in a boiling water bath for 20 min. Three-four months old stock plants were used for the cultivation of the new plants. 3-4 cm long cuttings were made from the tip of the young stem. All other leaves were carefully removed from cuttings' end leaving 2-3 leaves near the apical bud and the cuttings were then transferred to the MS6 agar medium by carefully pushing the stem about 0.5 cm into the agar. The jars were then closed using cling film and clip followed by incubation at 16 h light/8 h dark cycle ($25 \mu\text{molm}^{-2} \text{s}^{-1}$) at 18 °C.

2.4 Molecular biological methods

2.4.1 Quantification of gene expression

2.4.1.1 *P. tremula × tremuloides* mRNA samples

Previously isolated (sample numbers 125-783 thanks to Prof. Dr. Uwe Nehls and 1163 - 1195 isolated by Annette Hintelmann, group Nehls) mRNA samples from non-mycorrhized (*P. tremula × tremuloides*) and mycorrhized fine roots (*P. tremula × tremuloides* / *Amanita muscaria* - strain ME II, AM4) were used for the experiment. The mRNA batches used for cDNA synthesis are listed in table 2.

Table 2: List of mRNA samples from mycorrhized and non-mycorrhized fine roots used for qRT-PCR.

The sample id denotes the identification number given by research group Nehls.

Serial number	mRNA id	Source	Concentration (ng/μl)
1	125	Mycorrhized root	2500
2	727	Mycorrhized root	2161
3	728	Mycorrhized root	2605
4	750	Mycorrhized root	986
5	752	Mycorrhized root	1252
6	774	Mycorrhized root	4700
7	783	Mycorrhized root	1390
8	1194	Mycorrhized root	847
9	1195	Mycorrhized root	891
10	729	Mycorrhized root	2990
11	738	Non-mycorrhized root	379
12	1163	Non-mycorrhized root	1064
13	1176	Non-mycorrhized root	543
14	1177	Non-mycorrhized root	1292
15	1189	Non-mycorrhized root	324
16	1190	Non-mycorrhized root	478

2.4.1.2 DNase treatment

Prior to cDNA synthesis, removal of genomic DNA contamination (if any) is necessary to avoid any erroneous RT-qPCR result. 1 μ l 10x reaction buffer (with MgCl₂), 1 μ l RNase inhibitor (RiboLock™ RNase, 40 U/ μ l) and 1unit peqGold DNase I (RNase free) were added to 100-200 ng RNA. The final volume was filled to 10 μ l with diethyl pyrocarbonate (DEPC) treated H₂O. The solution was mixed carefully by pipetting and incubated in a PCR machine set at 20 °C constant temperature for 15 minutes. 1 μ l 50 mM EDTA, pH 8.0 was added and incubated at 70 °C for further 8 minutes to ensure the inactivity of DNase at the higher temperature. The mixture was immediately placed onto ice. Aliquots of the DNase treated RNA were frozen in liquid nitrogen and stored at -80 °C.

2.4.1.3 cDNA synthesis

50-100 ng DNA free total RNA, 100 pmole Oligo d(T)18 primer, DEPC treated H₂O to a total volume of 12.5 µl were mixed together in a PCR tube and incubated at a constant temperature of 70 °C for 5 minutes. The tubes were immediately placed on ice for 5 minutes and spun down. 1 µl dNTPs (25 mM), 0.5µl RiboLock™ RNase (40 U/µl), 1 µl RevertAid Premium reverse transcriptase (200U/µl) and 4 µl 5x RevertAid buffer were mixed together carefully by pipetting and incubated at 25 °C for 10 minutes followed by 30 minutes at 50 °C. For the denaturation of reverse transcriptase, the reaction was finally terminated by heating the reaction at 85 °C for 5 minutes. Thereafter, 30 µl of 5 mM Tris-HCl, pH 8.0 was added into the solution, aliquots were made, frozen in liquid nitrogen and stored at -80 °C.

2.4.1.4 Real time quantitative PCR

Real time quantitative PCR is a very powerful tool for gene expression quantification, determination of the expression of a particular gene is possible by measuring the amount of available mRNA (Schmittgen & Livak, 2008). SYBR green I binds to the amplified double stranded DNA irrespective of their DNA sequence in the continuous PCR cycles (Ponchel *et al.*, 2003). In a Real time PCR the C_p is referred to the PCR cycle at which the fluorescent signal of the DNA binding reporter dye i.e. SYBR green I crosses the background fluorescence (Schmittgen & Livak, 2008).

10 µl 2x real time mix (Thermo Scientific), 0.2 µl primer mix (forward and reverse, 100 pmol/µl each), 9.3 µL DEPC treated H₂O and 0.5 µl prepared cDNA were mixed together in a 96 well qPCR plate (Roche Deutschland Holding GmbH, Grenzach-Wyhlen, Germany) and the plate was covered with transparent film. The RT-qPCR reactions were carried out in a Light Cycler 480 II (Roche Deutschland Holding GmbH, Germany) PCR machine. The thermocycler program was set for 42 cycles of the denaturation at 95 °C for 15 s, annealing at 52 °C – 60 °C for 30 s, elongation at 72 °C for 10 s; an initial denaturation and a final elongation was also set at 95 °C for 15 s and 72 °C for 10 s respectively. A dissociation curve (melting curve) was set in a range of 60 °C to 95 °C. Technical and biological replicates were maintained. Software Light Cycler 480 (version 1.5.1) was used for data analysis.

2.4.1.5 Quantification of gene expression using ubiquitin as standard

Primer pairs Pt_UBQ-for 5' GTTGATTTTTGCTGGGAAGC 3' and Pt_UBQ-rev 5'GATCTTGGCCTTCACGTTGT3' targeting 193 bp fragment of housekeeping poplar Polyubiquitin gene (Brunner et al. 2004) was used for calibration of mRNA content.

2.4.1.6 Detection of primer efficiency

The amplification efficiencies of the sample and reference genes are required to be similar for determination of relative gene expression (Liu and Saint 2002; Wong and Medrano 2005). It can be determined from standard curves and linear regressions (Pfaffl 2001; Hands et al. 2006).

PCR products of 4 replicates were combined, 1/10th volume 4 M LiCl, 2 volumes of 100% EtOH was added to it and carefully mixed by vortex. The samples were incubated for 30 min at room temperature followed by centrifugation at 16000 g for 20min. The supernatant was discarded and 500 µl 70 % ethanol was added, mixed and centrifuged at 16000 g for 15 min (room temperature). The pellet was dissolved in 20 µl Tris/HCl (5 mM, pH 8) and the DNA fragments were isolated by gel purification using NucleoSpin Gel and PCR Clean-up kit (Macherey and Nagel, Dueren, Germany). Concentration of the DNA fragment was measured using Nanodrop 1000 Spectrophotometers (Peqlab, Erlangen, Germany) and ImageJ (2014, version 1.48v, Wayne Rasband, National Institutes of Health). Dilutions of 10⁻³ -10⁻⁸ were generated and qPCR was performed. From the initial concentration and the dilution factors a standard curve was generated. The amplification efficiency was calculated (figure 7) from the slopes of linear regressions using the formula $E_{\text{efficiency}} = 10^{(-1/\text{slope})}$. Slopes ranging between -3.2 to 3.5 indicate optimal PCR conditions giving nearly 100 % amplification efficiency (Wittwer et al. 2001; Pfaffl2001; Hands et al. 2006).

$$\text{Relative gene expression ratio} = \frac{\text{Efficiency}_{\text{target}}^{\text{Cp target (calibrator - sample)}}}{\text{Efficiency}_{\text{reference}}^{\text{Cp reference (calibrator - sample)}}$$

$$\text{Relative gene expression ratio of } Potri.007G095000 = \frac{1.919^{(34.28 - 25.63)}}{1.742^{(26.95 - 26.91)}} = 275.42$$

Figure 7: An example of calculating relative gene expression ratio.

Shown is the calculation of relative gene expression ratio for *Potri.007G095000*. Cp values and amplification efficiencies of *Potri.007G095000* and reference gene polyubiquitin are used. Sample and calibrator are mycorrhiza and non-mycorrhized fine roots, respectively.

2.4.2 Isolation of nucleic acids

2.4.2.1 Isolation of total RNA

Total RNA was isolated from singly picked transformed and non-transformed poplar roots. RNase free lab space and materials were used for the entire isolation procedure. The roots were flash frozen in liquid N₂ and grounded to fine powder using mortar and pestle. Total RNA was isolated using NucleoSpin RNA Plus (Macherey and Nagel, Dueren, Germany) according to manufacturer's user manual. The isolated RNA was checked by gel electrophoresis.

2.4.2.2 Isolation of genomic DNA

A modified pre-established method of J J Doyle (Doyle, 1987) was used for genomic DNA isolation. Around 0.5 g leaf tissue was grounded to fine powder using mortar and pestle under liquid nitrogen. Preheated (60⁰C) 2.5 ml 2 x CTAB-isolation buffer (2 % CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, pH 8.0 and freshly added β-mercaptoethanol) was added to the grounded powder and homogenized by pestle agitation. The suspension was equally distributed into three 1.5 ml reaction tubes and incubated at 60⁰C for 30 min under gentle agitation. 600 μl chloroform/isoamylalcohol (v/v: 24/1) was added, mixed carefully and centrifuged at 3800 g for 10 min at room temperature. Thereafter, the DNA containing upper aqueous phase was transferred to a fresh reaction tube using a wide-bore pipette tip to avoid DNA shearing. 2/3 volume (of the previously transferred suspension) ice cold isopropanol was added and the reaction tube was inverted gently for nucleic acid precipitation. The DNA was spun down at 240 g for 1 min at room temperature. The supernatant was then carefully removed and 600 μl wash buffer (76 % ethanol and 10 mM ammonium acetate) was added, incubated for 20 min at room temperature with a gentle agitation, followed by centrifugation at 240 g for 20 min at 4⁰C. The supernatant was carefully discarded, the pellet was air dried at room temperature for 5 min and re-suspended thereafter in 150 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). RNase A was added to remove any RNA contamination, to a final concentration of 10 μg/ml and incubated at 37⁰C for 30 min. Nucleic acid was precipitated by incubating at -20⁰C for 30 min after adding 15 μl of 4 M LiCl and 300 μl

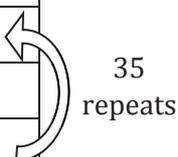
ice cold ethanol. DNA was then spun down at 4 °C, 3800 g for 5 min; the supernatant was discarded, followed by removal of salts by washing the DNA with 500 µl 70 % ice cold ethanol and centrifuging at 4 °C, 3800 g for 5 min. The ethanol was removed, the pellet was air dried at room temperature and finally re-suspended in 50 µl TE buffer. The isolated genomic DNA was stored at 4 °C for future usage.

2.4.3 PCR amplification of DNA fragments

Duplicate PCR reactions of 25 µl each were carried out in a thermocycler machine (Peqlab Biotechnologie GmbH, Erlangen, Germany or Biometra GmbH, Goettingen, Germany). The PCR reactions were prepared as followed: 5 µl 5x reaction buffer (New England Biolab, Frankfurt, Germany), 0,5 µM of each forward and reverse primer, 0.2 mM dNTP mix, 100 ng of genomic DNA or 20 ng of plasmid DNA/cDNA and 0.02 U/µl proof-reading Taq polymerase (Phusion® DNA polymerase, New England Biolab, Frankfurt, Germany) were mixed carefully in 0.2 ml PCR tubes. The PCR tubes were kept on ice during the preparation until they were placed in a preheated thermocycler. PCR conditions were optimized for maximum PCR efficiency. An online melting temperature (T_m) calculator tool (New England Biolab, Frankfurt, Germany) was used for first basic calculation of the annealing temperature. Elongation time was calculated according to the length of the fragment. Approximately 20 s of elongation time per 1 Kb fragment (but never exceeding 3 min) were used for the PCR reaction. The PCR product was checked by agarose gel electrophoresis.

Table 3: Condition for PCR amplification. For individual product PCR was further optimized.

Cycle	Function	Time (min)	Temperature (°C)
1	Initial denaturation of template DNA	00:30 (for genomic DNA: 3 min max)	98
2	Denaturation	00:15	98
3	Primer annealing	00:20	52-60
4	Elongation	Maximum 03:00	72
5	Final Elongation	05:00	72



2.4.4 Agarose gel electrophoresis

Gel electrophoresis was performed in 1 % agarose gels (2 % agarose gels for qPCR shorter fragments). 1 g of agarose (Biozym Scientific GmbH, Oldendorf, Germany) was

molten in 100 ml of 0.5 x TAE buffer. The dissolved agarose was cooled down approximately to 60 °C before pouring it into a gel casting tray with a comb to create wells for loading the samples. The comb was removed after the agarose gelatinized and the gel was placed in an electrophoresis chamber filled with 1 x TAE buffer. 1 µl of each of the PCR products (5 µl in case of qPCR fragments) were mixed with 1 µl of 6 x loading dye and 4 µl of dist. H₂O before running them into an 1 % agarose gel with a voltage of 60 V for 40 min. Low and high molecular weight DNA marker (“Gene Ruler™ 100 bp DNA ladder plus” from Fermentas and “Phage Lambda DNA/*Sty*I Marker” from Bioron) was also run along with the samples.

After gel electrophoresis, the DNA was visualized by incubating the gel in freshly prepared 0.5 µg/ml Ethidium Bromide bath for 15 min. Ethidium bromide being an intercalating agent, enters the grooves of the DNA molecule, fluoresces when exposed under UV light (252-366 nm) and emits at 590 nm (Lottspeich and Engels 2012). An UV transilluminator was used for the visualization of the resulting DNA bands and then photographed using an image capturing device (Doc Print, Vilber Lourmat) at 312 nm wavelength.

2.4.5 Determination of nucleic acid concentration

2.4.5.1 Densitometric quantification of DNA

Quick and easy quantification of small amounts of DNA is possible with agarose gel analysis. The concentration of the DNA can then be estimated by comparing the band intensity either visually (Sauer *et al.*, 1998) or using a imaging system or software, i.e. brighter electrophoretic bands will indicate higher yield of the nucleic acid (Bruns *et al.*, 2007).

The documented agarose gel after the electrophoresis was analysed using the software ImageJ (National Institutes of Health) (<https://imagej.nih.gov/ij/>) for DNA quantity estimation. The image was first edited using Adobe Photoshop Elements 2.0 (Adobe Systems Incorporated, California, USA) to adjust the height of the bands such a way that a band of known quantity (e.g. a band of the DNA marker) can lie exactly next to the sample band, followed by colour inversion. Area of the bands was selected, the lanes were plotted and background was eliminated. Lastly the software determines the area of

the peaks which then can be used to calculate the DNA concentration simply by comparing it with a DNA marker band of known concentration.

2.4.5.2 Photometric quantification of DNA

Nucleic acids (e.g. DNA and RNA) absorb UV light maximally at 260 nm wavelength owing almost completely to its constituent bases. Therefore, the concentration of DNA or RNA molecules can be determined by spectrophotometric measurement of the absorbance at 260 nm ($OD_{260} = 1$ equals to 50 $\mu\text{g/ml}$ double stranded (ds) DNA and 40 $\mu\text{g/ml}$ RNA); higher the absorbance values (A_{260}), greater are the yields (Bruns *et al.*, 2007).

The photometric quantification was done using a spectrophotometer (Nanodrop 1000 - Peqlab Biotechnologie GmbH, Erlangen, Germany) and analysis was done using the software NanoDrop ND-1000 V3.7.1. The blank was set using the same solution used to dissolve the purified DNA to ensure an accurate calculation, then 1.5 μl of the sample was loaded and the absorbance (A_{260}) was measured. The automatically calculated $A_{260/280}$ ratio was also noted for confirming the purity of isolated fragments. The fragments were stored in $-20\text{ }^{\circ}\text{C}$ for future use. Products revealing an $A_{260/280}$ ratio of approximately 1.8 and 2.0 indicating purity (i.e., protein free) of DNA and RNA respectively (Wilfinger *et al.*, 1997) were used.

2.4.6 Cloning of DNA fragments

2.4.6.1 DNA digestion

Analytic restriction digestion was performed in a total volume of 20 μl with approximately 1 μg of plasmid DNA, 4-10 U of selected restriction enzyme per reaction (Thermo Scientific GmbH, St. Leon-Rot, Germany and New England Biolabs GmbH, Frankfurt, Germany) and 2 μl 10 x enzyme specific buffer at respective temperature for 2-3 hours. For preparative purpose 3-5 μg plasmid DNA was digested with 10-15 U restriction enzyme in a total volume of 50 μl and was incubated at desired temperature for 4-5 hours.

2.4.6.2 Isolation of the DNA fragments from agarose gel

For purification of DNA bands, a fresh 1 % agarose gel was prepared with a broad comb on a cleaned casting tray. The total volume of the PCR reaction was loaded onto the gel

and high molecular weight marker was loaded leaving at least one lane gap between the product and the marker ensuring the ease of subsequent excision. After separation of the DNA bands at 60 V for 40 minutes, the gel was incubated in a freshly prepared Ethidium bromide bath for 10 min. DNA fragment was carefully excised from the agarose gel, keeping under 312 nm UV illumination using a fresh scalpel. The cut out gel pieces were transferred into a 2 ml reaction tube (Sarstedt, Nümbrecht Germany) and purified using the NucleoSpine Extract II kit (MACHEREY-NAGEL GmbH & Co. KG, Dueren Germany) according to the manufacturers' user instruction.

2.4.6.3 Ligation of DNA fragments into plasmids

In order to prevent self-ligation of a linearized plasmid, removal of 5' phosphate is necessary in case of single enzyme based integration. De-phosphorylation of linearized plasmid DNA was conducted with approximately FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific GmbH, Germany) according to manufacturer's protocol. DNA containing a 5' hydroxyl terminus (for e.g. PCR products amplified using primers without a 5' phosphate) had to be phosphorylated prior to ligation. Phosphorylation was conducted using T4 Polynucleotide kinase (New England Biolab GmbH, Frankfurt, Germany) according to manufacturer's user manual.

DNA fragments with sticky ends were ligated at 21 °C for 2-3 hours in a total reaction volume of 20 µl with T4 DNA ligase according to user manual (New England Biolabs GmbH, Frankfurt, Germany). Depending on the size, vector/insert ratios between 1:3 and 1:4 were used. Sticky ends were modified to blunt ends when required, using blunting enzymes provided in CloneJET PCR cloning Kit (Thermo Fisher Scientific GmbH, Germany) according to user manual. Blunt ended ligation was performed using vector/insert ratios in a range of 1:1-1:3 and the ends were ligated using T4 DNA ligase in a total volume of 20 µl according to manufacturers' user instruction (CloneJET PCR cloning Kit, Thermo Fisher Scientific GmbH, Germany).

2.4.6.4 Isolation of Plasmid

Plasmid isolation from *Escherichia coli*

Single *E. coli* colonies grown on antibiotic resistant plate were inoculated in 3 ml LB medium supplemented with selected antibiotics and incubated overnight at 37 °C in a shaker (200 rpm). Around 12 single plasmids carrying colonies were used per construct.

Alkaline lysis is most frequently used for lysing the bacterial cells prior to plasmid isolation (Birnboim & Doly, 1979; Birnboim, 1983). 2 ml of each overnight culture was centrifuged in 2.2 ml reaction tubes for 5 min at 8000 \times g and the precipitated cells were re-suspended in 300 μ l solution 1 (50 mM Tris-HCl, pH 7.5; 10 mM EDTA and 0.1 mg/ml RNase A). After that 300 μ l of solution 2 (1% w/v SDS and 200 mM NaOH) was added to the cell suspension, carefully mixed by inverting the tubes several times followed by 5 min incubation at room temperature. Sodium dodecyl sulphate (SDS) leads to cell lysis and release of the cell contents by solubilising the phospholipid and protein components of the cell membrane; NaOH present in the solution denatures proteins, chromosomal and plasmid DNA; on the other hand RNase A digests the released RNA during the cell lysis. Then 200 μ l of ice cold buffer 3 (1.5 M potassium acetate, pH 4.8 adjusted with acetic acid) was added and incubated for 25 min on ice after mixing well by carefully inverting the tubes several times. Acidic potassium acetate neutralizes the lysate; potassium dodecyl sulfate (KDS) precipitates due to the high salt concentration. Denatured proteins, chromosomal DNA along with cell debris are then co-precipitated in the insoluble salt detergent complexes by centrifuging for 10 min in 4 $^{\circ}$ C at V_{max} (18,000 \times g). Being circular and covalently closed, plasmid DNA renatures therefore resides in the supernatant. This clear lysate is then transferred into fresh 1.5 ml reaction tube, 600 μ l of ice cold 100% (v/v) isopropanol was added to it, mixed well by vortexing. After incubating for 1 hour at -20 $^{\circ}$ C, the tubes were centrifuged at 4 $^{\circ}$ C for 10 min at V_{max} (18,000 \times g). Isopropanol concentrates the DNA by precipitation. The supernatant was discarded, and the pellet was washed with 500 μ l 70 % (v/v) ethanol by centrifuging at room temperature for 5 min at V_{max} (18,000 \times g). Ethanol was discarded, and the pellet was air dried approximately for 15-20 min. The pellet was then dissolved in 50 μ l of 5 mM Tris-HCl (pH 8.0) and incubated at 65 $^{\circ}$ C for 5 min to remove any remaining DNases. The plasmids were then checked by agarose gel electrophoresis and the concentration of plasmid was estimated by spectrophotometric quantification. The plasmids were stored at -20 $^{\circ}$ C for future use.

Further salt precipitation was performed using 4 M Lithium chloride (LiCl) to obtain better quality of plasmid DNA for Sanger sequencing. 1/10 and 2 volumes of 4 M LiCl and 100 % (v/v) ethanol were added to the dissolved plasmid respectively. The mixture was then mixed well and incubated at room temperature for 1 hour followed by centrifugation at V_{max} (18,000 \times g). The supernatant was discarded and the precipitated

plasmid was then washed with 500 μ l 70 % (v/v) ethanol and centrifuged at room temperature for 5 min at V_{max} (18,000 \times g). Ethanol was removed, the pellet was air dried and finally dissolved in 30 μ l of 5 mM Tris-HCl (pH 8.0). The isolated plasmids were tested for quality by gel electrophoresis before storing at -20 $^{\circ}$ C.

Plasmid isolation from *Agrobacterium sp.*

Plasmids were isolated from *Agrobacterium sp.* to confirm the insertion of the desired construct. The above mentioned method was used for the plasmid isolation, followed by transformation of chemically competent *E. coli* cells with the isolated plasmids. Plasmids were then isolated from these transformed *E. coli* cells and checked with restriction digestion (with respective restriction enzymes) for further confirmation of the inserted clones.

2.4.7 Confirmation of isolated plasmid

2.4.7.1 Restriction analysis

Suitable construct was checked by performing restriction digestion. The virtual constructs in Geneious software were analysed for the best fitting restriction enzyme that digest both inside the multi cloning site (containing insert) and vector back bone, preferably once each. In some cases, more than one restriction enzyme was used for the analysis.

2.4.7.2 DNA sequencing

For further confirmation, the selected constructs were sequenced, e.g. pJet1.2 forward and reverse primers were used for sequencing any insert cloned into pJet1.2. 500 ng plasmid DNA (250 ng in case of PCR product) and 25 pmol primer were mixed carefully in a fresh 1.5 ml reaction tube and samples were sent to Macrogen Europe (Amsterdam, Netherland) for sequencing. The delivered sequencing result was later verified with the desired virtual construct sequence using Geneious software package. The quality of the sequencing result was checked using BioEdit software (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>).

2.4.8 Transformation of *Escherichia coli*

2.4.8.1 Preparation of chemically competent cells

Three *E. coli* strains (Top10 F', BL21 DE3 and Rosetta-gami) were made chemically competent for future transformation according to the following protocol. A single colony was inoculated in 3 ml LB medium supplemented with selected antibiotics and incubated over night at 37 °C under continuous agitation (200 rpm). 250 µl of the overnight culture was added to 250 ml LB medium and incubated at 37 °C in a rotary shaker (220 rpm) until the OD₆₀₀ reaches approximately 0.4-0.6. The bacterial culture was then centrifuged at 5000 rpm at 4 °C for 5 min followed by the addition of 100 ml ice cold TFBI (30 mM KOAc, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂, 15 % glycerol). The cells were gently re-suspended and incubated on ice for 5-10 min. All the used tubes, flasks etc. were chilled on ice for further steps. The suspension was centrifuged again at 5000 rpm for 5 min at 4 °C, the supernatant was discarded, and the pellet was re-suspended in 10 ml TFBII (10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl, 15 % glycerol). The cells were incubated on ice for 60 min; aliquots of 50-100 µl were made, flash frozen in liquid N₂ and stored at -80 °C.

2.4.8.2 Transformation of chemically competent *E. coli*

Aliquots of competent *E. coli* cells were thawed on ice and the following mixture was prepared on ice: 20 µl KCM solution (100 mM KCl, 30 mM CaCl₂, 50 mM MgCl₂), up to 20 µl of the ligation reaction (or 100 ng of plasmid), the total volume was made up to 100 µl with autoclaved double distilled H₂O and was added to the cells by carefully pipetting up and down few times. The tube was then incubated on ice for another 20 min, followed by a 2 min heat shock at 42 °C in a preheated thermo block (Helmut Saur Laborbedarf, Reutlingen, Germany or Eppendorf AG, Hamburg, Germany). 500 µl LB medium was added and cell suspension was incubated at 37 °C with low shaking. After one hour of incubation, the cells were precipitated at 8000 ×g for 5 min, 700 µl of the supernatant was removed; the pellet was re-suspended in rest of the supernatant and the suspension was spread onto LB agar plate containing selected antibiotics using a sterile glass rod, finally the plates were incubated over night at 37 °C.

2.4.9 Transformation of *Agrobacterium sp.*

2.4.9.1 Preparation of electrocompetent cells

Agrobacterium sp. cells were made electrocompetent for transformation. A single colony was used for the inoculation of 3 ml LB medium (supplemented with strain specific antibiotic) and incubated at 28 °C continuous shaking at 180 rpm. 500 ml LB-medium was inoculated with the 3 ml pre-culture and was incubated at 37 °C (180 rpm) until an OD₆₀₀ of 0.8. The culture was centrifuged at 4000 rpm for 10 min at room temperature. The pellet was suspended in 50 ml sterile ice cold 10 % glycerol, followed by a spin down for 20 min at 4000 rpm. This step was repeated thrice, and the pellet was finally suspended in 10 ml ice-cold 10 % glycerol, aliquots (100 µl each) were made in previously chilled 1.5 ml tubes, flash frozen in liquid nitrogen and stored at -80 °C for future use.

2.4.9.2 Transformation of electrocompetent *Agrobacterium sp.*

Aliquot of electro-competent *Agrobacterium sp.* was thawed on ice and 1 µg of plasmid DNA was carefully mixed by pipetting. The competent cell-DNA mix was transferred into pre-cooled electroporation cuvette kept on ice. Electroporation was performed at 400 Ohm, 25 µF and 1.8 KV (using Bio-Rad gene Pulser II and Pulse controller plus, *Bio-Rad* Laboratories GmbH, München, Germany) for around 5 sec and 600 µl LB medium was added to it. The mixture was then transferred to a fresh tube and incubated at 28 °C in a shaker. After 1 h the culture was spread onto CPY medium supplemented with 50 µg/ml Kanamycin and incubated at 28 °C overnight.

2.5 Heterologous expression of protein

2.5.1 Overexpression of strep-tagged recombinant proteins by *E. coli*

Proteins were overexpressed using pET28a-Strep tag vector (kindly provided by Dr. Frank Dietz, Biochemistry, University of Bremen), where the protein is expressed under the control of T7 promoter which can be controlled using IPTG. 50-100 µl BL21-DE3 and Rosetta-gami *E. coli* was transformed with 50 ng plasmid DNA. 5 ml culture supplemented with selected antibiotics (50 µg/ml Kanamycin for BL21-DE3; 50 µg/ml Kanamycin and 25 µg/ml for Rosetta-gami) was grown at 37 °C in a rotary shaker until OD₆₀₀ of 0.7-0.8. Protein over-expression was induced using different IPTG

concentration before incubating the tubes at different temperatures for a varied time span (table 4). Uninduced controls were maintained for comparison. Post induction cells were harvested at 5000 ×g for 10 min at 4 °C. Cells were then either stored at -20 °C or further processed to obtain clear lysates.

Table 4: Different conditions used for protein overexpression expression.

Conditions	Parameter Range
Incubation time	4 hours – Over night
Incubation temperature	14 °C- 37 °C
IPTG concentration	0.005 μM-1 mM
Cell lysis technique	French Press and sonication

2.5.2 Preparation of cell lysate

The cell pellets harboring the expressed protein were suspended in chilled Tris-HCL buffer (25 mM Tris-HCl, 300 mM NaCl, 0.1% Triton X100, pH 8) unless otherwise. Suspensions were sonicated (using an Ultraschall Desintegrator Branson Sonifier II W 250, Branson) multiple times at 30-watt output, 10 s each keeping on ice. The tubes were allowed to cool down by in between each sonication. 20 μl crude extract was collected and stored for comparison before centrifuging at 16000 ×g for 10 min at 4 °C. Cell lysate was also collected using French press (French Press SLM Aminco). The cell suspension (as mentioned above) was placed inside a pre-chilled pressure-cell of French press and was exposed to 76 Mpa pressure for breaking the cells. The lysate was collected back in tube, 20 μl crude extract was taken for storage and cell debris was pelleted out via centrifugation (16000 ×g, 10 min, and 4 °C).

2.5.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

10 % polyacrylamide separating gel (using Rotiphorese® Gel 30 37, 5:1) was first poured carefully into the gap between two glass plates (Biometra GmbH, Göttingen, Germany) sealed along bottom and sides, allowing the gel to polymerize. A layer of dist. H₂O was added on the top to minimize evaporation. After the gel was polymerized, the H₂O layer was discarded, the 5 % polyacrylamide stacking gel was poured on top and a comb was inserted for loading wells formation.

The stacking gel was allowed to polymerize before the protein samples were loaded. Protein samples were prepared for loading using 4x loading dye (8 % w/v SDS, 12 %

w/v Sucrose, 0.008 % w/v Bromophenol blue and freshly added 2 % v/v 2-Mercaptoethanol) to a final concentration of 1x. Samples were heated at 80 °C for 5-10 min before loading on to the SDS polyacrylamide gels. A lower molecular weight marker (GE Healthcare Europe GmbH, Freiburg, Germany) was also added for comparison. Gels were run at 100-130 V for 1-2 h in a protein gel electrophoresis chamber (Biometra GmbH, Göttingen, Germany).

Table 5: Composition of 10 % separating and 5 % stacking gel

	10 % Separating gel	5 % Stacking gel
Tris-HCl (pH 8.8)	375 mM	--
Tris-HCl (pH 6.8)	--	125 mM
Acryl/bisacrylamide (30:0.8)	10 % (v/v)	5 % (v/v)
SDS	0.1 % (w/v)	0.1 % (w/v)
Tetramethylethylenediamine (TEMED)	0.08 % (v/v)	0.1 % (v/v)
Ammonium persulfate (APS)	0.05 % (w/v)	0.05 % (w/v)

2.6 Generation of composite plants

The ability of *Agrobacterium rhizogenes* to induce root formation was utilized to create so-called composite poplar plants where transgenic roots are induced from untransformed shoots (Neb et al. 2017). These plants were propagated on MS6 agar plates following sterile conditions under a clean bench. Half of the MS6 medium was removed from the petri dish to make room for a poplar cutting. Sterile poplar shoot cuttings containing 2-3 leaves were then dipped into a freshly grown *Agrobacterium rhizogenes* colony and inserted into halved MS6 medium. After three days of incubation (16 h light, 23 °C), the cuttings were then transferred into fresh MS6 agar containing 1.18 mM carbenicillin and 0.52 mM cefotaxime to inhibit *Agrobacteria* growth. Two sterile cotton plugs were also provided to soak the extra moisture inside the petri dish. The plates were then secured with parafilm and incubated in an upright position at 23 °C (16 h light/8 h dark cycles).

2.7. Ectomycorrhization

5-7 small agar blocks from freshly grown *A. muscaria* plates were placed on a cellophane membrane resting on MMN medium with reduced glucose concentration (10 mM), such a way that the fungus can grow accessing the nutrients of the medium through the membrane, which can later be easily transferred. For mycorrhization, sugar free MMN medium with reduced concentration of phosphate was used, upon which the *A. muscaria* grown membrane was transferred and a hole was made into the petri dish with the help of a sterile hot scalpel. Poplar cuttings with small roots were transferred on to the membrane such a way that the shoot can pass through the hole letting only the root system enclosed inside the petri dish; a sterile charcoal paper and cotton plugs were placed; finally the hole (around the shoot) was sealed using sterile Silicones paste (GE Bayer Silicones GmbH & Co. KG, Leverkusen, Germany) before securing the plate with parafilm. The plates were then incubated (12 h light, 18 °C) in up righted position inside a mini greenhouse flooded with 1 % copper sulphate which maintains a sterile moist condition.

2.8. Leaf infiltration of *Nicotiana benthamiana*

Sub-cellular localisation of a protein is a fundamental step in protein functional analysis. *Agrobacterium sp.* is known to mediate transfer of transgenes from the T-DNA of bacterial Ti plasmid into the plant cells when infiltrated into plant leaves as a liquid culture and expresses the transgene in the infiltrated region (Kapila *et al.*, 1997).

Constructs were introduced into *Agrobacterium sp.* (*Agrobacterium tumefaciens* strain C58C1 and GV3101, *Agrobacterium rhizogenes* K599) by electroporation. Along with the gene of interests, transgenic *A. tumefaciens* C58C1 containing a plasma membrane located protein fused to a red fluorescent protein (DsRed) was used as a positive marker. The level of transgene expression is known to peak at 60-72 h post infiltration and declines thereafter due to post transcriptional gene silencing (PTGS) in plants (Johansen & Carrington, 2001); therefore p19, a tomato bushy stunt virus protein (Voinnet *et al.*, 2003) was co-infiltrated to reduce RNA interference.

Single colonies of these recombinant bacteria were grown for 24 h at 28 °C (180 rpm) in 50 ml conical flask containing 10 ml CPY medium supplemented with respective antibiotics (50 µg/ml Kanamycin for *Agrobacterium tumefaciens* strain C58C1 and *Agrobacterium rhizogenes* K599; 2.5 µg/ml Tetracyclin, 40 µg/ml Gentamycin,

100 µg/ml Rifampicin and 50 µg/ml Kanamycin for *Agrobacterium tumefaciens* GV3101). Cells were then harvested by centrifugation (4000 ×g for 20 min at 22 °C) and suspended in a freshly prepared activation medium (10 mM MES, 10 mM MgCl₂ and 150 µM acetosyringone) to a final concentration corresponding to an optical density (OD) of 0.3 at 600 nm (Voinnet *et al.*, 2000). Cultures were incubated at 28 °C in a shaker (180 rpm) for 3 h, followed by mixing the different constructs in a 1:1:3 ratios for plasma membrane positive control, p19 and gene of interest respectively before infiltration. Bottom side of a three-four weeks old healthy *N. benthamiana* leaf was infiltrated using 5 ml syringe, applying counter pressure on the upper side of the leaf. Two leaves per plant were used for each construct and the plants were incubated for two-three days at 22 °C with 12 h light.

2.9 Microscopic analysis

2.9.1 Preparation of root samples

4 % agarose was prepared and placed onto a preheated plate at 60 °C until the agarose cools down to reach the plate temperature. Holes were made at the bottom of a 50 µl PCR tube and it was filled with 4 % agarose using a Pasteur pipette. A selected root was placed inside the tube and kept in a vertical position using a needle. The tube was then placed into a pressure vial and pressure was applied turning the screws clockwise to remove air bubbles adjacent to the root. After 10 min the agarose embedded root was taken out and the upper and lower part of the agarose was removed using a scalpel for a parallel surface. A drop of 4 % agarose was placed onto a preheated vibratome fixation plate and the agarose block containing root was placed on it such that the root stays vertical to the plate surface. The root was inspected under a microscope after the agarose had solidified and placed onto the object carrier of microtome Leica VT1000S (Wetzlar, Germany). 50 µm slices were made, placed onto glass slide together with a drop of water, cover slips were placed on top and checked under microscope. The prepared slides were then stored inside a petri dish consisting a wet tissue paper to keep the root cross section from drying.

2.9.2 Preparation of leaf samples

Small square sections were cut near the infiltration site of a leaf using a scalpel. These sections were then placed on a glass slide containing a drop of water and cover slips

were placed on top to keep the section in place. The slides were then placed inside a petri dish containing a wet tissue paper to avoid leaf tissue drying until checked under the microscope.

2.9.3 Visualization of samples under microscope

Transformed leaves or roots were checked with YFP and RFP channel (co-infiltrated leaves) under the Stereo microscope (Leica-MSV269, Germany) for transformed roots and potential area of transformation around the infiltrated region on tobacco leaf. Selected leaf/root sections were then illuminated under confocal laser scanning microscope (LSM 780/ ELYRA PS.1, thanks to Andreas Ellrott, Max Plank Institute of Marine Microbiology, Bremen) at 488 nm and 561 nm for the excitation of sYFP and RFP respectively. sYFP was detected within 499-561 nm, RFP 588-623 nm. For the detection of chlorophyll derived autofluorescence in leaves, a detection window of 663- 706 nm was used.

3. Results

3.1 Development of a protocol for the formation of composite poplar plant (collaboration with D. Neb, group Nehls)

An important prerequisite for gene function analysis is the generation of transgenic plants. In the present study, an *Agrobacterium rhizogenes* mediated root transformation protocol was developed for further root centred research.

3.1.1 Root induction by different *Agrobacterium rhizogenes* strains

Although the host specificity of *Agrobacterium rhizogenes* is limited to few plant species in nature, it can further be extended to other plant species under laboratory conditions (Porter, 1991). To test strain dependent differences in transformation efficiency, four different *A. rhizogenes* strains - 1724, a mikimopine strain (Shiomi et al., 1987); 2659, a cucumopine strain (K599) (Daimon et al., 1990); 8196, a manopine strain (Hansen et al., 1991) and 15834, an agropine strain (Mankin et al., 2007) were analysed. The strains were tested for their capacity of root formation in about one-month old *Populus tremula* × *alba* cuttings. Two independent batches of 20 plants each were analysed. All four *A. rhizogenes* strains were first transformed with binary vector pBIN19-YFPPTS1 (Nowak, et al. 2004), where the strong double 35S promoter of Cauliflower mosaic virus (CaMV) controls the expression of peroxisomal targeted yellow fluorescent protein (YFP). After inoculation with single bacterial strains, the plants were monitored for 8 weeks for the following factors- a) hairy root phenotype of the roots, b) root formation rate, c) number of formed roots per plant and d) transformation efficiency of the binary vector T-DNA.

3.1.1.1 Analysis of the hairy root phenotype induced by *A. rhizogenes*

Roots revealing a 'hairy root' phenotype can easily be distinguished from the wild roots by their reduced gravity perception (figure 7). All the tested four *A. rhizogenes* strains were able to induce hairy roots in poplar cuttings and developed microcalli at the infected shoot surface prior to root formation. Incidence of micro callus formation was further tested with *Agrobacterium* strain K599 (figure 8).

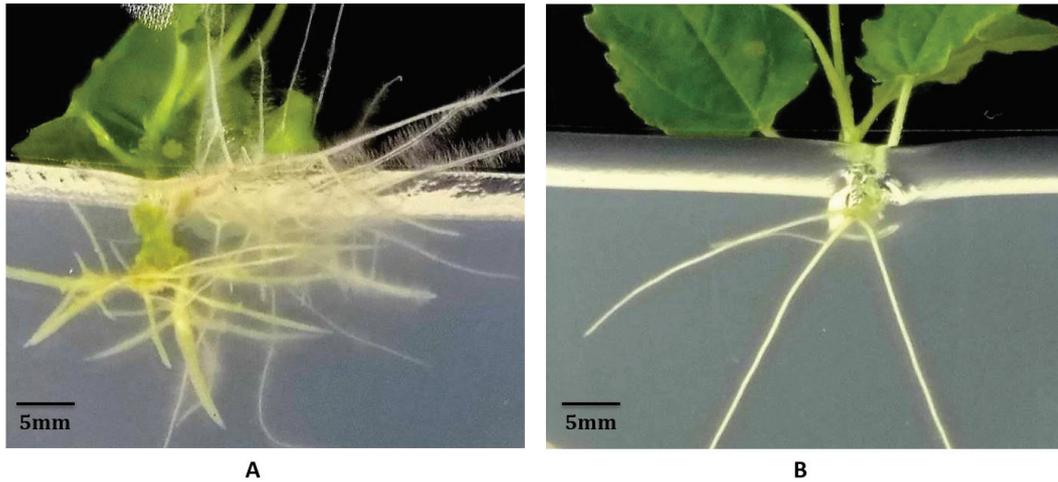


Figure 7: Comparison of roots formed by *A. rhizogenes* infected (A) and wild type (B) poplar.

Shown are typical cuttings of *P. tremula x alba* either *A. rhizogenes* infected (A – strain 15834) or non-inoculated (B) after four weeks of incubation.

Microcalli development prior to transgenic root formation

One-month old *P. tremula x alba* cuttings were inoculated with *Agrobacterium* strain K599 containing pBIN19-YFPPTS1 (Nowak, et al. 2004) along with sterile water inoculated controls, 20 cuttings each. The cuttings were inspected twice a week for YFP signals over a period of 8 weeks. After two weeks, all K599 inoculated poplar cuttings (100 %) formed micro callus at the infected shoot surface, while only few (25 %) control plants showed clear micro callus formation. YFP signals were easily detected from the developed microcalli, from which transgenic roots appeared (figure 8).

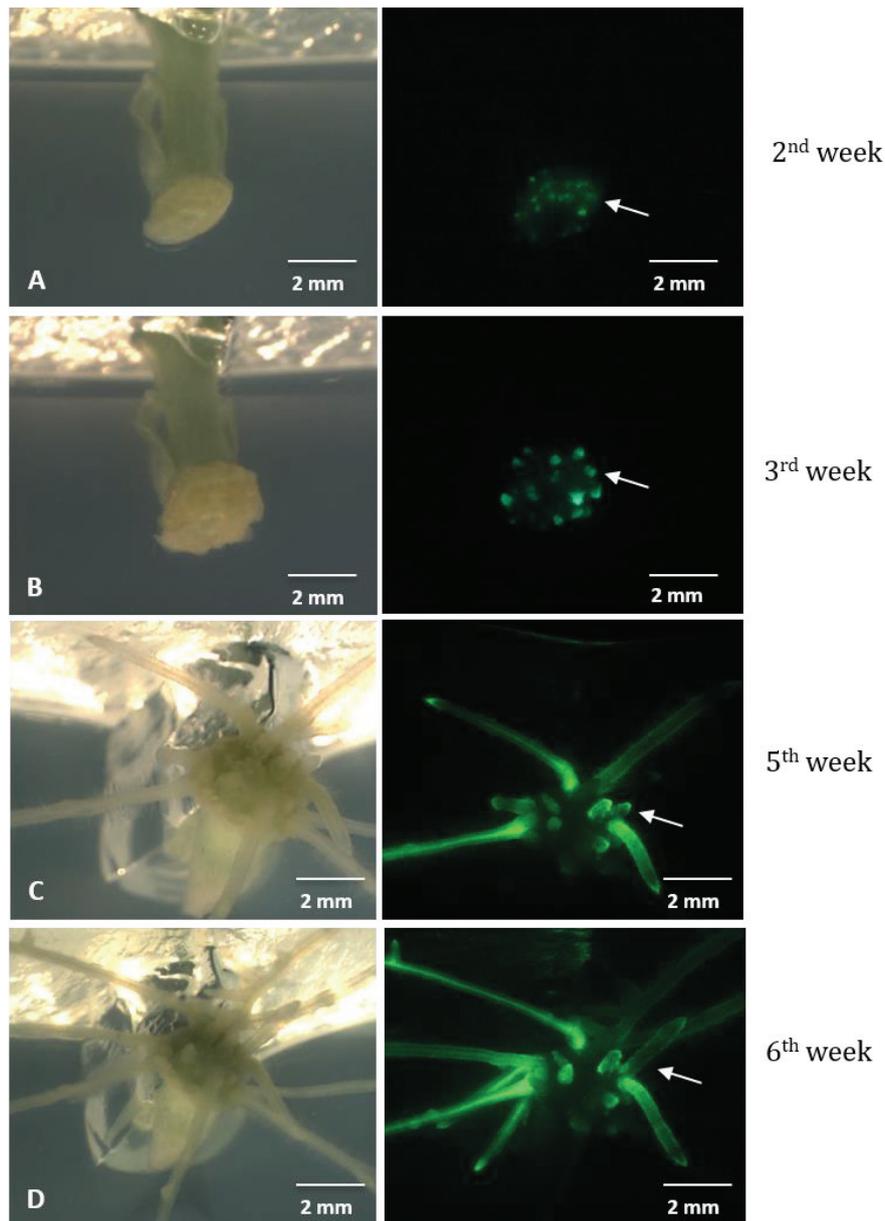


Figure 8: Development of microcalli at the shoot surface of poplar cuttings inoculated with *Agrobacterium rhizogenes* strain K599.

Rooting by a typical *Populus tremula* × *alba* cutting inoculated with *A. rhizogenes* strain K599 harboring pBIN19-YFPPTS1 (Nowak, et al. 2004) was followed over a period of 8 weeks. Development of microcalli and roots at the surface of the cuttings was examined twice a week by a stereo microscope (Leica MZ10F) under transmission light (left) and UV light with YFP filter set (right). A transgenic microcallus from which a root is formed, is marked with arrows.

3.1.1.2 Root induction by different *Agrobacterium rhizogenes* strains

In addition to the four different *A. rhizogenes* strains, plants inoculated with sterile water were maintained as negative controls. After inoculation the plants were observed weekly under a stereo microscope (Leica MZ10F) for root formation. A similar pattern was observed for root induction in both poplar batches (figure 9). Root formation was the fastest in control and strain 15834 inoculated plants, where roots formed between

the first and fourth week. First roots appeared at least two weeks after inoculation with other three agrobacterial strains and strain 1724 showed the slowest root formation rate. After 8 weeks of incubation, 90 %, 84 % and 59 % plants inoculated with *A. rhizogenes* strains 8136, K599 and 1724 respectively formed at least one root, while the negative controls and strain 15834 infected plants revealed 100 % root formation.

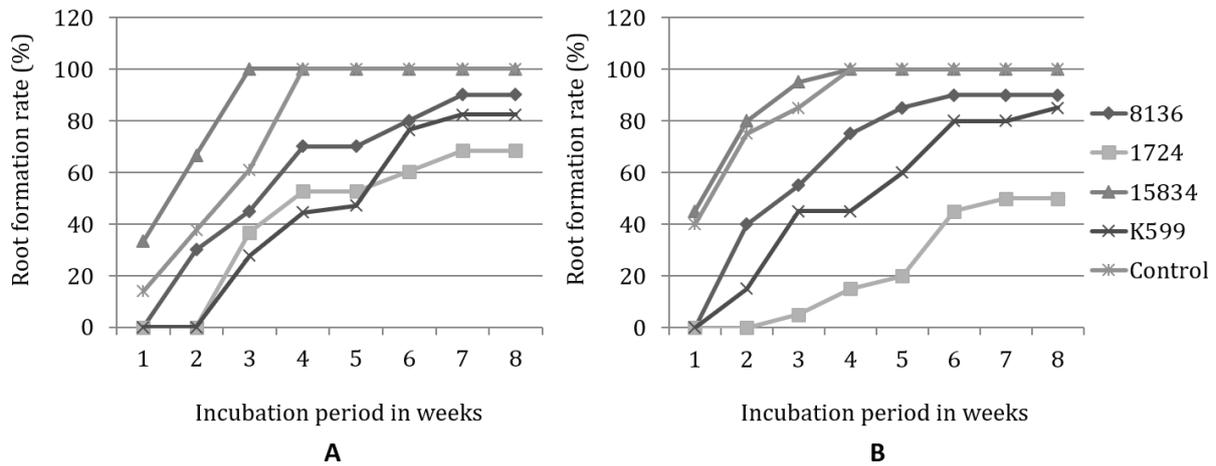


Figure 9: Root formation in poplar cuttings inoculated with different *Agrobacterium rhizogenes* strains.

About one month old *Populus tremula × alba* cuttings were inoculated with the following *A. rhizogenes* strains: 8136, 1724, 15834 and K599 along with sterile water as control, in two batches (A and B) 20 plants each. The root formation rate was monitored for a period of 8 weeks. Shown are the percentages of plants that formed per week in two independent batches (A and B). Results from two different batches show similar root formation pattern, on average after 8 weeks of incubation 19 ± 1.41 (n=2), 13.5 ± 4.94 (n=2), 16.5 ± 0.70 (n=2) cuttings per batch formed at least one root for strain 8136, 1724 and K599 respectively. Strain 15834 and sterile water inoculated all plants formed roots within the first four weeks for both batches.

Not only the proportion of plants that formed roots but also the number of roots formed by different *A. rhizogenes* strains differed. Plants inoculated with strain 1724 revealed 7.71 ± 2.64 roots per plant which did not differ significantly ($p= 0.08$) from water control (6.94 ± 1.68) (figure 10). In contrast, plants inoculated with strain 8136 (9.62 ± 2.04) and K599 (10.22 ± 2.07) showed a slight but significant increased ($p= 1.02 \times 10^{-8}$ for strain 8136 and 1.69×10^{-10} for strain K599) number of roots. Plants inoculated with *A. rhizogenes* strain 15834 revealed the highest number of roots (15.02 ± 3.70) per plant, which is significantly higher ($p= 1.17 \times 10^{-19}$) compared to control plants.

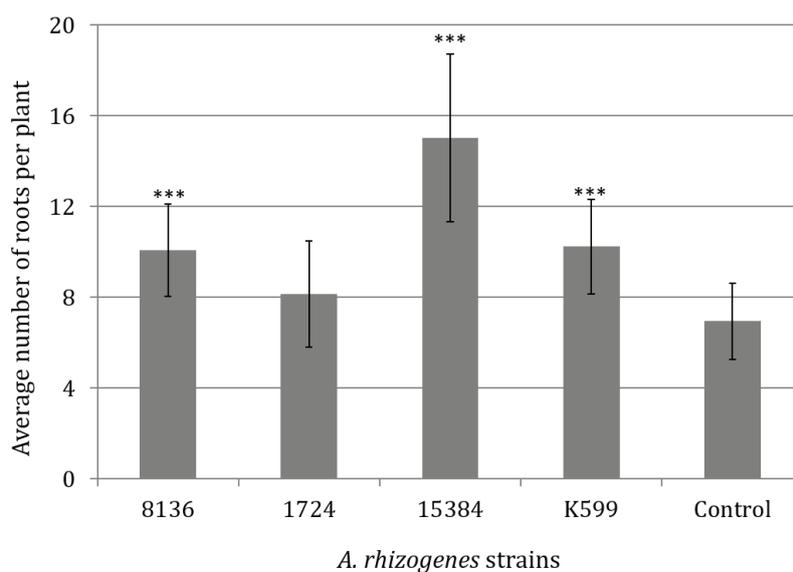


Figure 10: Average number of roots formed per poplar cuttings inoculated with different *Agrobacterium rhizogenes* strains.

Plants inoculated with different *A. rhizogenes* strains along with the controls were assessed for the number of roots formed after 8 weeks of inoculation. Shown are the mean values of 40 plants from two independent batches and the respective standard deviations. The number of roots per plant differed significantly (Student t-test) from plants inoculated with *A. rhizogenes* strains 15384 ($p= 1.17 \times 10^{-19}$), K599 ($p=1.69 \times 10^{-10}$) and 8136 ($p= 1.02 \times 10^{-8}$) compared to control plants. Only exception was with strain 1724, where the root number per plant did not differ significantly ($p= 0.08$) from the control plants. Columns marked with *** indicates p values ≤ 0.001 .

3.1.1.3 Determination of root co-transformation efficiency

A. rhizogenes used in this work contained two T-DNA harbouring plasmids: the Ri plasmid and the shuttle vector pBIN19-YFPPTS1 (Nowak, et al. 2004). The *A. rhizogenes* strains were investigated for their ability to transfer the T-DNA of both vectors, into the infected plants. The T-DNA of pBIN19-YFPPTS1 (Nowak, et al. 2004) contained a marker cassette that allows a strong, constitutive expression of a peroxisome targeted yellow fluorescent protein (YFP). Plant roots containing the T-DNA of the Ri- plasmid are expected to reveal a hairy root phenotype.

Roots were examined weekly for visible YFP signals and for hairy root phenotype over a period of 8 weeks. Out of all four tested strains, only *A. rhizogenes* K599 allowed easy detection of strong YFP signals by epifluorescence microscopy (figure 11 and 12). Roots expressing YFPPTS1 could be identified as the 'hairy roots'. After 8 weeks, 36 out of 40 poplar cuttings showed at least one root with a detectable YFP signal and a total of 87 % roots showed YFP expression (table 06). Peroxisomal targeted yellow fluorescence protein could easily be identified only for K599 inoculated plants (figure 12 D), whereas

only cell wall autofluorescence was detected for other roots (figure 12 A, B, C and E) at increased laser intensity.

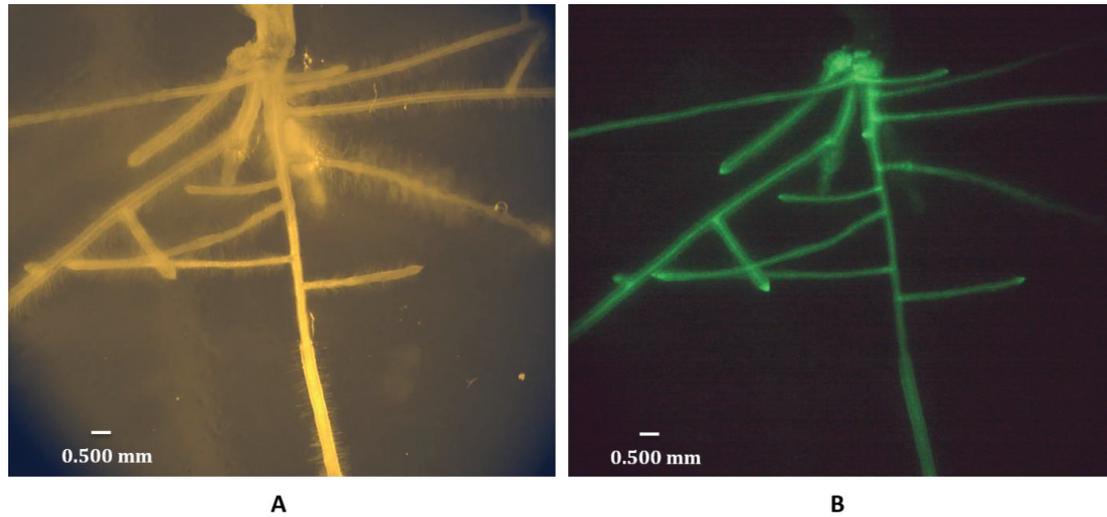


Figure 11: Detectable YFP fluorescence of transgenic poplar roots.

P. tremula x alba cuttings were inoculated with *A. rhizogenes* K599 containing the binary vector pBIN19-YFPPTS1 (Nowak, et al. 2004). Shown are roots formed three weeks after inoculation under bright light field (A) and UV-light using the YFP filter set (B). Visualization was performed using a stereo microscope (Leica MZ10F). Roots showing YFP signal can be identified as the ‘hairy roots’.

Table 6: Comparison of T-DNA transferability of *A. rhizogenes*.

40 *P. tremula x alba* cuttings each were inoculated in two independent batches with four *A. rhizogenes* strains containing the binary vector pBIN19-YFPPTS1 (Nowak, et al. 2004). After 8 weeks of incubation the formed roots were investigated under by epifluorescence microscopy for detectable YFP signals. Shown are the total number of formed roots and the number of roots showing YFP signal. The transformation efficiency is calculated in percentage.

<i>A. rhizogenes</i> strains	Total number of roots	Number of roots showing YFP signal	Transformation efficiency in %
K599	379	333	87
8136	387	0	0
1724	301	0	0
15384	603	0	0

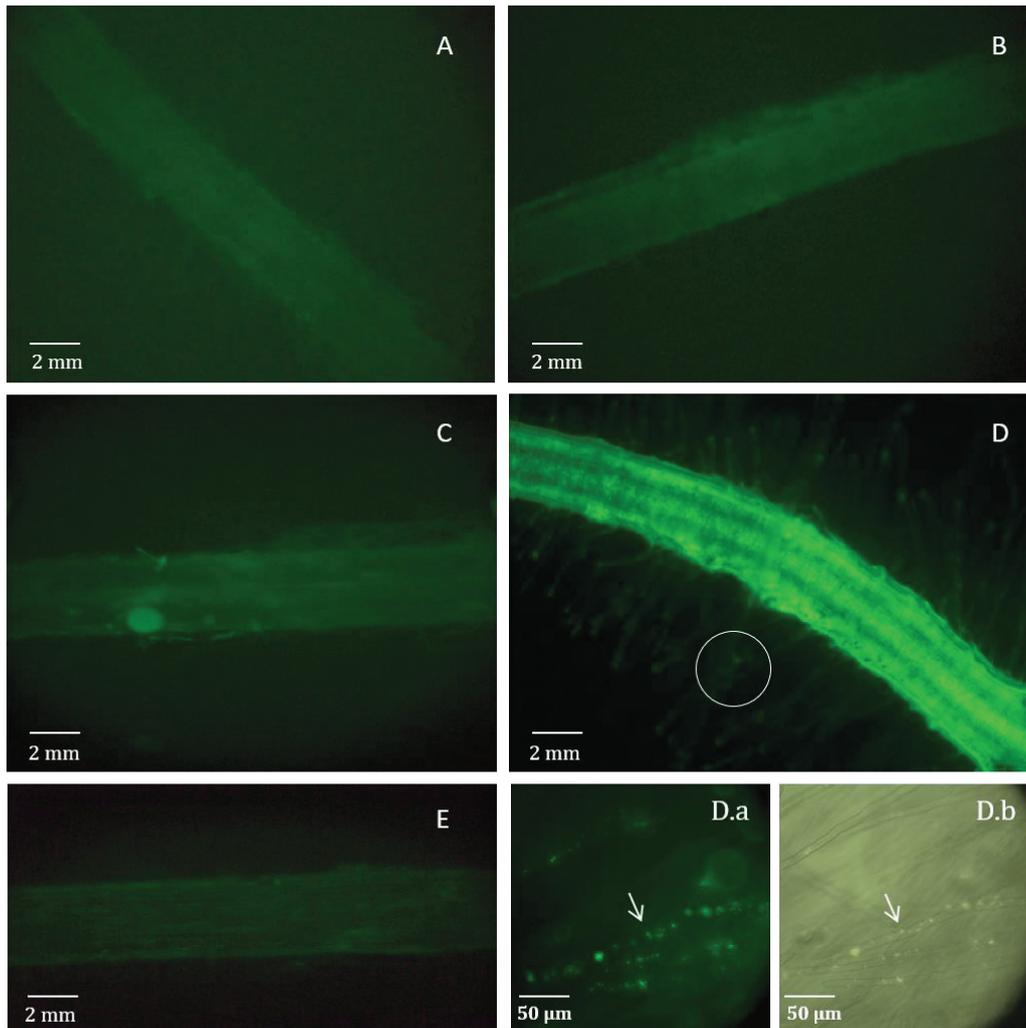


Figure 12: Detection of YFPPTS1 expression by epifluorescence microscopy.

At higher magnification single roots of *P. tremula x alba* plants inoculated with *A. rhizogenes* strains 8136 (A), 1724 (B), 15834 (C) and K599 (D) along with control (E) were observed under UV light with a YFP filter set. The circled area of image D was further magnified in Da and Db. YFPPTS1 (arrow marked) signals can be seen in the root hairs (D.a- view with YFP filter, D.b – overlapping view of a bright field light and YFP filter). Slight cell wall autofluorescence was observed for all roots including the negative control.

3.1.2 Development of transgenic roots in other *Populus sp.*

In order to investigate root induction in other *Populus sp.*, 15 one month old *Populus trichocarpa* and *Populus tremula x tremuloides* cuttings each were inoculated with *A. rhizogenes* K599 harbouring binary vector pBIN19-YFPPTS1 (Nowak, et al. 2004). 12 out of 15 *P. tremula x tremuloides* composite plants formed at least one root that showed peroxisomal targeted YFP and 79 % of all roots were transgenic. However, no fluorescent roots were observed for *A. rhizogenes* inoculated *P. trichocarpa*.

3.1.3 Induction and growth properties of K599-mediated composite poplar roots

Sucrose, known as a potent root morphogen in *Arabidopsis* (Freixes, et al. 2002; Lee-Ho, et al. 2007) is present (1 %) in the standard rooting medium (Hamp et al. 1996) used for poplar cuttings. Therefore, a comparison of root formation and growth properties between water control and *A. rhizogenes* K599 mediated composite poplars were performed on sucrose containing (10 g l^{-1}) and sucrose free agar medium.

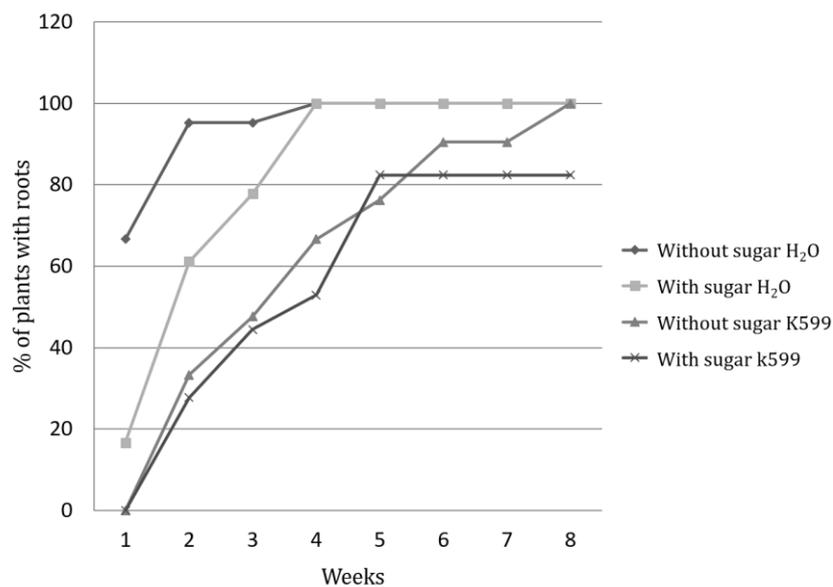


Figure 13: Effect of sucrose on root formation by poplar cuttings.

20 *Populus tremula × alba* cuttings were inoculated with *A. rhizogenes* strain K599 and sterile water. The cuttings were inspected once a week for a period of 8 weeks for their root formation. Shown is the percentage of poplar cuttings that formed at least one root per plant at a given time point.

For the control plants, the initial root formation was faster in sucrose free medium forming roots in 66.67 % cuttings, whereas only 16.67 % cuttings started to form roots in sugar containing medium after one week (figure 13). However, all control plants were rooted after four weeks of incubation irrespective of the presence of sucrose in their growth medium. Although no obvious differences in root formation kinetics were observed in K599 inoculated plants grown in absence or presence of sucrose, after eight weeks of incubation the number of poplar cuttings that formed roots was slightly higher when grown on sucrose free medium (figure 13).

Control plants grown in the presence of sugar formed significantly ($p=0.0004$) higher root number (9.6 ± 3.3) per plant compared to plants grown in sucrose free medium (6.2 ± 1.9). In contrast, when grown on sucrose containing medium the number of K599

mediated transformed roots per plant were slightly but non-significantly ($p=0.38$) higher (7.4 ± 4.2 versus 5.7 ± 2.3) than plants grown on sucrose free media.

No significant difference ($p \geq 0.05$) was observed in root growth rate of the transformed and control plants. Root growth rate per week was the highest in the first three weeks of incubation and after the fourth week the rate reduced to 2.64 ± 0.89 mm per week. The presence of sucrose had the greatest impact on root branching in both water control and K599 inoculated poplar cuttings. Branching started after 2-3 weeks of incubation for most of the developed roots. Sucrose present in the growth medium significantly elevated (control cuttings: $p = 1.7 \times 10^{-9}$ and K599 treated cuttings: 7.32×10^{-6}) the number of branched roots per plant for water control (11 ± 7.1 versus 0.5 ± 0.8) and K599 treated plants (8.3 ± 7 versus 2.0 ± 2.4).

3.1.4 YFP signal detection and cell wall autofluorescence by laser scanning microscopy

To confirm the autofluorescence observed under epifluorescence microscope, cross sections of poplar fine roots were inspected under laser scanning microscope with 5 % - and higher light intensities. Results showed that irrespective of different independent laser lines (458, 488 and 561 nm) used, the strong cell wall autofluorescence was detected over a broad emission spectrum between 500-650 nm (figure 14).

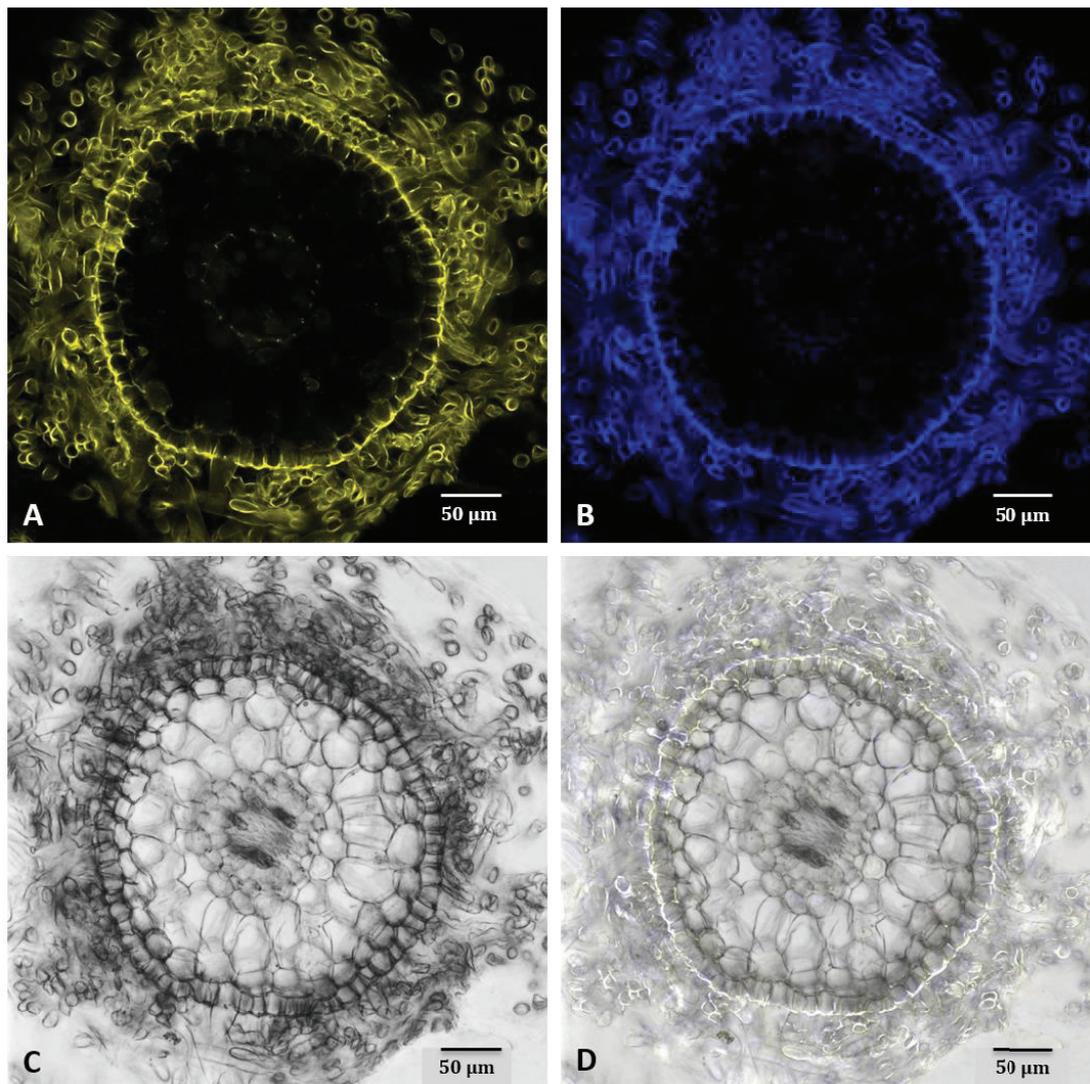


Figure 14: Autofluorescence of poplar fine roots.

Radial cross sections of non-transgenic control *Populus tremula* × *alba* composite plants were analyzed by laser scanning microscopy (excitation: argon laser at 488 nm with 10 % laser intensity). Shown are (in false color) the YFP channel (detection range 510-560 nm, A), the DsRed channel (detection range 560-650 nm, B), the transmission light (C) and an overlay of the three channels (D).

To confirm, the successful transformation by pBIN19-YFPPTS1, roots were analysed by confocal laser scanning microscopy. Peroxisomes were easily detectable in whole transgenic roots as well as in cross sections due to their small size and frequent localization far away from the cell wall and peroxisomes being much smaller compared to cytoplasm, fluorescence proteins are concentrated in a small compartment and give a strong signal at lower laser intensities even below 2 %, at which on the other hand the cell wall autofluorescence is much weaker compared to the specific YFP signal.

Furthermore, the peroxisomal signals were also confirmed to be lying in the YFP emission spectrum (figure 15) by using lambda-scale mode of laser scanning

microscope. It detects and specifies any signal obtained from a sample over a broad range of emission spectrum for any given laser line.

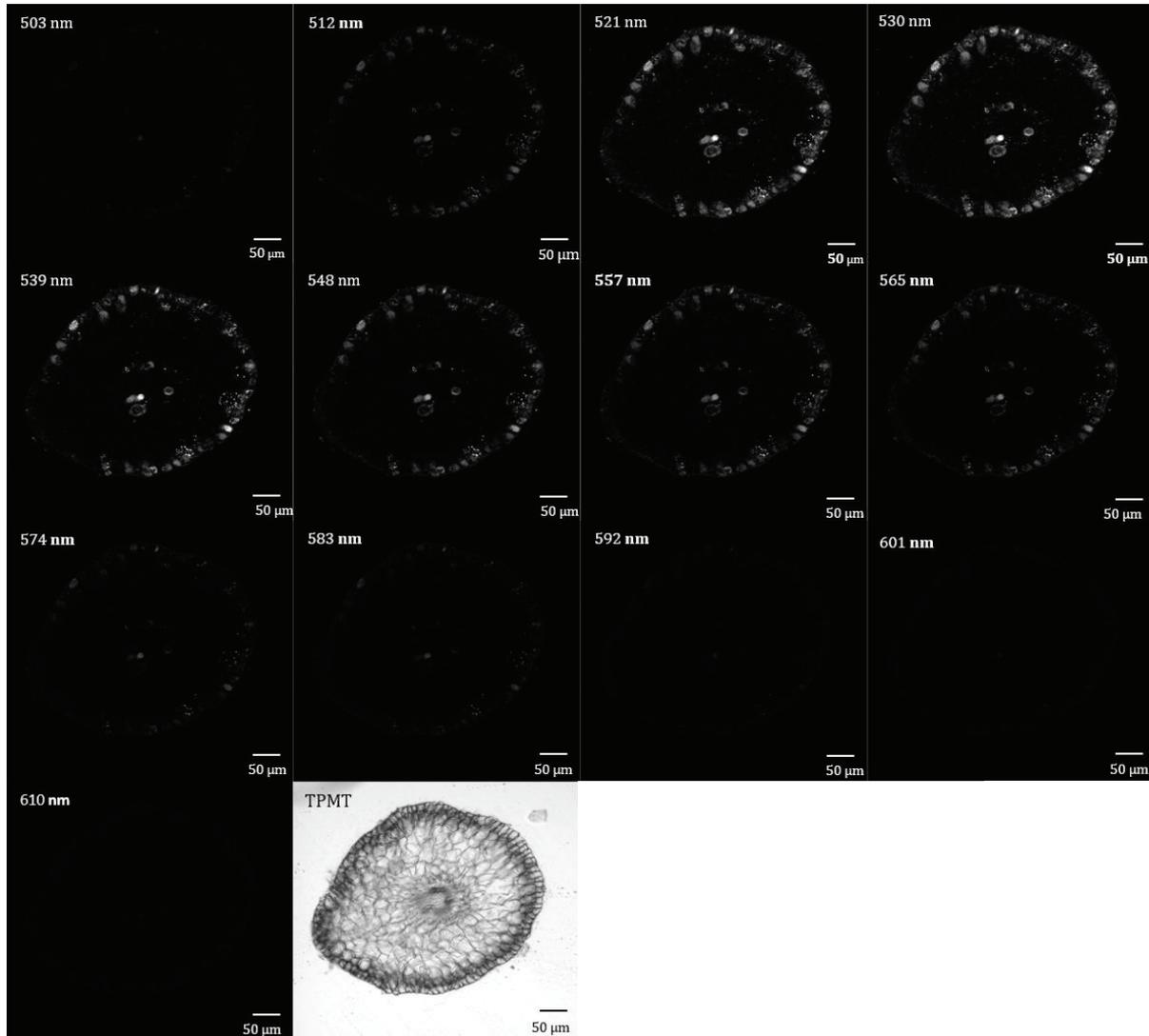


Figure 15: Detection of fluorescence signal in λ -mode (lambda scan) by cLSM.

Shown are the cross sections of a *P. tremula* \times *alba* transgenic root harboring the T-DNA of the binary vector pBIN19-YFPPTS1 (Nowak, et al. 2004) analyzed in λ -mode (lambda scan) using a broad range detection window from 503 nm to 610 nm separated into 32 single windows and transmission light (TPMT). The respective detection wavelength of the individual window is indicated on the upper left corner of each image.

No chimeric roots, containing fluorescent and non-fluorescent branches were ever observed in composite poplar plants. Furthermore, 50 μ m thick cross sections were generated along transgenic roots from root tip to the base and were inspected by laser scanning microscopy. A total of 8 independent roots of 4 different composite poplars showed peroxisomal localized YFP signals. An example of such a cross section is shown in figure 16.

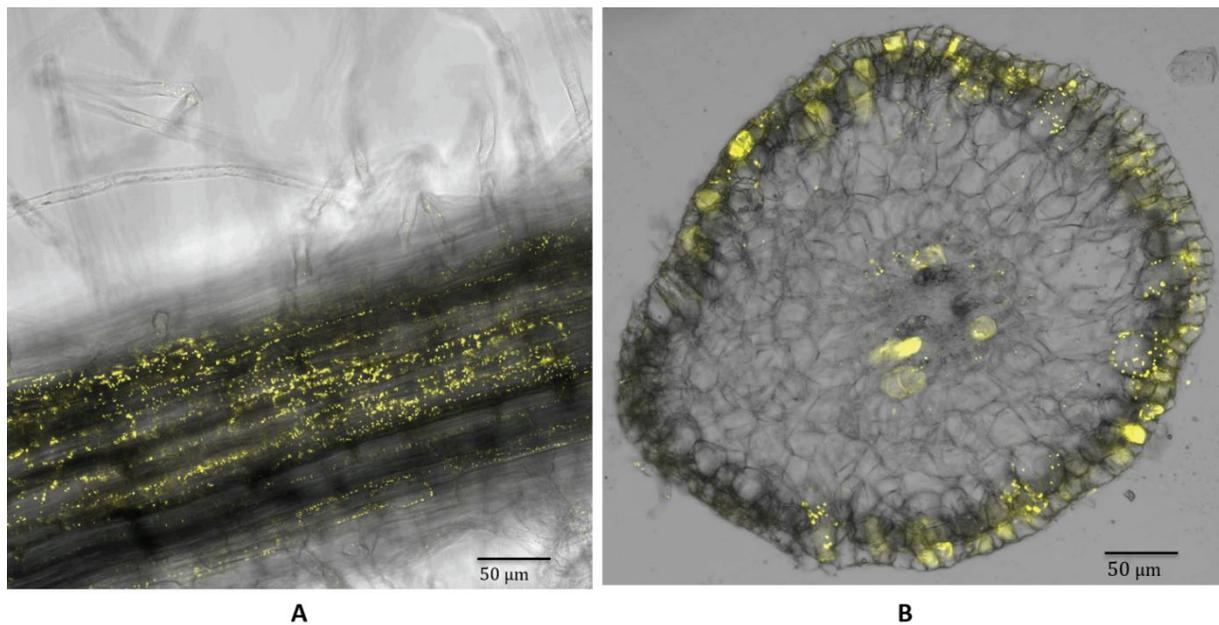


Figure 16: Peroxisome targeted YFP fluorescence of *A. rhizogenes* K599 mediated transgenic root.

Seven weeks old *A. rhizogenes* K599 induced transgenic roots of *Populus tremula* × *alba* composite plants were analyzed by laser scanning microscopy (excitation: argon laser at 488 nm with 2 % laser intensity). Shown are overlays of the YFP channel (detection range 510-560 nm) and the transmission light channel of a whole mount view (A) and a cross section of a transgenic root (B). Peroxisomes are shown in yellow.

3.2 Determination and comparison of gene expression in mycorrhized and non-mycorrhized roots

3.2.1 Identification of homologs in different *Populus sp.* databases (collaboration with L. Hodrea, group Nehls)

Initially, the genes targeted in this work were chosen based on a whole genome expression analysis using 60 bp oligonucleotides (Nehls, unpublished). In the DNA microarray cDNA from ectomycorrhized and non-mycorrhized *P. tremula* × *tremuloides* fine roots were used. However, the 60 bp oligonucleotides were designed using the *P. trichocarpa* genome database (Tuskan et al., 2006) as *P. tremula* × *tremuloides* genome databases were not available. In the meantime, genome data of *P. tremula*, *P. tremuloides* and *P. tremula* × *tremuloides* are available (UPSC draft genome release: <http://popgenie.org>). As the expression analysis in this thesis was also based on *P. tremula* × *tremuloides* mRNAs, the best matching *P. trichocarpa* homologs were first identified in genome libraries of *P. tremula*, *P. tremuloides* and *P. tremula* × *tremuloides*. In this project, a total of 50 ectomycorrhiza upregulated poplar genes were studied.

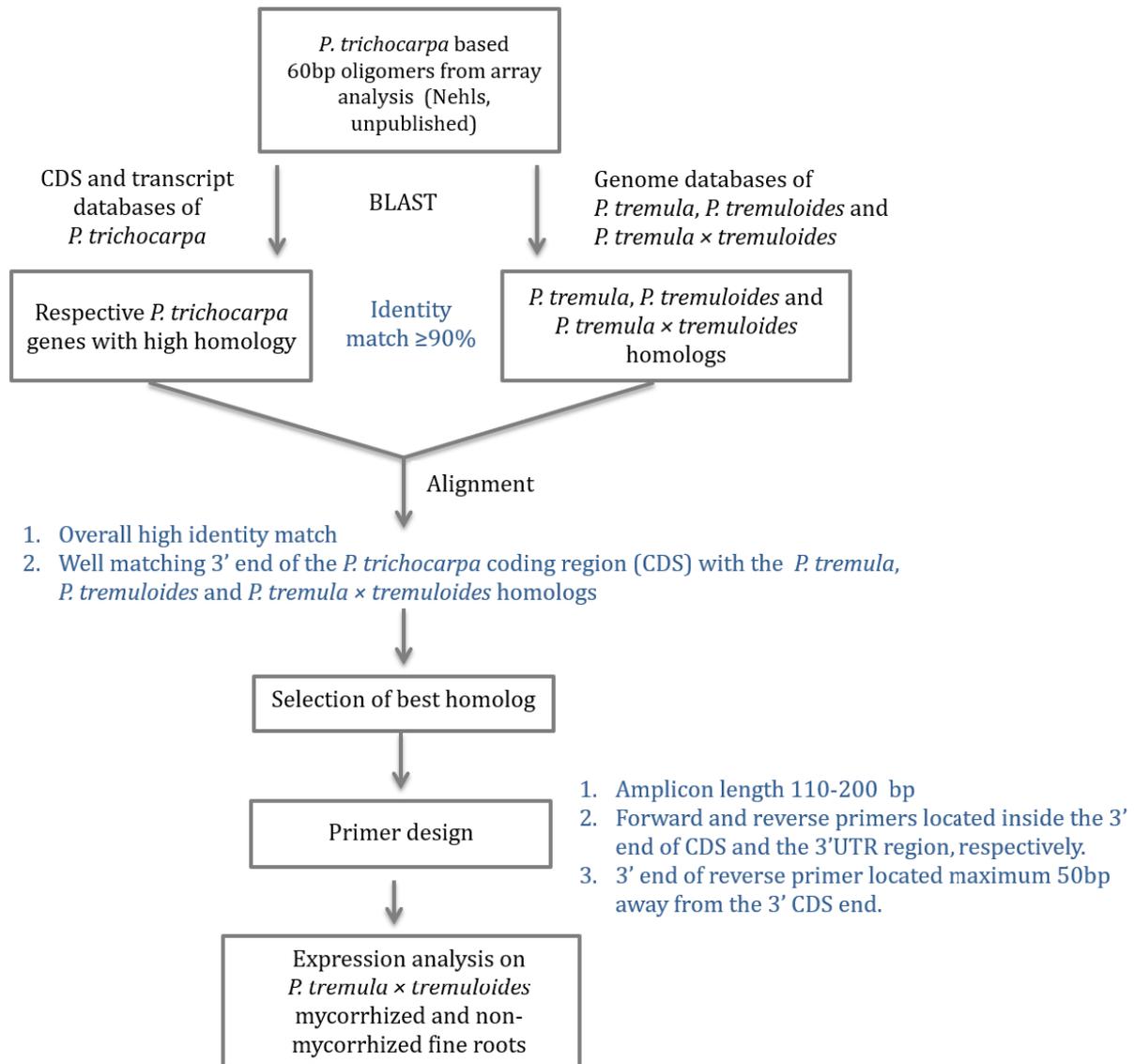


Figure 17: Flow chart of identifying the best homolog for *P. trichocarpa* genes in the *P. tremula*, *P. tremuloides* and *P. tremula x tremuloides* genome database.

Strategies used are shown in blue. 60 bp oligomers based on *P. trichocarpa* sequence from array analysis (Nehls, unpublished) was used to find the best matching sequences in the CDS and transcript databases of *P. trichocarpa* and genome databases of *P. tremula*, *P. tremuloides* and *P. tremula x tremuloides*. The respective *P. trichocarpa* genes were then aligned with the homologs from other poplar species and based on selected criteria the best matching homologs were identified for primer designing. Real time qPCR-based gene expression analysis was performed on *P. tremula x tremuloides* cDNA.

For the identification of the best matching homolog, the 60 bp oligomers of selected genes were used to search for homologous genes in the coding region (CDS) and transcript databases of *P. trichocarpa* using the Basic Local Alignment Search Tool (BLAST) programme within the Geneious software package. For 45 oligomers 100 % identity matches were observed. More than one possible match with varied identity percentage (in a range of 95.2 % - 98.3 %) was identified for 5 genes

(POPTR_0004s08250_1¹, POPTR_0019s03920_1, POPTR_0002s18140_1, POPTR_0002s18120_1 and POPTR_0019s02830_1). In such cases, the sequence with highest identity match has been chosen for further analysis. Genes (*Potri.016G034600*, *Potri.016G034400*², *Potri.017G057800*, *Potri.003G21600*, *Potri.018G131600* and *Potri.005G072600*) for which no further homologs were found in other *Populus sp.* genome databases were discarded from further analysis. Finally, suitable homologs were identified for 42 genes in the genome databases of *P. tremula*, *P. tremuloides* and *P. tremula × tremuloides* revealing identity matches between 93.3 % and 100 %.

The short 60 bp oligomers were only used to identify the potential homologs, however for the final confirmation the *P. trichocarpa* CDS, a longer highly conserved sequence was thereafter aligned with the obtained homolog sequences of other poplar species. The analysis was done using a Megablast (Morgulis et al., 2008) in the Geneious software package and the best homolog was selected based on the following criteria

1. **The identity match:** An overall good match i.e. higher 'identity percentage' (90-100 %) of the alignment was important.
2. **The consensus similarity:** A well matching 3' end of the coding region (CDS) of *P. trichocarpa* with the homologous sequences from other three poplar species was mandatory. 3' end of CDS including 3'-UTR region was targeted in the expression analysis. Therefore, minimal nucleotide changes at the 3' end of the CDS between different poplar species, especially at least a 160 bp- 200 bp region upstream from the 3' end of the coding sequence was necessary.

As for *P. tremula*, *P. tremuloides* and *P. tremula × tremuloides* only genome databases were available, presence of introns was expected in the respective homolog sequences. In such cases, although the alignment resulted in relatively low consensus identity due to presence of the introns, the homologs were chosen for further analysis if the above criteria matched. A frequently observed phenomenon was a good homology between a *P. trichocarpa* CDS and the corresponding poplar homolog only in particular regions, whereas the 5' and 3' end of the CDS, did not match well. For such cases, a second homolog search was performed using only the 5' and 3' end of the CDS.

Out of the 42 genes, homologs showing best homology in the genome sequences of *P. tremula*, *P. tremuloides* and *P. tremula × tremuloides* were found to be suitable for primer designing for 21 genes. Designed forward and reverse primers of the selected genes are listed in appendix table 14.

² Potri represents the *P. trichocarpa* genes based on the nomenclature of GenBank version 3.

3.2.2 Verification of gene expression by qRT-PCR for selected genes

First strand cDNA was checked for quality using reference gene (polyubiquitin) primer pair (Brunner et al, 2004) by qRT-PCR. Low crossing point values (C_p) and product specific melting curves (figure 39 appendix) confirmed successful cDNA synthesis. Thereafter, PCR conditions were optimized using one mycorrhized and non-mycorrhized fine root sample each. PCR products were obtained only for 14 genes (Table 7), while the rest did not reveal any product in either of the cDNA samples. C_p values varied between 22 and 37 for all the tested primers. The difference in C_p values between the mycorrhized and non-mycorrhized samples were expected; e.g. for *Potri.006G009300*, C_p value (36.48 ± 0.73 , n=3) was significantly higher ($p= 4.00 \times 10^{-5}$) for fine root compared to the mycorrhized sample (27.81 ± 0.15 , n=3). Primer-dimer formation leading to a faulty C_p value for such SYBR based detection, can easily be detected and distinguished from the product specific melting peaks. Gel electrophoresis confirmed the expected PCR product lengths (figure 41 appendix).

PCR conditions were optimized for genes revealing reliable results in the initial PCR approach (table 20 appendix). Genes that revealed unexpected and unsatisfactory results or no bands, qRT-PCR was repeated at reduced annealing temperature of 51 °C to increase primer-template binding. For primer pairs for which smaller DNA fragments (110-130 bp) were expected, the fluorescence was detected at reduced temperature 78 °C – 80 °C as elevated temperature may affect the real time quantification. Further, gel electrophoresis confirmed the qRT-PCR results. Only six genes, *Potri.007G081200*, *Potri.003G210600*, *Potri.002G188900*, *Potri.006G009300*, *Potri.007G095000* and *Potri.008G071100* confirmed significantly higher gene expression in mycorrhiza compared to non-mycorrhized fine roots as previously shown in the DNA microarray analysis (Nehls, unpublished). The amplification efficiencies of the selected genes were also determined by calibrating to reference polyubiquitin (table 23 appendix).

Table 7: List of the selected ectomycorrhiza upregulated *Populus trichocarpa* genes for which suitable homologs were found and primers were successfully designed.

Shown are the selected genes that were primarily chosen by their higher expression of at least 10 fold in array analysis (Nehls, unpublished) in mycorrhiza than fine roots, represented by their *P. trichocarpa* gene id, supposed function (Nehls, unpublished), poplar species for which the best matching homolog was found, amplification result in the primary real time quantitative PCR and final selection based on reproducibility in different biological and technical replicates.

Sequence ID of <i>P. trichocarpa</i>	Supposed function (MapMan) (Nehls, unpublished)	Species database of chosen homolog	Successful amplification in both mycorrhized and fine root samples (primary test) ^B	Selection based on biological and technical replicates ^C
<i>Potri.007G095000</i>	misc.UDP glucosyl and glucoronyl transferases	<i>Populus tremulooides</i>	Yes	Selected
<i>Potri.011G132100</i>	Protein synthesis initiation	<i>Populus tremula</i>	Only primer-dimer formation	Rejected
<i>Potri.009G028600^A</i>	Not assigned. Unknown	<i>Populus tremula x tremulooides</i> and <i>Populus tremulooides</i>	Yes	Rejected
<i>Potri.001G085900</i>	Not assigned. Unknown	<i>Populus tremulooides</i>	Yes	Rejected
<i>Potri.004G045600</i>	Secondary metabolism. phenylpropanoids. lignin biosynthesis.	<i>Populus tremula x tremulooides</i>	Only primer-dimer formation	Rejected
<i>Potri.014G106600^A</i>	Secondary metabolism. phenylpropanoids. lignin biosynthesis.	<i>Populus tremula</i> and <i>Populus tremulooides</i>	Several bands. No-specific amplification	Rejected
<i>Potri.003G210600</i>	Secondary metabolism. phenylpropanoids. lignin biosynthesis.	<i>Populus tremulooides</i>	Amplification in mycorrhiza sample. Primer-dimer formation in fine root.	Selected
<i>Potri.002G180600</i>	Secondary metabolism. phenylpropanoids. lignin biosynthesis.	<i>Populus tremula x tremulooides</i>	Only primer-dimer formation	Rejected
<i>Potri.009G127900</i>	Transport. Major Intrinsic Proteins.	<i>Populus tremula</i>	Only primer-dimer formation	Rejected
<i>Potri.004G068800</i>	Not assigned. Unknown	<i>Populus tremula</i>	Only primer-dimer formation	Rejected

<i>Potri.011G046600</i>	Protein degradation. serine protease	<i>Populus tremula</i>	Yes	Rejected
<i>Potri.006G009300</i>	Not assigned. Unknown	<i>Populus tremula</i>	Yes	Selected
<i>Potri.012G117100</i>	GTP binding	<i>Populus tremulooides</i>	Several bands. No-specific amplification	Rejected
<i>Potri.002G188900</i>	transport.ABC transporters and multidrug resistance systems	<i>Populus tremula x tremulooides</i>	Yes	Selected
<i>Potri.007G012400</i>	Protein degradation. AAA type	<i>Populus tremula x tremulooides</i>	Yes	Rejected
<i>Potri.007G081200</i>	misc. cytochrome P450	<i>Populus tremulooides</i>	Amplification in mycorrhiza sample. Primer-dimer formation in fine root.	Selected
<i>Potri.016G104300</i>	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	<i>Populus tremula</i>	Only primer-dimer formation	Rejected
<i>Potri.010G244400</i>	Not assigned. Unknown	<i>Populus tremula x tremulooides</i>	Only primer-dimer formation in fine root. Faint band of expected size in mycorrhiza sample.	Rejected
<i>Potri.004G019300</i>	misc. gluco-, galacto- and mannosidases	<i>Populus tremula</i>	Yes	Rejected
<i>Potri.008G071100</i>	RNA regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	<i>Populus tremula</i>	Yes	Selected
<i>Potri.010G196900</i>	Protein degradation. subtilases	<i>Populus tremulooides</i>	Only primer-dimer formation	Rejected

^A *P. trichocarpa* genes for which more than one homolog were found in the genomic libraries.

^B Primary real time qPCR was performed using one mycorrhized and non-mycorrhized fine roots each. PCR products were obtained only for 14 genes, while the rest did not reveal any product in either of the cDNA samples. PCR conditions were optimized for genes that either revealed unexpected results or no specific bands.

^C Selected genes were chosen for further analysis and were tested using different biological and technical replicates of mycorrhized and fine root samples. Genes that revealed comparable Cp values between the replicates were chosen for further analysis, while the rest were rejected due to high differences in Cp values between biological replicates.

3.2.3 Comparative gene expression analysis of mycorrhized and non-mycorrhized *Populus tremula* × *tremuloides* fine roots

The difference in gene expression between the mycorrhized and non-mycorrhized root samples was determined based on the average relative gene expression ratios (Pfaffl 2010) of different biological replicates and are summarized in table 9. Cp values and amplification efficiencies of both targeted and reference genes were used for the calculation.

Gene expression was significantly ($p \leq 0.001$) higher in mycorrhized roots than non-mycorrhized fine roots supporting previous DNA microarray analysis by Nehls. Variation in gene expression ratios between individual biological replicates was observed for mycorrhiza samples (figure 18). However, a general gene expression pattern was observed - compared to other biologicals replicates mycorrhized *P. tremula* × *tremuloides* root samples with id -125, 728, 750 and 752 showed an overall low relative gene expression ratio (still higher expression compared to fine roots) in all the tested genes.

Table 8 : Gene expression difference in mycorrhized sample compared to non-mycorrhized fine roots.

Shown are *P. trichocarpa* ids of selected genes and the differences in gene expression. Gene expression difference, determined from the average relative gene expression ratios (Pfaffl 2010) of different biological replicates, was significantly higher (Student t-test) in mycorrhized roots. Gene expression difference marked with *** represents significant difference with p values ≤ 0.001 .

Gene id	Difference in gene expression between mycorrhized and fine root samples
<i>Potri.002G188900</i>	78 ***
<i>Potri.007G081200</i>	57 ***
<i>Potri.006G009300</i>	76 ***
<i>Potri.003G210600</i>	40 ***
<i>Potri.007G095000</i>	104 ***
<i>Potri.008G071100</i>	33 ***

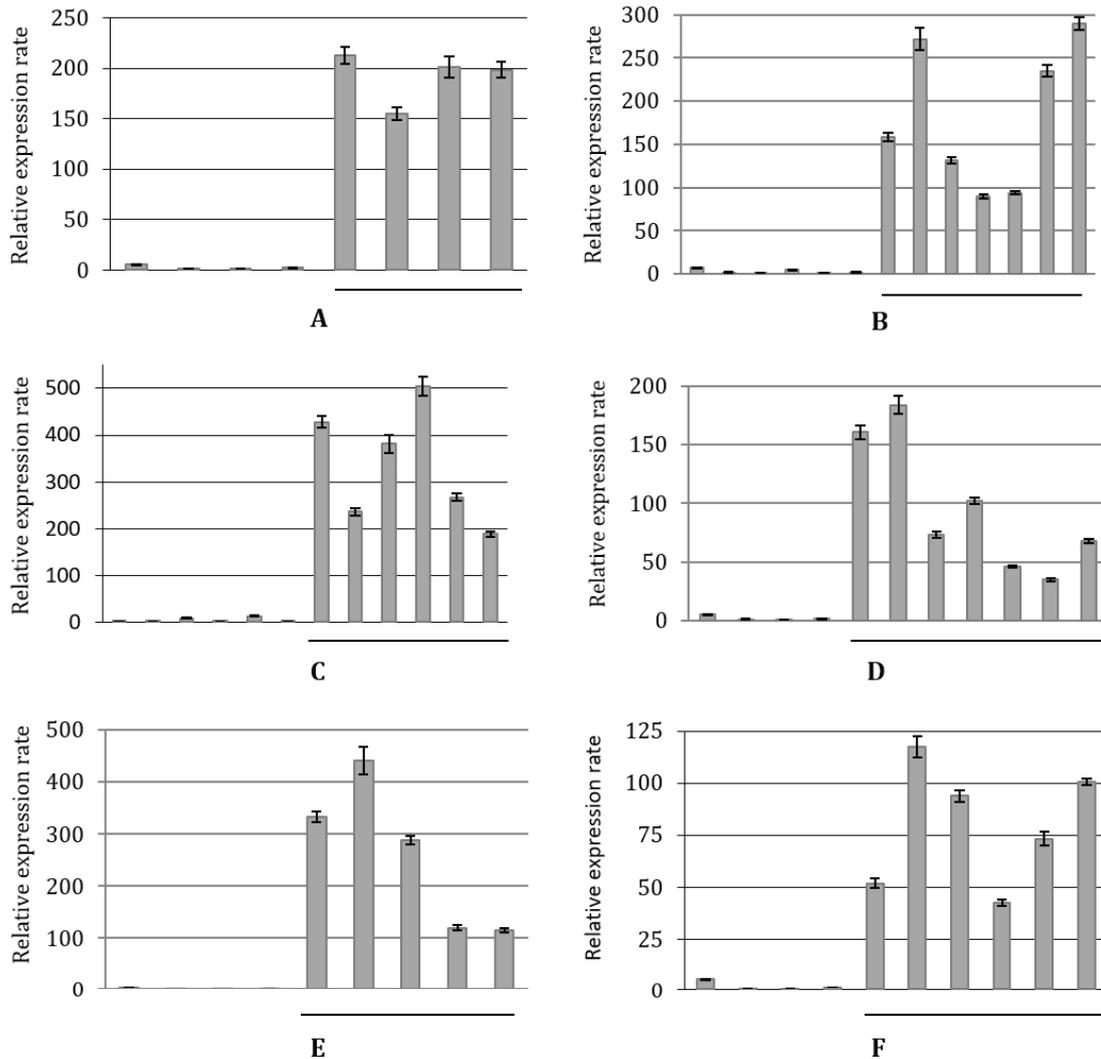


Figure 18: Relative gene expression rates of six selected *P. trichocarpa* genes.

Shown are the relative gene expression ratios of the *P. tremula* × *tremuloides* genes: *Potri.002G188900* (A), *Potri.007G081200* (B), *Potri.006G009300* (C), *Potri.003G210600* (D), *Potri.007G095000* (E) and *Potri.008G071100* (F) in various mycorrhized and non-mycorrhized samples represented by different columns. The mycorrhized samples are underlined. Each column represents average value of 6-8 technical replicates.

Two ectomycorrhiza dependent highly upregulated genes were selected for further analysis: *Potri.007G095000* that encodes an UDP glucosyl/glucuronyl transferase revealing the highest gene expression difference in qRT-PCR and *Potri.008G071100*, an ethylene responsive transcription factor that belongs to the large plant specific transcription factor super family AP2/ERF.

Potri.008G071100 (referred as *PtrDREB1* or *Pt-ABI4.1* in many literatures) belongs to subgroup A3 within DREB subfamily, where two members *Potri.008G071100* and *Potri.010G186400* (known as *PtrDREB2/ Pt-ABI4.2*) are present (Chen et al., 2013, Zhuang et al., 2008). To confirm *Potri.008G071100* specific amplification, the qPCR

product of was purified (8GqPCR) and cloned into pJet1.2 vector. A suitable clone (8GqPCR-pJet) was sequenced and resulting sequence matched well with the respective homolog (figure 19).

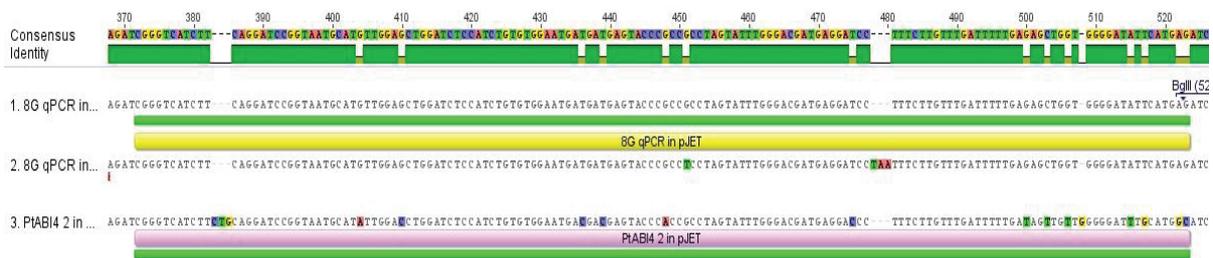


Figure 19: Multiple alignment of qPCR amplified fragments for *Potri.008G071100*.

The qPCR amplified fragment of *Potri.008G071100* from *Populus tremula × tremuloides* was cloned into the pJet1.2 and sequenced using pJet forward and reverse sequencing primers. Shown is the multiple alignment of the expected sequences of *Potri.008G071100* (2, annotated in yellow) and other DREB-A3 subgroup member (3, annotated in purple) and the obtained sequence of amplified fragment (1).

3.3 Glycosyltransferase

3.3.1 Subcellular localization of the putative glycosyltransferase *Potri.007G095000*

3.3.1.1 Vector construction for subcellular protein localization

The *Potri.007G095000* coding sequence (CDS) along with extra few bases at the 5' and 3' ends was amplified from *P. tremula × tremuloides* cDNA using the primers 13 and 14 (table 18 appendix). Optimized PCR conditions are listed in appendix table 21. The amplified fragment 7GCDS (figure 20A) was cloned into pJet1.2/blunt vector and verified by restriction endonuclease analysis (figure 20B - pJet-7GCDS) and sequencing.

Following binary vectors were used: a) pCXUN-sYFP, and b) pPLV11-Sharinal. The stop codon depleted coding region of *Potri.007G095000* is cloned such a way that an in frame *Potri.007G095000*-sYFP fusion protein is produced. Primer pairs 15/16 and 17/18 (table 18 appendix) were used to amplify CDS without stop codon from pJet-7GCDS (figure 20). Amplified fragments 7GCDSLIC and 7GCDSblunt were cloned into *HpaI* digested vectors pCXUN-sYFP and pPLV11-SharinalI using ligase independent and blunt end cloning strategies respectively. The clones were verified by restriction endonuclease analysis (figure 20 - pCX7GCDS and pLV7GCDS) and sequencing.

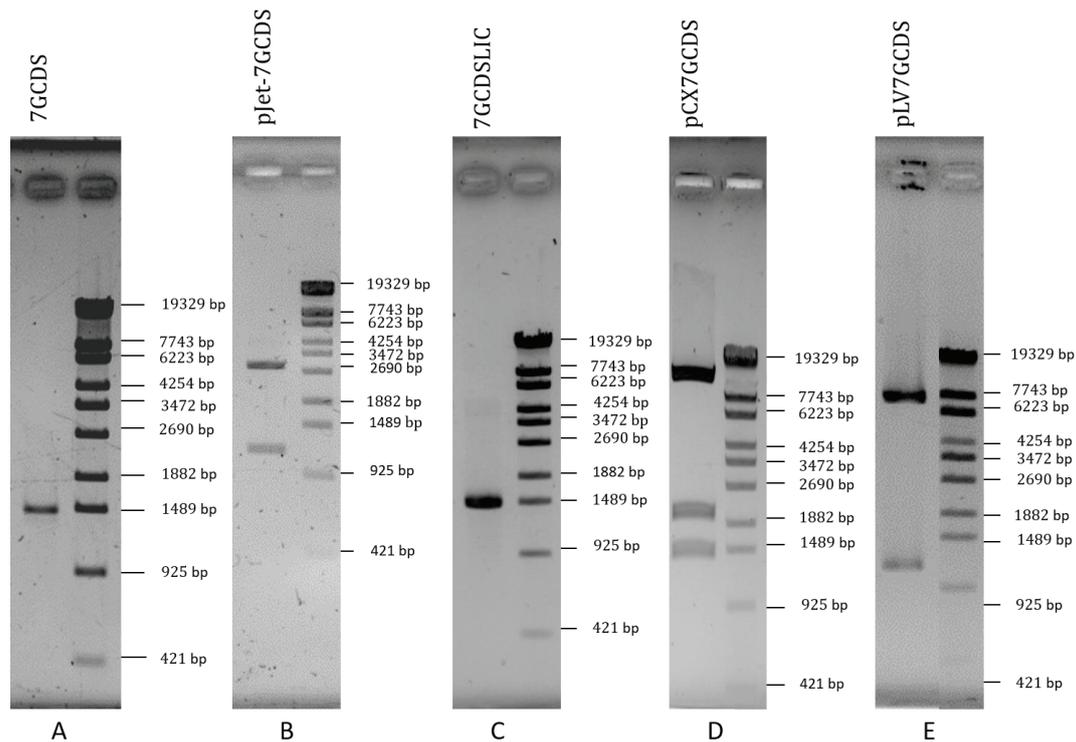


Figure 20: Amplification and vector construction of *Potri.007G095000* coding region for subcellular localization.

The amplified 1465 bp CDS fragment (A) was cloned into pJet1.2/blunt vector and insertion was checked by restriction endonuclease *HindIII* digestion (B). Two bands were expected – 1048 bp and 3391 bp. The clone was used as a template to amplify a stop codon depleted CDS containing ligation independent cloning (LIC) overhangs (C- expected size 1483 bp). A blunt end and ligation independent cloning strategies were followed. Restriction digestion by endonuclease *PstI* and *Bsp120I* on clone pCX7GCDS (D) and pLV7GCDS (E) respectively verified the correct clones releasing the expected band pattern (D- 1388 bp, 1985 bp and 10119 bp; E- 1127 bp and 6920 bp). Lambda DNA/*Eco130I* (*StyI*) was used as a DNA marker.

3.3.1.2 Transient expression of *Potri.007G095000*-sYFP in *Nicotiana benthamiana* leaves

Along with *A. tumefaciens* C58C1 strains containing a plasma membrane localized DsRed and P19- a post-transcriptional gene silencing inhibitor, *Nicotiana benthamiana* leaves were co-infiltrated with the following constructs – a) *A. rhizogenes* containing pCX7GCDS and b) pLV7GCDS transformed into *A. rhizogenes* K599 and *A. tumefaciens* GV3101.

While *A. rhizogenes* K599 infiltrated tobacco leaves revealed no sYFP signal, *A. tumefaciens* GV3101 infected samples did. Co-localization of the *Potri.007G095000*-sYFP fusion protein and DsRed indicated a most likely subcellular localization of *Potri.007G095000* in the nucleus and cytoplasm (figure 21). The sYFP signal was

verified with a 32-channel detector (lambda scan) of cLSM (data not shown). The experiment was repeated thrice and revealed similar results.

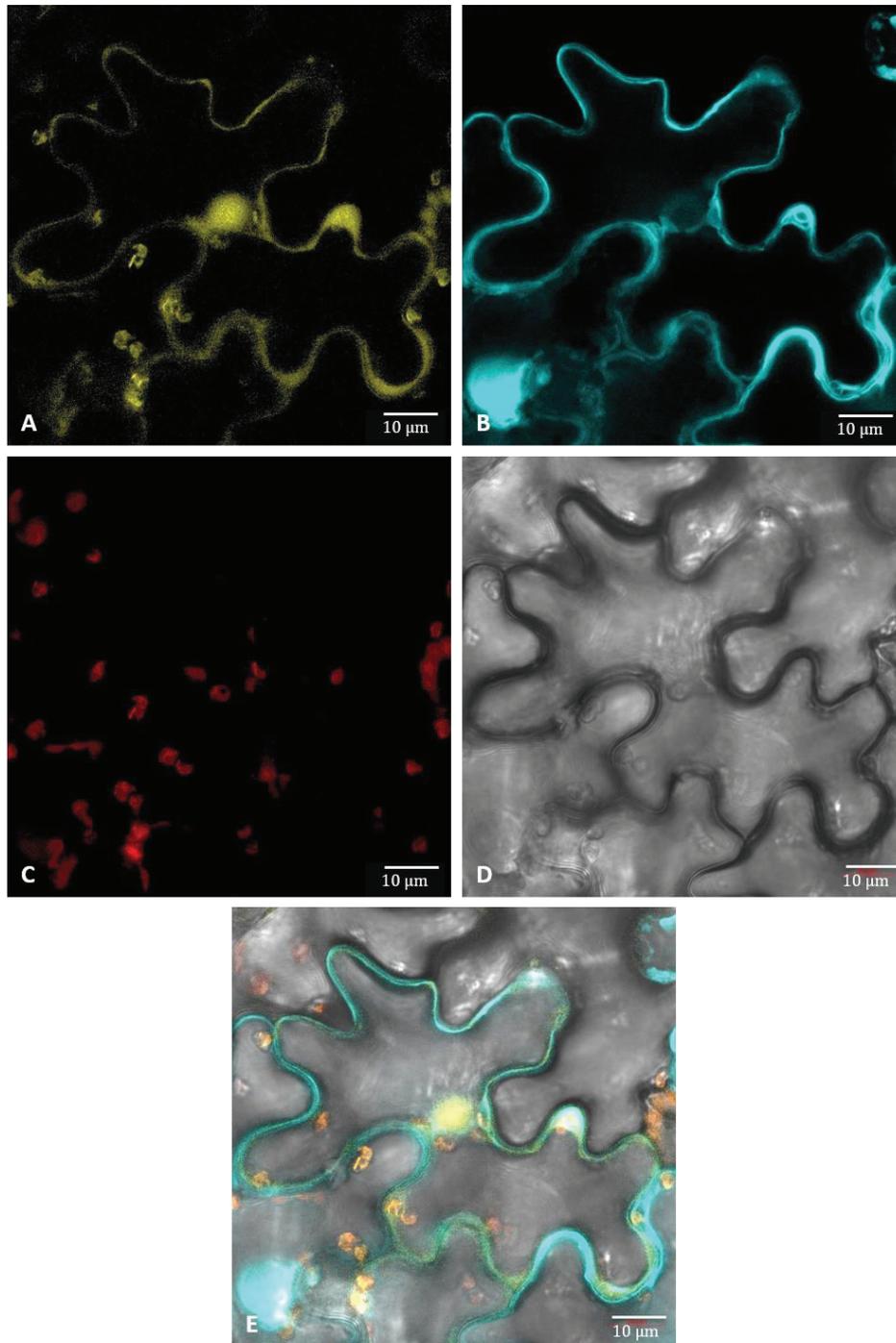


Figure 21: Co-localization of plasma membrane localized DsRed and Potri.007G095000-sYFP in *Nicotiana benthamiana* leaf epidermis cell.

3 days after infection, leaf sections were analyzed by cLSM (Zeiss LSM 780) argon laser at 488 nm and 2 % laser intensity. Shown are the subcellular localization of Potri.007G095000-sYFP (in yellow, A: 510-560 nm) and plasma membrane localized DsRed (turquoise, B: 580-650 nm), chlorophyll autofluorescence (red, C: 660 -730 nm), the transmission light channel (D) and an overlay of all the four channels (E) in false color.

3.3.2 Promoter Analysis

3.3.2.1 Generation of a promoter reporter construct of *Potri.007G095000*

Two forward- reverse primer pairs were designed according to the *P. tremuloides* and *P. tremula* genome sequence to allow PCR amplification of about 4 kb and 3 kb upstream of the *Potri.007G095000* CDS. Several attempts of PCR optimization resulted in amplification of only 3248 bp fragment (figure 22A - 7GPr3Kb) using primers 22 and 23 (table 19 appendix). The purified PCR fragment was cloned into pJet1.2/blunt vector and was verified by restriction endonuclease digestion (Figure 22B - pJet-7GPr3Kb), followed by sequencing of selected clone using pJet1.2 forward/reverse primers.

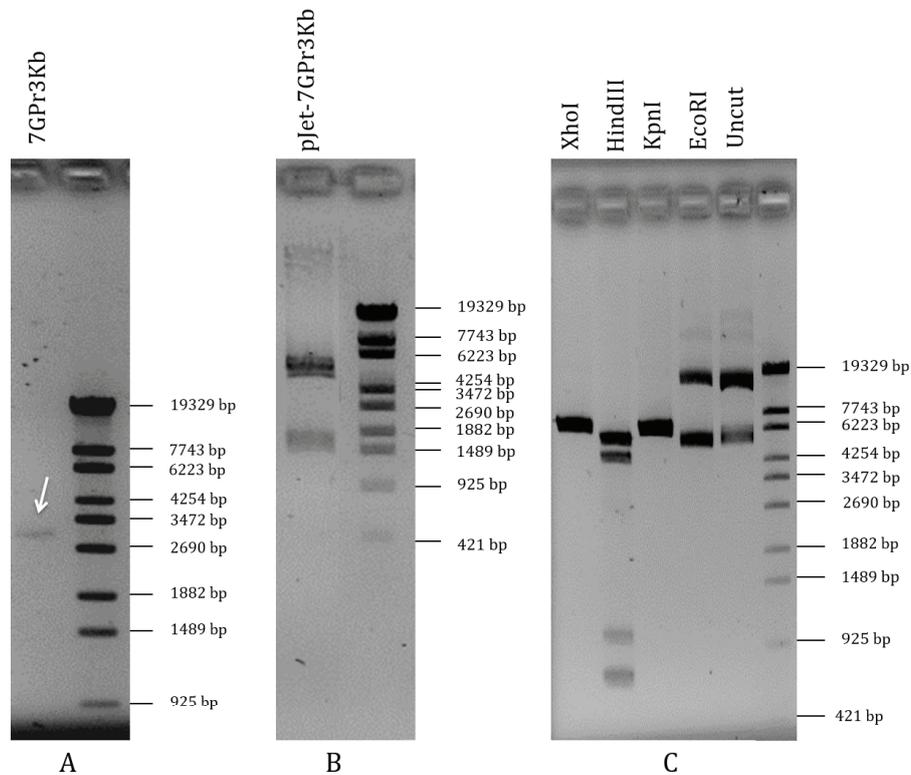


Figure 22: Gel electrophoresis separation of the amplified promoter fragment of *Potri.007G095000* and clone verification.

3248 bp long promoter fragment (A) was cloned into pJet1.2/blunt vector and insertion was verified by restriction endonuclease *NcoI* digestion (B, expected fragments – 1680 bp and 4542 bp). The clone was digested with different restriction enzymes *XhoI* (cuts once inside pJet backbone and linearizes 6222 bp plasmid), *KpnI* (single digestion inside insert and linearizes the 6222 bp plasmid), *EcoRI* (no digestion) for selection of restriction enzyme recognition sites to be used in sticky end cloning strategy. Undigested and *HindIII* digested plasmids were maintained as positive and negative controls respectively (C). Lambda DNA/*Eco130I* (*StyI*) was used as a DNA marker for size estimation.

For directed insertion of the *Potri.007G095000* promoter fragment into the plant transformation vector pPLV11-SharinaII (Nehls, unpublished), a conventional

restriction endonuclease based strategy was chosen. *XhoI* and *EcoRI* recognition sites of the vector multiple-cloning-site were selected. A 3080 bp fragment 7GPr3KbpLV (figure 23A) was amplified using primer pair 24/25 (table 19 appendix) and was cloned into pJet1.2/blunt. As the chosen restriction sites are separated in the target vector (pPLV11-SharinaII) in short distance, a shutter DNA fragment was introduced in a *HindIII* site centred between both restrictions sites used for directional cloning (figure 23C). A verified clone (figure 23B) and the modified pPLV11-SharinaII vector were digested with *EcoRI* and *XhoI* endonucleases. The ends of the vector backbone were dephosphorylated before ligating with the promoter fragment and was verified by restriction endonuclease digestion and sequencing with sYFP specific primer.

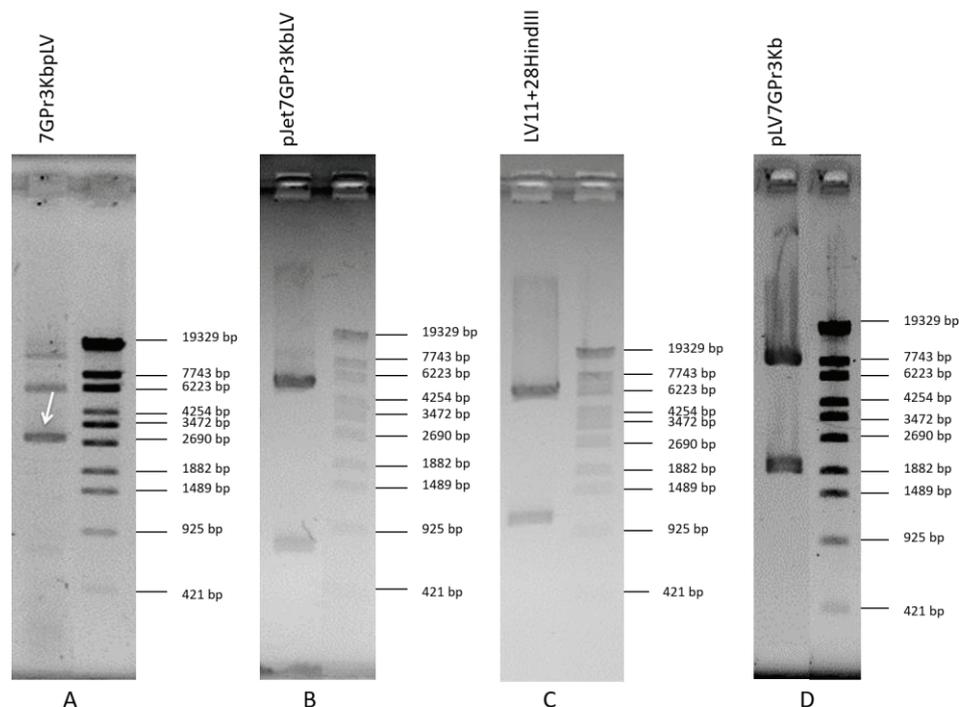


Figure 23: Amplification and verification of pPLV11-SharinaII containing 3 kb *Potri.007G095000* promoter fragment.

3080 bp upstream of *Potri.007G095000* was amplified with primer pair containing *XhoI* and *EcoRI* recognition site overhangs (A) was inserted into pJet1.2/blunt vector and the clone was confirmed by restriction digestion analysis with *HindIII* (expected bands – 306 bp-faint band, 541 bp and 5375 bp) (B). For ease of cloning, a shutter fragment was inserted into pPLV11-SharinaII and verified by restriction enzyme digestion with *HindIII* (expected bands – 1056 bp and 5806 bp) (C). The 3kb promoter fragment was cloned into pPLV11-SharinaII and verification of insertion was done by *KpnI* digestion (expected fragments - 2113 bp and 6914 bp) (D). Lambda DNA/*Eco130I* (*StyI*) was used as a DNA marker for size estimation.

3.3.2.2 *Populus tremula* × *alba* transformation, ectomycorrhiza formation and cLSM analysis

Construct pLV7GPr3Kb was transformed into *Agrobacterium rhizogenes* strain K599 and used for *Populus tremula* × *alba* transformation. The empty pPLV11-Sharinall and water dipped plants were maintained as negative controls. After three weeks of incubation composite poplar formed roots, no YFP signal was detected by stereo microscopy. Cross sections analysed by cLSM revealed only cell wall autofluorescence and nonspecific background at high laser intensity above 10 % (figure 24). Co-transformed plants were inoculated with *Amanita muscaria* and after 3-4 months ectomycorrhiza formed. Cross sections of the transgenic ectomycorrhized roots revealed similar results to non-mycorrhized and ectomycorrhized control plants by cLSM (figure 24 and 25).

50 µm thick cross sections from root tip to the base of transgenic and control ectomycorrhized roots were further tested with a 32-channel detector (lambda scan) by cLSM. The nonspecific background (small dots) and the cell wall autofluorescence was detected over a broad detection window of 500-660 nm. Moreover, the thorough scan from root tip to the base revealed appearance of the background 'dots' to be random in every focal plane, which is unlikely in case of peroxisomes.

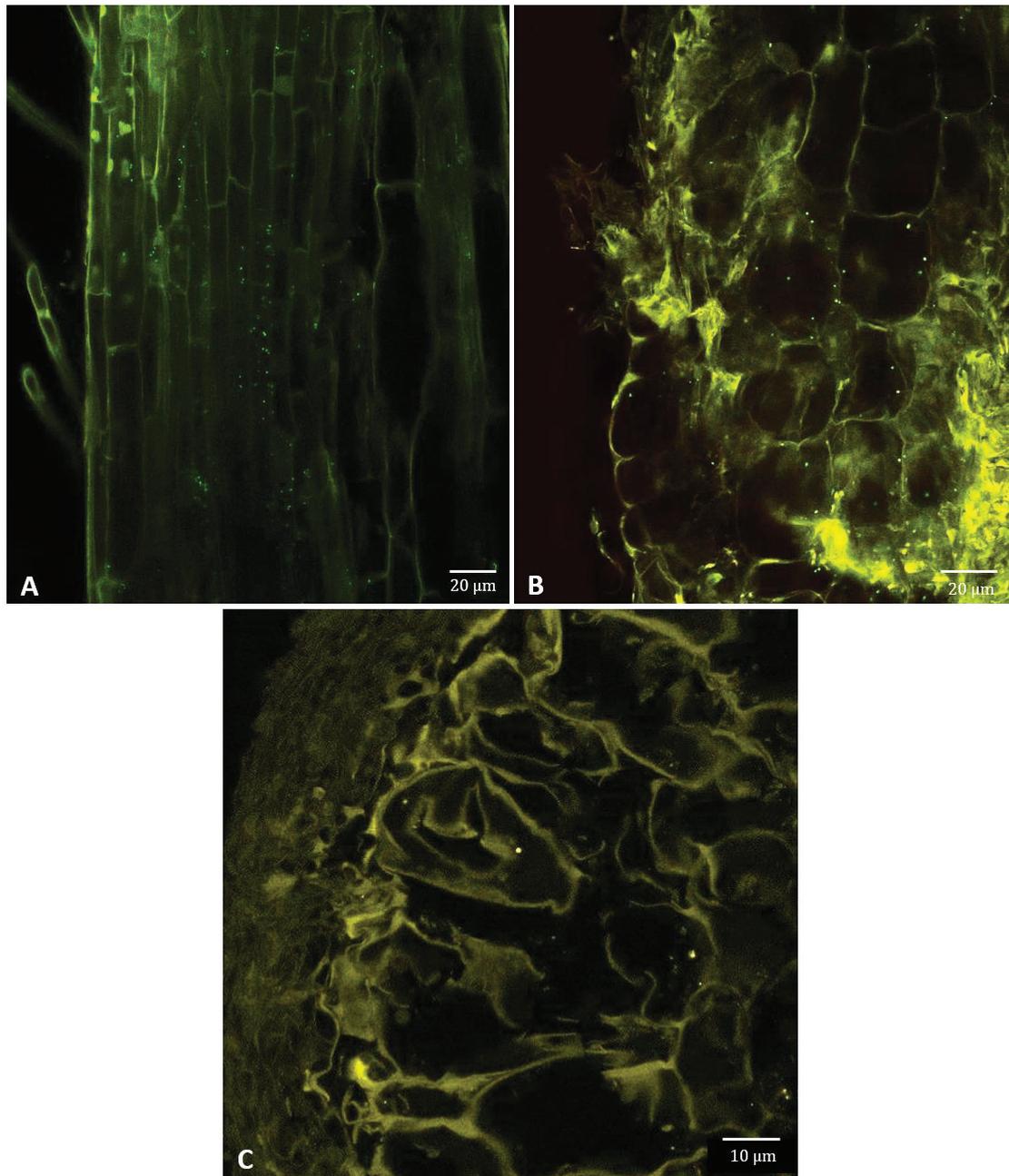


Figure 24: Analysis of transgenic ectomycorrhized and non-mycorrhized fine roots.

Populus tremula × *alba* cuttings were transformed with *A. rhizogenes* strain K599 harboring pLV7GPr3Kb (3 kb promoter fragment of *Potri.007G095000*, B). Shown are images of non-mycorrhized water control (A) and pLV7GPr3Kb transformed non-mycorrhized roots (B) by confocal laser scanning microscopy (Zeiss LSM 780) with argon laser 488 nm at 10 % intensity. Ectomycorrhized poplar roots transformed with the empty vector pPLV11-Sharinall was used as a negative control (C). Strong cell wall autofluorescence and non-specific background fluorescence is visible in transgenic and control roots in YFP channel (detection 510-560 nm).

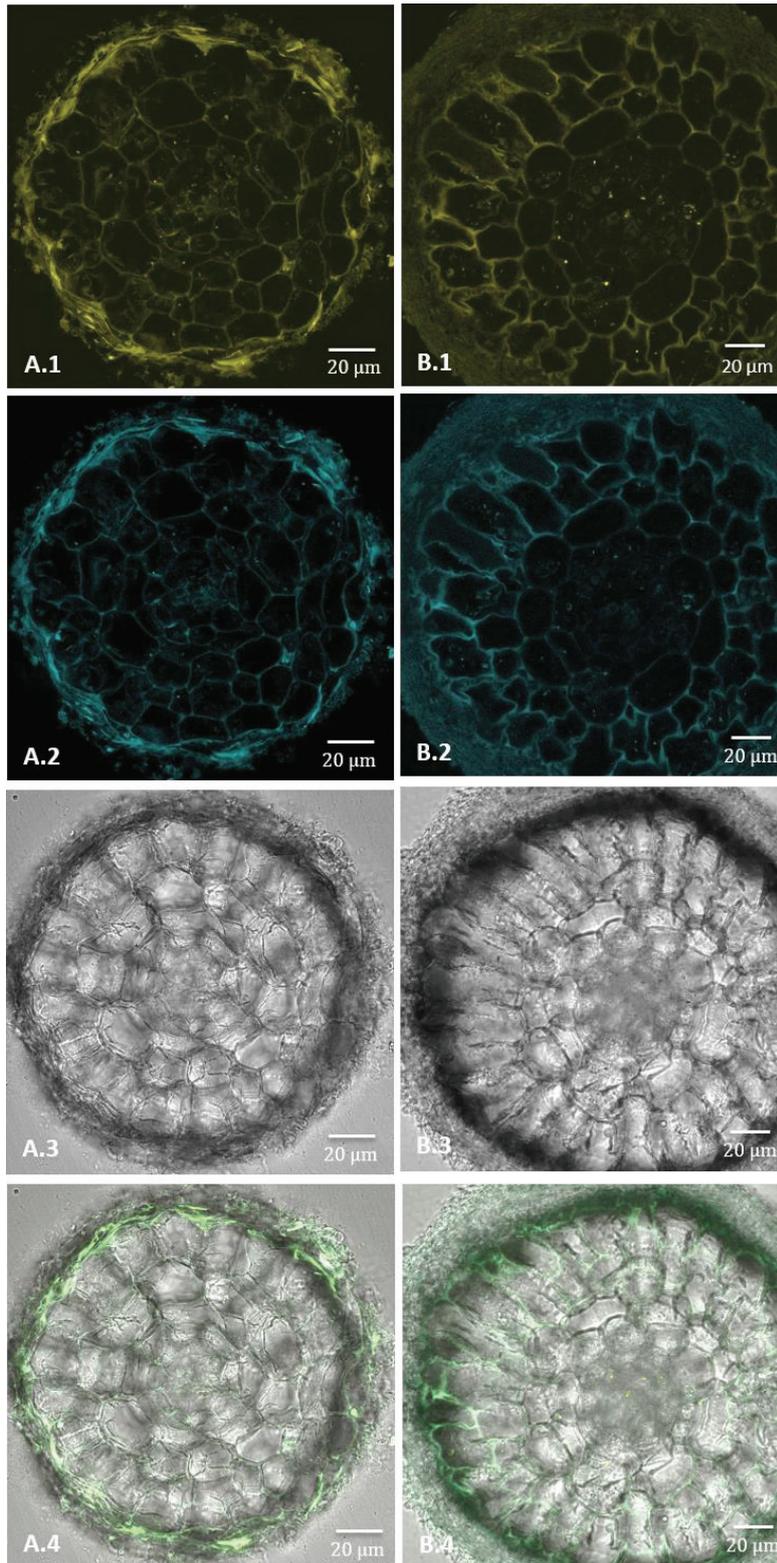


Figure 25: Comparison of cell wall autofluorescence and background fluorescence of ectomycorrhizized control and transgenic composite poplar roots.

P. tremula × *alba* cuttings inoculated with *A. rhizogenes* strain K599 harboring a 3 kb promoter fragment of *Potri.007G095000* and non-transgenic plants were inoculated with *Amanita muscaria* after root formation. Cross sections of ectomycorrhizas were analyzed by cLSM microscopy (Zeiss LSM 780) with argon laser 488 nm at 10 % intensity after 3-4 months of incubation. Shown are sections of transformed (A) and non-transgenic roots (B), YFP channel (yellow, detection range 510-560 nm, A1 and B1), DsRed channel (blue, detection range 560-650 nm, A2 and B2), the transmission light image (A3 and B3) and overlays of the three channels (A4 and B4). The cell wall autofluorescence and non-specific background fluorescence was detected over a broad range of detection window (510-650 nm).

3.3.2.4 Vector construction for truncated promoter fragments

For identification of *cis* acting elements, the initial promoter construct was truncated from its 5' end. pLV7GPr3Kb was digested with *Xho*I/*Hind*III and *Xho*I/*Kpn*I to generate truncated promoter fragments of 2656 bp and 1151 bp respectively (figure 26). The digested vector constructs were re-ligated and were verified by restriction endonuclease analysis (figure 27).

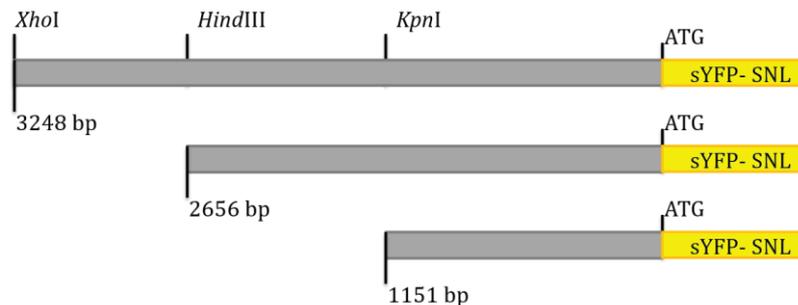


Figure 26: Scheme of promoter fragments generated in this study.

pLV7GPr3Kb (3 kb promoter fragment of *Potri.007G095000* harbouring pPLV11-Sharinall) was used to create shorter promoter fragments (2656 bp and 1151 bp) by double restriction digestion followed by purification and re-ligation.

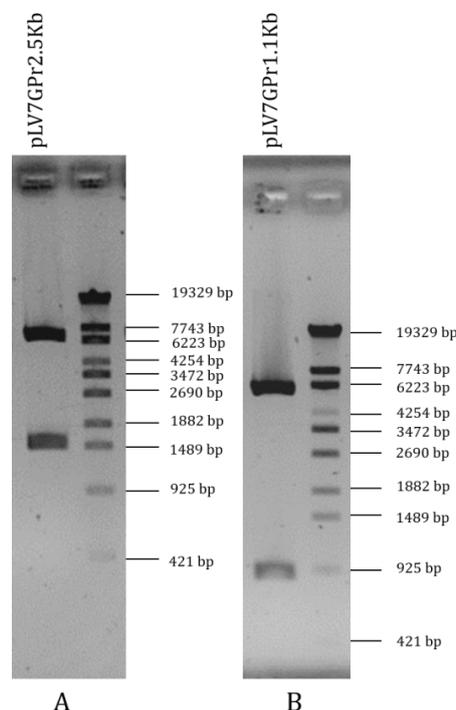


Figure 27: Verification of clones containing 5' truncated promoter fragments of *Potri.007G095000*.

pPLV11-Sharinall binary vector containing 2656 bp and 1151 bp fragments of the *Potri.007G095000* were verified by restriction digestion analysis using *Kpn*I (expected fragments – 1517 bp and 6914 bp) and

KpnI/EcoRI (expected fragments – 1152 bp and 5762 bp) respectively. Lambda DNA/*Eco130I* (*StyI*) was used as a DNA marker for size estimation.

pLV7GPr2.5Kb and pLV7GPr1.1Kb containing *Agrobacterium rhizogenes* K599 were used to transform 20 *Populus tremula* × *alba* each. The empty binary vector pPLV11-Sharinall and non-transgenic plants were used as negative controls. Roots formed after three-four weeks of incubation and no YFP signal was visible under epifluorescence microscope apart from cell wall autofluorescence. The co-transformed plants were inoculated with *Amanita muscaria* for ectomycorrhiza formation. However, no ectomycorrhiza formation occurred after three months of incubation. Due to time limitation the experiment could not be repeated.

3.4 Ethylene responsive transcription factor

Potri.008G071100 was predicted as a potential ethylene responsive transcription factor (Nehls, unpublished), of the *AP2/ERF* superfamily. These TFs are characterized by the presence of the *AP2/ERF* domain involved in DNA binding, consisting of about 60-70 amino acids (Nakano et al., 2006) (figure 28).

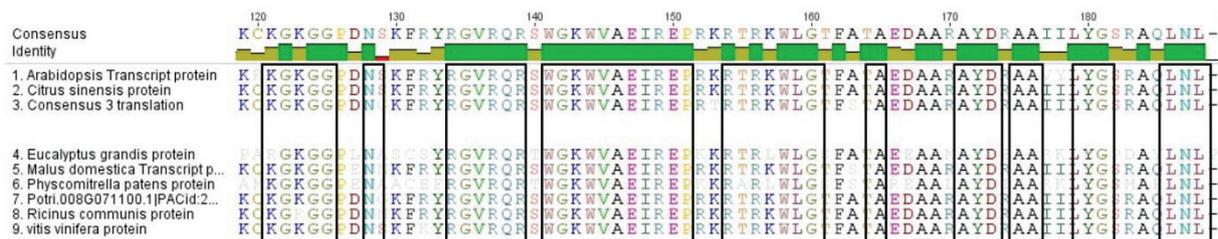


Figure 28: Conserved AP2/ERF DNA binding domain in different putative *Potri.008G071100* homologs.

Shown is a protein alignment of putative *Potri.008G071100* homologs. Conserved regions are highlighted.

3.4.1 Subcellular Localization of *Potri.008G071100*

3.4.2.1 Vector construction for in-frame fusion with a super yellow fluorescent protein (sYFP)

The CDS with few extra bases at the 5' and 3' end was amplified (8G-CDS) from *P. tremula* × *tremuloides* cDNA as a first step using the primers 1 and 2 (table 15 appendix). The optimized PCR condition is shown in (table 21 appendix). The fragment was cloned into the pJet1.2/blunt vector and selected clones (pJet-8GCDS) were verified by restriction analysis and sequencing. A virtual translation of the obtained sequence

was performed using the Geneious software package and was compared with the expected sequence.

For subcellular localization the stop codon depleted coding region of *Potri.008G071100* was amplified (8GCDS-SL) from pJet-8GCDS with the primer pair 6/7 (table 16 appendix). A conventional blunt end cloning strategy was applied for cloning the fragment into binary vectors pCXUN-sYFP and pPLV11-Sharinal. Both vectors allow a C-terminal in-frame fusion of a super yellow fluorescent protein (sYFP). The phosphorylated 8GCDS-SL PCR fragment was integrated into the *HpaI* restriction site of the respective dephosphorylated vectors. The integration of the fragment was verified by restriction endonuclease analysis (figure 29). The clones were furthermore sequenced with a sYFP specific primer. The obtained sequences matched well with the expected fragments and revealed the successful in-frame fusion of *Potri.008G071100* with the sYFP CDS.

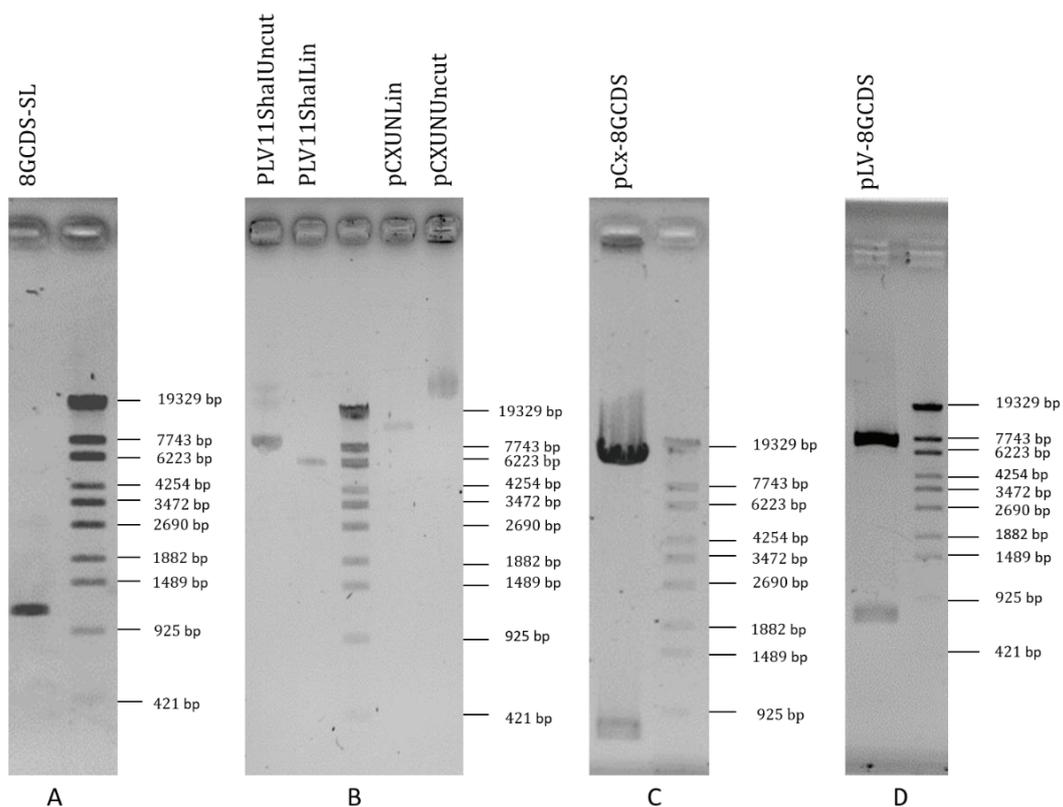


Figure 29: Verification of binary vectors containing *Potri.008G071100* CDS by restriction enzyme analysis.

Stop codon depleted coding region (8GCDS-SL) was amplified (A- expected size 1155 bp), cloned into *HpaI* restriction enzyme digested linearized binary vectors pCXUN-sYFP (pCXUNLin) and pPLV11-Sharinal (PLV11ShaIin) (B- undigested empty vectors PLVShaIUncut and pCXUNUncut as negative

control). Insertion of 8GCDS-SL into the vectors was verified by restriction endonuclease *Bam*HI digestion for clones pCx-8GCDS (C, expected bands – 84 bp, 760 bp, and 11743 bp) and pLV-8GCDS (D, expected bands – 39 bp, 84 bp, 727 bp and 6903 bp) – only large bands are visible. Lambda DNA/*Eco*130I (*Styl*) was used as marker for DNA size estimation.

3.4.2.2. Microscopic analysis of Potri.008G071100- sYFP fusion protein

For subcellular localization of the Potri.008G071100-YFP fusion, pCx-8GCDS transformed *A. rhizogenes* K599 and pLV-8GCDS transformed both *A. rhizogenes* K599 and *A. tumefaciens* GV3101 were used. Infiltration of tobacco leaves was performed with a mixture of three agrobacterial suspension containing – a) plasma membrane localized DsRed, b) a construct constitutively expressing P19 (RNAi suppressor) and c) Agrobacteria containing the construct of interest. *A. rhizogenes* strain K599 showed overall low transformation rate compared to *A. tumefaciens* GV3101. After 3 days of incubation, infiltrated leaves were first analysed under a stereo microscope for sYFP and DsRed fluorescence. Selected sections were further analysed by a laser scanning microscopy. Co-localisation of sYFP and DsRed indicated a most likely subcellular localization of Potri.008G071100 in the nucleus (figure 30).

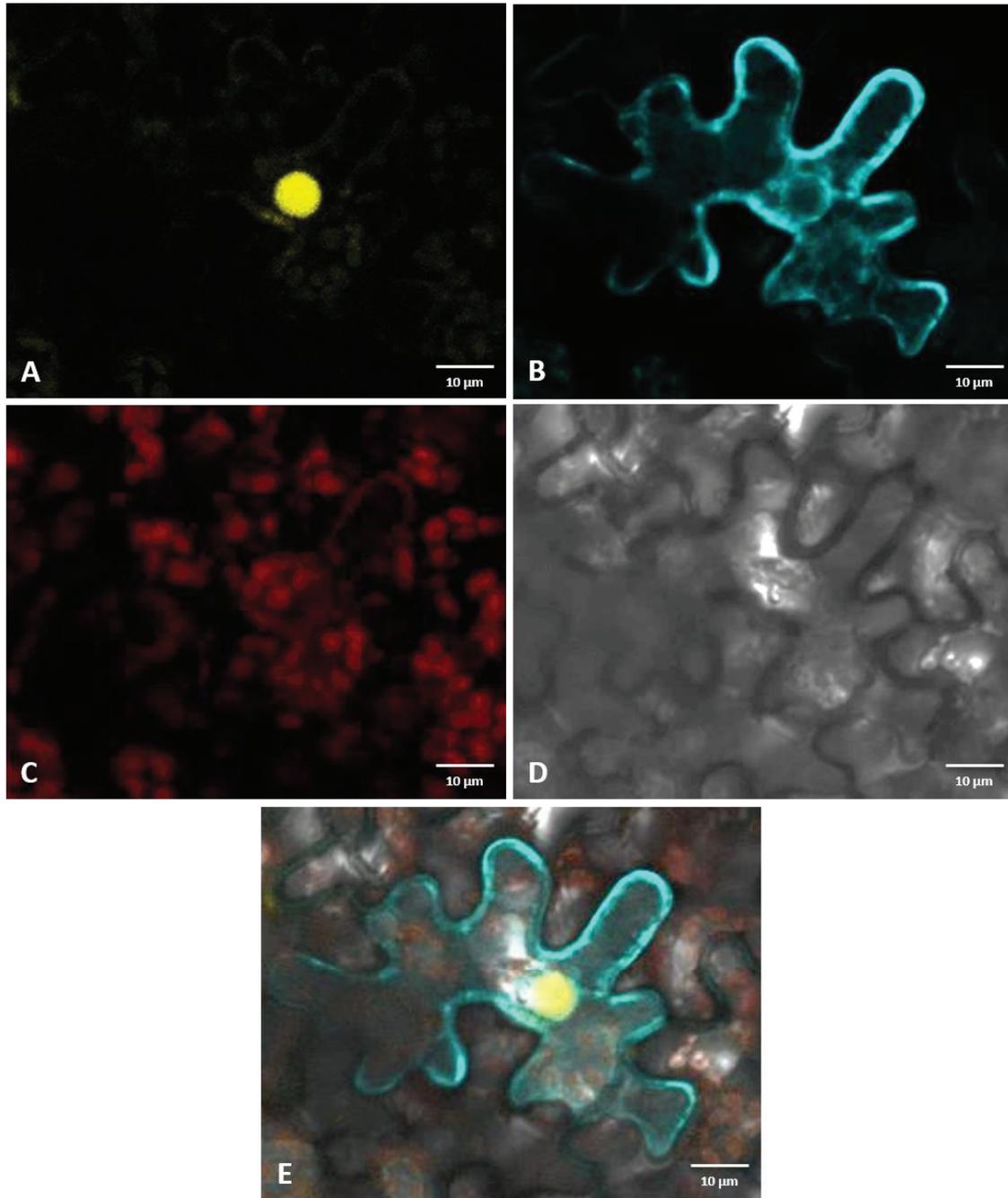


Figure 30: *Nicotiana benthamiana* leaf epidermis cells transformed with plasma membrane localized DsRed and Potri.008G071100-sYFP.

After 3 days of incubation leaf sections were analyzed under cLSM. Samples were exposed with argon laser at 488 nm with 2 % laser intensity. The subcellular localization of Potri.008G071100-sYFP and plasma membrane localized DsRed are shown in (false color) yellow (A: 510-560 nm) and turquoise (B: 580-650 nm) respectively. Detected chlorophyll autofluorescence (660 -730 nm) is shown in red (false color) (C), leaf epidermis cells are illuminated by transmission light (D) and an overlay of all the four channels (E).

3.4.2 Promoter analysis

It has been shown that 3 kb upstream sequence of *ABI4*, the *Arabidopsis* homolog of *Potri.008G071100* contains the *cis*- acting motifs necessary for sugar response (Arroyo et al., 2003), ABA signalling and regulating its own expression (Bossi et al., 2009). Therefore, a *Potri.008G071100* promoter fragment of around 3.5 kb was PCR amplified from *P. tremula x tremuloides* leaf gDNA. Two primer pairs 8/9 and 9/10 (table 17 appendix) were used to amplify 3498 bp and 3635 bp of the upstream regions (8GPr3.4/3.6Kb). DNA fragments of expected sizes were purified and subsequently cloned into pJet1.2/blunt vector. Digestion with restriction enzymes and sequencing analysis were used for verification (figure 31). Only the primer pair 8/9, resulted in the successful amplification of the promoter region pJet-8GPr3.4Kb.

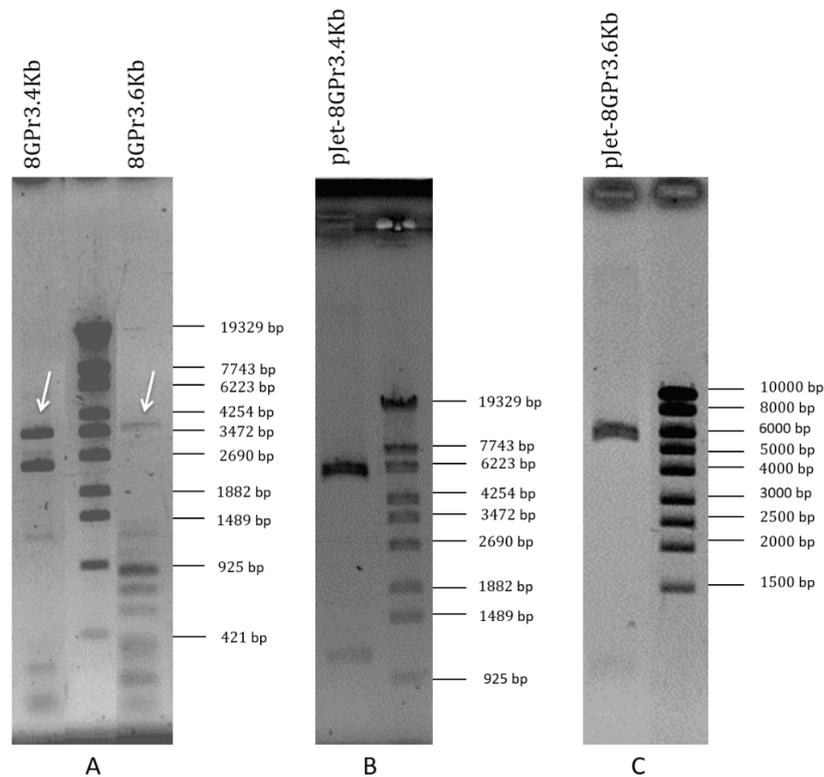


Figure 31: Amplification and cloning of 3.5 kb promoter fragment of *Potri.008G071100*.

3498 bp and 3635 bp promoter fragments were PCR amplified from *P. tremula x tremuloides* genomic DNA (A- arrow marked). The fragments were purified and cloned into pJet1.2 blunt vector. The clones were verified by restriction endonuclease analysis with *NcoI* and *XhoI* for 3.4kb (B, expected bands- 980 bp and 5492 bp) and 3.6 kb (C, expected bands - 475 bp and 6147 bp) fragments respectively. Lambda DNA/*Eco130I* (*StyI*) was used as marker for DNA size estimation.

For functional testing whether the amplified promoter fragment contained all regulatory elements, the promoter fragment was inserted into a binary vector. A PCR based strategy was chosen by which two restriction enzyme recognition sites were introduced at the 5' and 3' ends for a site directed sticky end insertion into the vector. The promoter fragment was amplified from pJet-8GPr3.4Kb using primer pair 11/12 (table 17 appendix). The amplified fragment (8G11Pr3.4Kb) was cloned into pJet1.2/blunt vector (pJ8G11Pr3.4Kb). The suitable clone (pJ8G11Pr3.4Kb) and modified pPLV11-SharinaII were digested with *KpnI* and *HindIII* and the ends of the vector backbone were dephosphorylated before ligation with the promoter fragment. The final construct was proven by restriction endonuclease digestion (figure 32 pL118GPr3.4Kb) and sequencing with M13 forward/reverse primers.

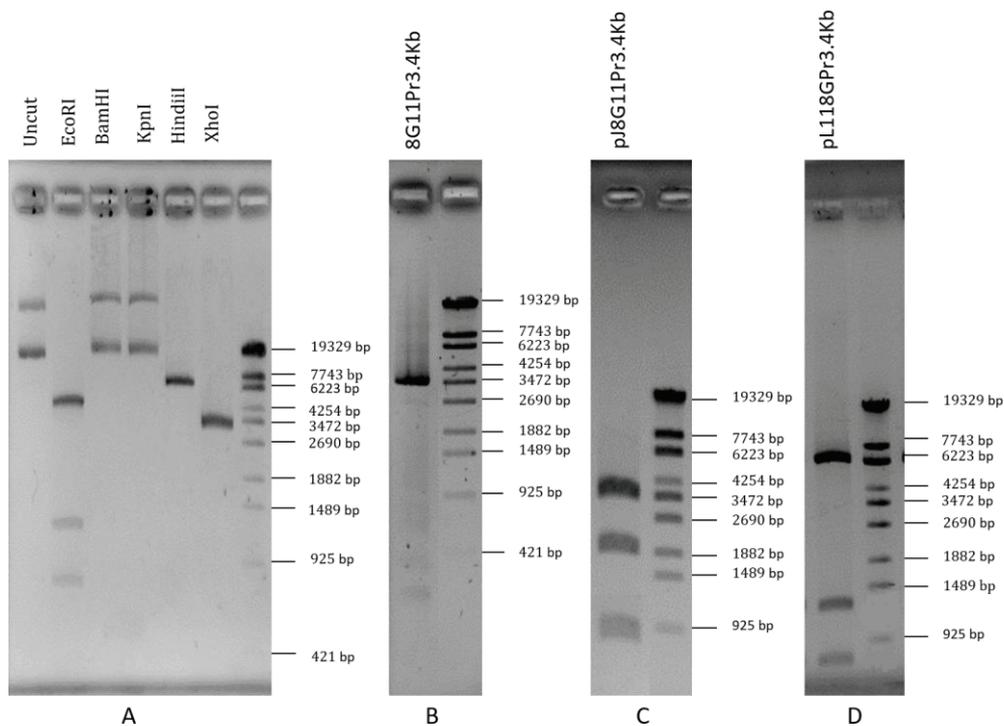


Figure 32: Verification of pPLV11-SharinaII containing 3.5 kb *Potri.008G071100* promoter fragment by restriction endonuclease analysis.

pJet-8GPr3.4Kb was digested with selected endonucleases *BamHI* (no digestion), *KpnI* (no digestion), *HindIII* (cuts once inside pJet backbone, linearizing 6484 bp plasmid) *XhoI* (expected fragments- 3069 bp and 3415 bp) for selection of restriction enzyme recognition sites to be used in conventional sticky end cloning; undigested and *EcoRI* digested plasmid were maintained as positive and negative controls (A) respectively. The 3510 bp promoter fragment was amplified (8G11Pr3.4Kb) with primer pair containing *KpnI* and *HindIII* recognition site overhangs (B), cloned into pJet1.2/blunt, verified by restriction enzyme analysis with *EcoRI* (C, expected fragments- 703 bp, 1890 bp and 3891 bp) followed by cloning into pPLV11-SharinaII and verification by *EcoRI* digestion (D, expected bands -703 bp-not visible, 806 bp, 1228 bp and 6549 bp). Lambda DNA/*Eco130I* (*Styl*) was used as marker for DNA size estimation.

3.4.3.2 *Populus tremula* × *alba* transformation, ectomycorrhiza formation and cLSM analysis

Construct pL118GPr3.4Kb was transformed into *Agrobacterium rhizogenes* K599 and used for poplar transformation. The empty pPLV11-SharinaII vector along with sterile water dipped plants were maintained as controls. Composite *Populus tremula* × *alba* formed roots after approximately three weeks and were tested for YFP signals by stereo microscopy. Furthermore, selected root sections were examined by cLSM and peroxisomal located YFP were clearly detectable (figure 33A). This experiment was repeated twice with twenty plants each. YFP was detected in at least one root for 83 % (± 3.53 , n= 40) plants.

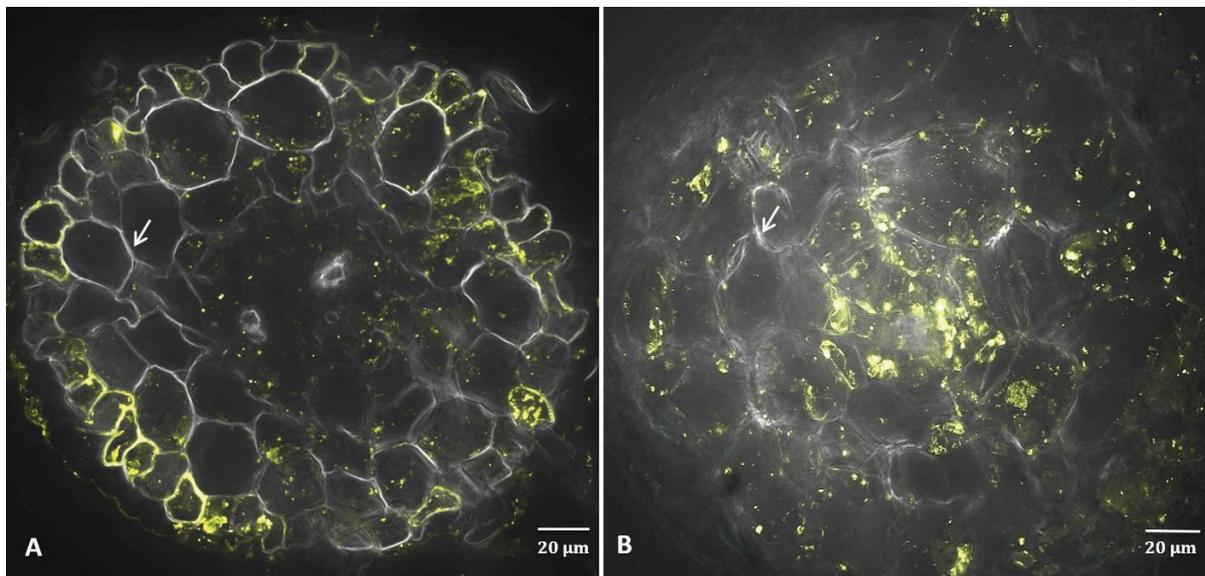


Figure 33: Comparison of fluorescence intensity between a transgenic non-mycorrhized and ectomycorrhized poplar composite plant harboring pL118GPr3.4Kb.

P. tremula × *alba* cuttings were inoculated with *A. rhizogenes* strain K599. After development of roots, the plants were inoculated with *Amanita muscaria*. Shown are cross sections of non-mycorrhized (A) and mycorrhized (B) roots analyzed by cLSM (Zeiss LSM 780, argon laser excitation wavelength 488 nm and 2 % laser intensity), peroxisomal targeted YFP (detection window 510-560 nm) and cell wall autofluorescence (detection window 580-650 nm, marked with arrows) are visible in (false color) yellow and white respectively.

Co-transformed composite plants were then inoculated with *Amanita muscaria* and ectomycorrhiza formed after around three-four months of incubation. No significant changes in YFP signal intensity in the ectomycorrhized roots were observed under the stereo microscope. Further verification by cLSM analysis of ectomycorrhized root

sections (figure 33B) confirmed this result. Cell wall autofluorescence was observed to be stronger in ectomycorrhized roots compared to non-transgenic poplar fine roots.

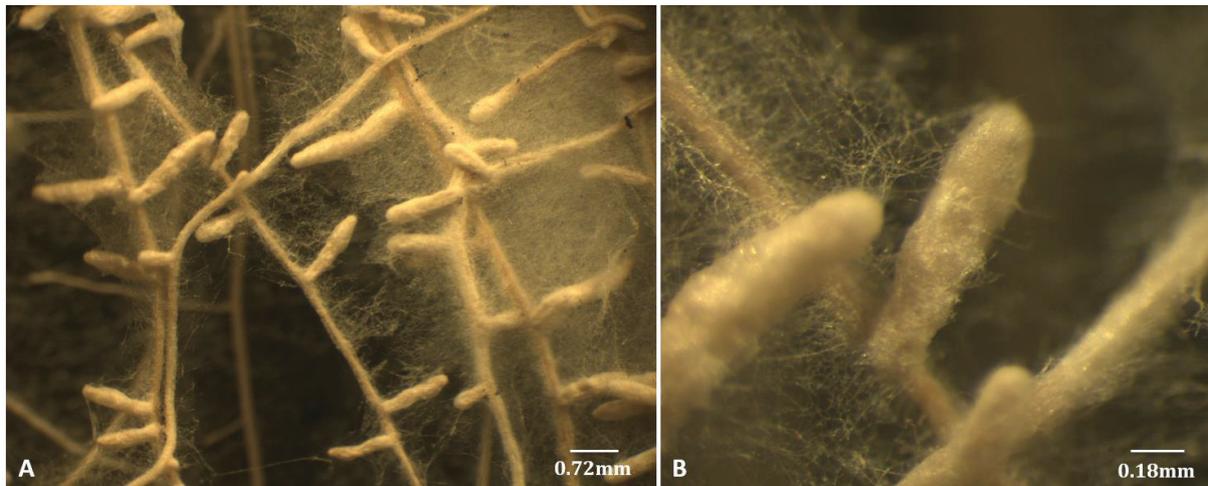


Figure 34: Ectomycorrhiza of a composite poplar with *Amanita muscaria*.

P. tremula × *alba* cuttings were inoculated with *Amanita muscaria* in a sugar depleted medium three-four weeks after roots formed. Ectomycorrhiza formed after 3-4 months of inoculation (A). General features of ectomycorrhiza such as swollen appearance, suppression of root hairs and presence of extra radical hyphae (Slankis, 1973) were observed (B, enlargement of A).

3.4.3.3 Vector construction of shortened promoter fragments

pL118GPr3.4Kb was digested with the following restriction enzyme combinations *KpnI/PmeI* and *KpnI/SphI*, followed by re-ligation to shorten the 3.4 kb promoter fragment from its 5' end. Verified clones (figure 35, pL118GPr1.6kb and pL118GPr400bp) were transformed into *Agrobacterium rhizogenes* K599. 20 *Populus tremula* × *alba* cuttings were inoculated per construct. Due to a high contamination rate of the plant batch, the transformation could not be repeated due to time limitation.

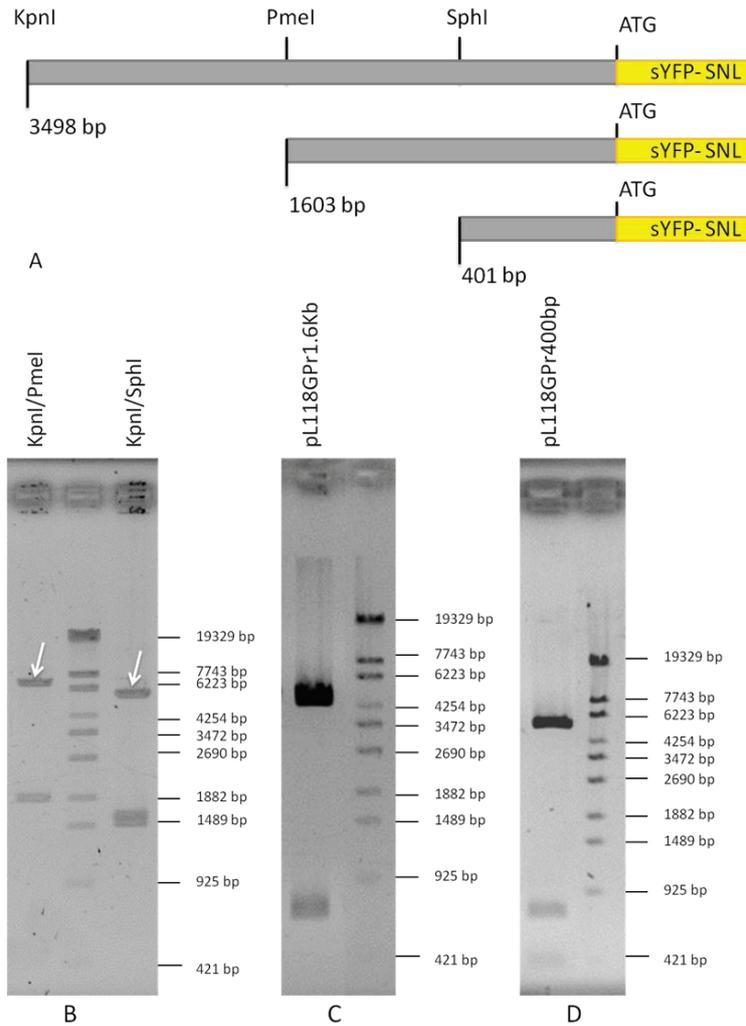


Figure 35: Verification of clones containing 5' truncated promoter fragments of *Potri.008G071100*.

pL118GPr3.4Kb was used to create shorter promoter fragments (1603 bp and 401 bp) by double restriction digestion, purification and re-ligation. pL118GPr3.4Kb was digested with *KpnI/PmeI* (B, expected size - 1908 bp, 7378 bp) and *KpnI/SphI* (B, expected size - 1541bp, 1568bp, 6177bp) (B). The marked fragments were isolated and re-ligated and the clones were verified by restriction enzyme digestion with *EcoRI* (C, expected bands- 743 bp, 766 bp and 5869 bp) and *KpnI/BamHI* (D, expected bands - 451 bp, 736 bp and 4990 bp) respectively.

3.4.3 Heterologous expression of *Potri.008G071100* in *E. coli*

The best *Arabidopsis* homolog *AT2G40220* (also known as *ABI4*) is known to be auto-regulated (Bossi et al., 2009). To identify potential *cis* elements in the promoter region of *Potri.008G071100*, electrophoretic mobility shift assay (EMSA) was planned. For this approach, however, the functional protein is requested. As the isolation from ectomycorrhiza is not feasible due to material limitation, heterologous expression in *E. coli* was targeted.

For heterologous expression, the vector pET28a was chosen. The cloning strategy was such that Potri.008G071100 will be attached to a C-terminal Strep-TactinII affinity tag. The CDS was amplified using a primer pair that introduces restriction sites of *NcoI* at the 5' end and *EcoRI* at the 3' end (table 15 appendix). The expected DNA fragment (8GCDS-pET28a) was first cloned into pJet1.2/blunt for initial verification (pJet-8GCDSpET). Thereafter the fragment was cloned into empty pET28a vector, which was linearized with the same enzyme combination. Restriction enzyme analysis was performed to confirm insertion of the fragment (pET28a-8GCDS - figure 36).

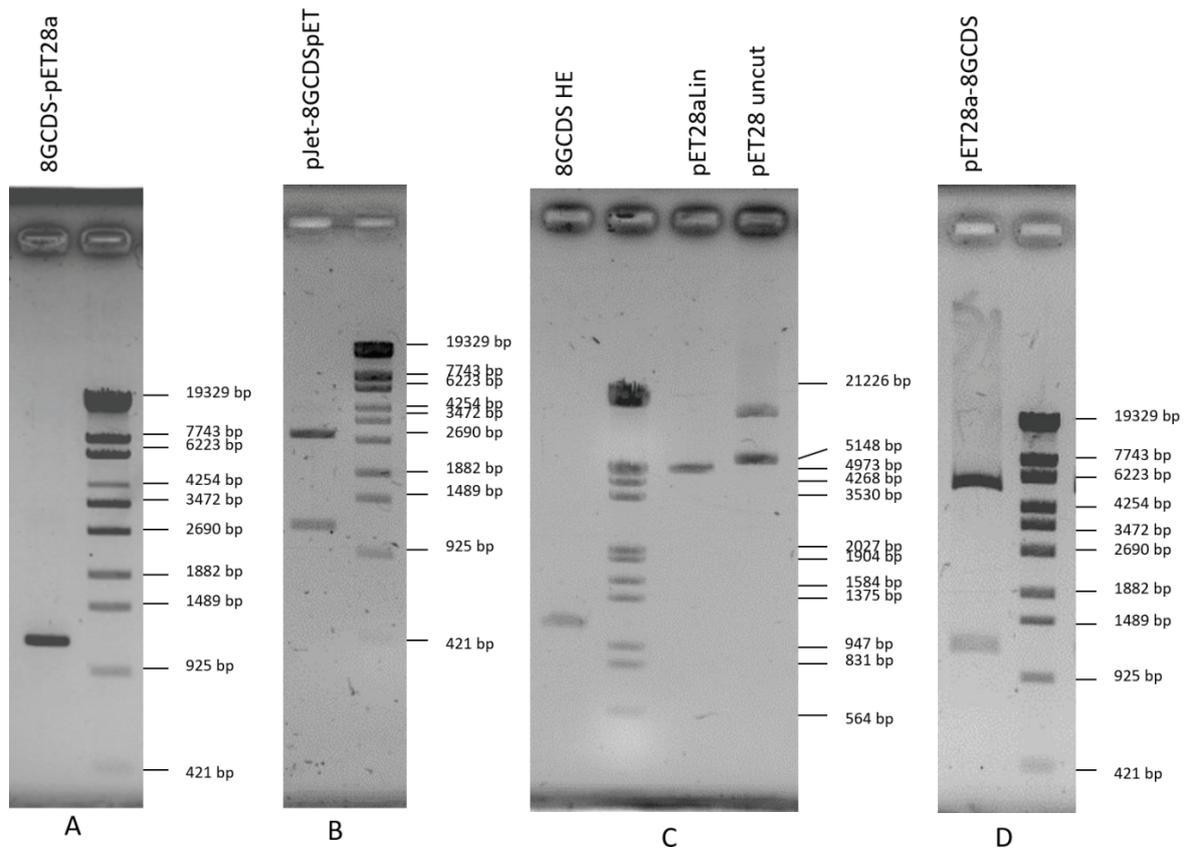


Figure 36: Amplification and verification of clone by restriction enzyme based analysis for Potri.008G071100 heterologous expression in *E. coli*.

Stop codon depleted CDS of *Potri.008G071100* was amplified with introduced 5' and 3' end restriction enzyme recognition sites (A, expected size 1170 bp). Clone was verified by restriction analysis with *NcoI* and *EcoRI* (B, expected bands 1155 bp and 2988 bp). Digestion with same enzyme combination on pJet-8GCDSpET and empty pET28a vector revealed *Potri.008G071100* CDS (C, 8GCDSHE expected size - 1155 bp) and linearized pET28a (C, pET28aLin expected size - 5257 bp), undigested empty pET28a was maintained as a negative control. 8GCDSHE integration into pET28a vector was confirmed with restriction analysis (D, expected bands- 1155 bp and 5263 bp (B)). Lambda DNA/*EcoRI-HindIII* (C) and Lambda DNA/*Eco130I (Styl)* were used as marker for DNA size estimation.

For further verification, the primer 5 (table 15 appendix) was designed for sequencing of selected clones and verify the in-frame fusion with the Strep-Tactin II affinity tag. Another primer, AM_peT28aseqfor (Alexander Makowka, 2015), was used for N-terminal sequence verification. The obtained sequences matched well with the expected clone and a virtual translation of it confirmed the Strep tag was in-frame to the stop codon depleted *Potri.008G071100* CDS (figure 37).

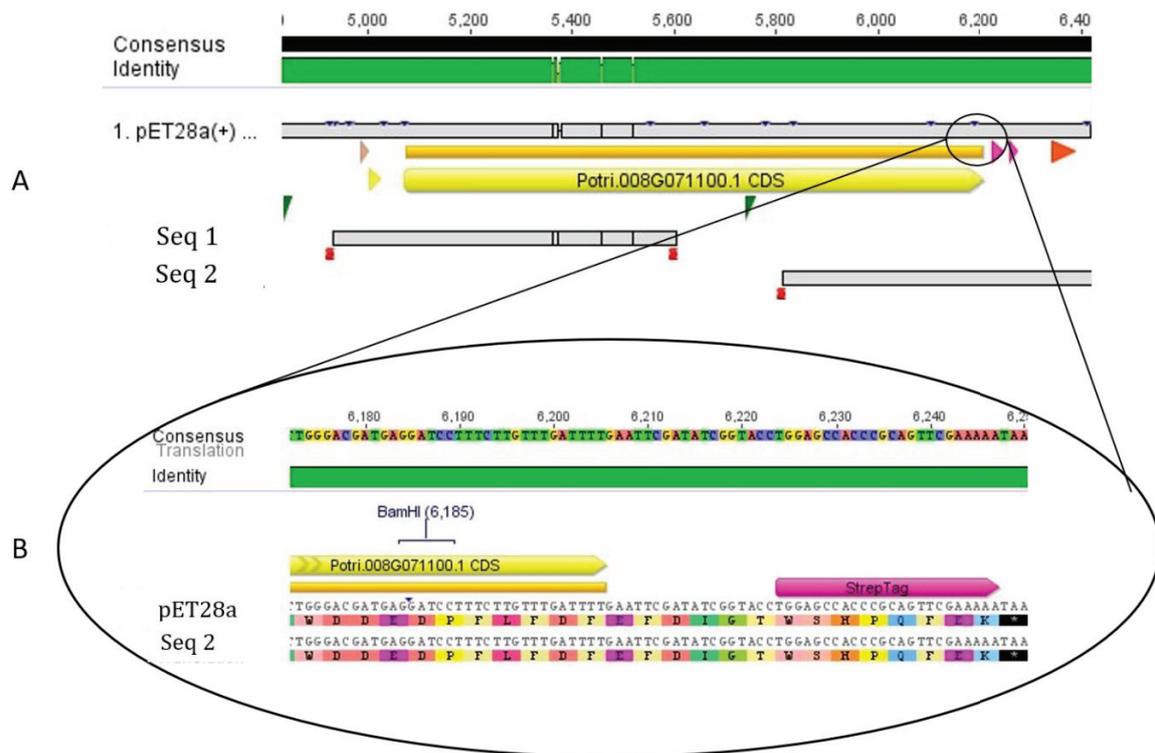


Figure 37: Multiple alignment of the sequenced fragments with the virtual construct of *Potri.008G071100* CDS in pET28a.

The PCR amplified stop codon depleted *Potri.008G071100* coding sequence was cloned into pET28a (pET28a-8GCDS). Shown is the multiple alignment of the sequencing results (Seq 1 and Seq 2) with the virtual construct (pET28a) (A). The resulted consensus sequence matched well with the construct and was verified by virtual translation, especially the stop codon depleted CDS end in-frame with the Strep-TactinII affinity tag (B). Coding region and the Strep Tag are annotated in yellow and pink respectively.

The final construct was transformed into two *Escherichia coli* protein expression cell lines - BL21-DE3 and Rosetta-gami and the protein was expressed with different conditions listed in table 9. Unfortunately, all attempts of receiving the natively folded protein in soluble form were unsuccessful (figure 38). Trials of solubilizing the protein from inclusion bodies by denaturing in 6 M urea and refolding by dialysis against 25 mM Tris-HCl pH 8 buffer was also unsuccessful.

Table 9: Conditions used for heterologous protein over expression of Potri.008G071100.

Shown are the different parameters – varied IPTG concentration used for induction, incubation temperatures, bacterial cell lines used as expression system, incubation time and cell lysis techniques.

IPTG induction	Temperature (°C)	Cell line	Incubation time	Cell lysis Buffer	Cell lysis
1 mM 0.3 mM	30	BL21/ Rosetta- gami	4 hrs, 6 hrs, 8 hrs and over night	25 mM Tris-HCl pH 8	Ultrasonication
	25				
	18				
	14				
0.1 mM	14 and 20	BL21/ Rosetta- gami	Over night		
0.5 mM					
0.1 mM	18	BL21/ Rosetta- gami			
100 µM	14	BL21			
50 µM					
25 µM					
10 µM					
5 µM					
0.5 µM					
0.05 µM					
0.005 µM					
0.1 mM	18			6 M Urea	Ultrasonication
				Dialysed against 25mM Tris-HCl pH 8	French Press

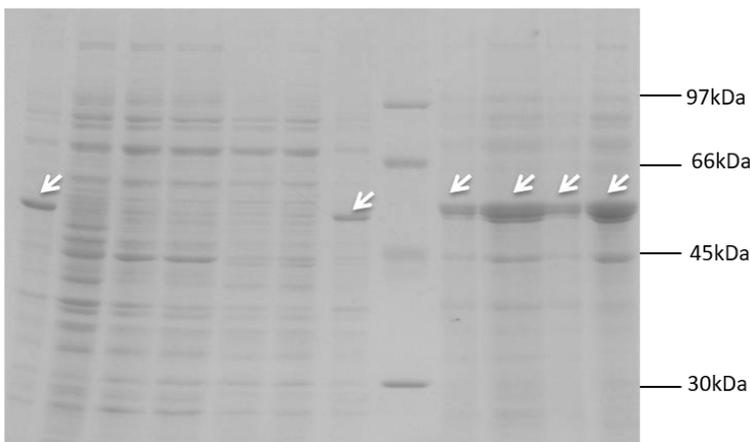
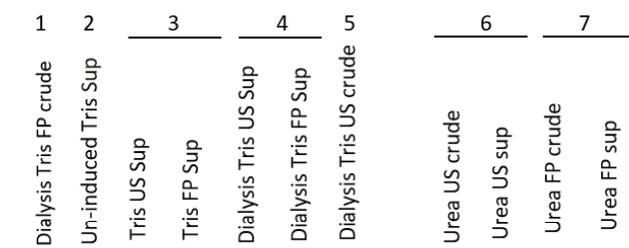
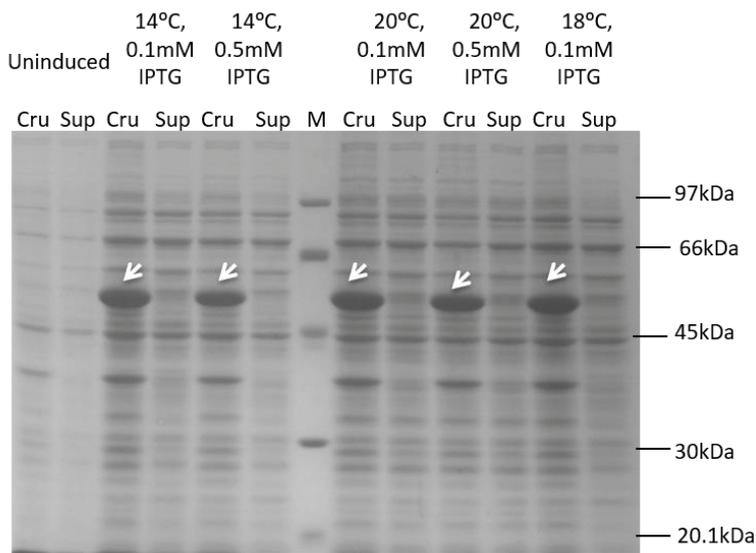
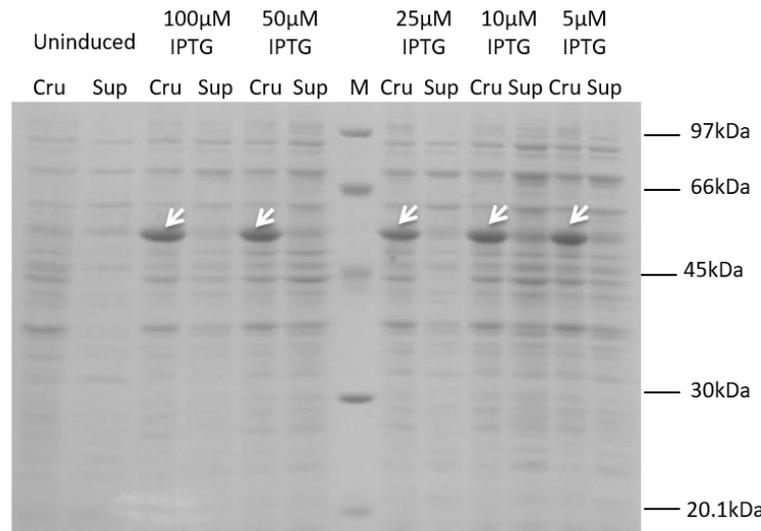


Figure 38: Coomassie blue stained SDS-PAGE gels of overexpressed Potri.008G071100.

Overexpressed protein of size around 46 kDa is obtained only in crude (Cru) after overnight incubation at 14 °C with varied IPTG concentrations 5 μM-100 μM (A) and in varied temperature (14-20 °C) with 0.1 mM and 0.5 mM IPTG induction (B). Attempts of refolding urea denatured overexpressed protein from both ultrasonicated (C6) and French pressed (C7) cell lysate in 25 mM Tris-HCl buffer pH 8 revealed insoluble proteins present in the crude (C1 and B5) rather than in the soluble supernatant fractions (C4). Un-induced culture was maintained as negative control and the expected protein band is marked with an arrow. Crude (Cru) is the raw cell lysate and Sup represents the supernatant obtained after centrifugation of the cell lysate. Cell lysis obtained using ultrasonication and French press is indicated as US and FP respectively. A low molecular weight protein marker was used for size estimation.

4. Discussion

4.1 Development of composite poplar plant: a protocol for obtaining fast transgenic roots

For the gene function analysis and further root centred research one of the aims of this thesis was to develop a fast method to generate transgenic poplar roots. Generation of fully transgenic plants in general is time consuming as it includes plant generation from callus cultures (Alpizar *et al.*, 2006). Hansen and co-workers developed an alternative method, so-called composite plants where transgenic roots are induced by *A. rhizogenes* infected non-transgenic shoots (Hansen *et al.*, 1989). The method has thus been used to generate composite plants in several species (Hansen *et al.*, 1989; Collier *et al.*, 2005; Alpizar *et al.*, 2006), however no such protocol has been developed for poplar.

Agrobacterium rhizogenes has a limited host specificity in nature (Chilton *et al.*, 1982) that however can further be extended to other plant species under laboratory conditions (Porter & Flores, 1991). It has been shown for other plants that different *A. rhizogenes* species may vary in plant transformation efficiency. In this work, four different *A. rhizogenes* strains: K599 (2659)- a cucumopine strain (Daimon *et al.*, 1990), 15834 - an agropine strain (Veena & Taylor, 2007), 8196 - a mannopine strain (Hansen *et al.*, 1991) and 1724- a mikimopine strain (Shiomi *et al.*, 1987), were therefore tested for transgenic roots induction in *Populus tremula* × *alba* cuttings. While all the tested strains induced root formation by non-transgenic poplar cuttings, they however differed from each other in efficiency. Formation of extended microcallus was observed at the cut surface of *P. tremula* × *alba* cuttings prior to rooting, which was then confirmed by repetition of the experiment and frequent analysis of the cuttings inoculated only with the suitable *A. rhizogenes* strain K599. The microcallus formation may also be an explanation of the retarded root formation and the fragile root harbouring microcalli also made the handling of the cuttings difficult.

Increased number of roots formed by strain 15834 can be due to its agropine type Ri plasmid that encodes two T-DNAs – TL-DNA and TR-DNA (Trovato & Linhares, 1999) carrying *rol* genes responsible for root induction (Veena & Taylor, 2007) and two genes *iaaM* and *iaaH* whose protein derivatives are responsible for auxin biosynthesis (De Paolis *et al.*, 1985). Auxin orchestrate almost every aspect of plant growth and

development including root initiation, growth and branching (Woodward & Bartel, 2005; Vanneste & Friml, 2009). Therefore, for strain 15834 infected plants locally increased auxin level enhances the root formation and branching, while other three strains (8196, K599 and 1724) harbouring only *rol* genes (Veena & Taylor, 2007) cannot induce further increment of auxin biosynthesis of transformed tissues. Transgenic 'hairy roots' showed loss of response towards gravity which is in agreement with other studies with suppressed geotropism in various *A. rhizogenes* transformed plants (Capone et al., 1989; Hansen et al., 1991; Chriqui et al., 1997; Tanaka et al., 2001; Tiwari et al., 2008).

4.1.1 Co-transformation efficiency of different *Agrobacterium rhizogenes* strains

A. rhizogenes is capable of transferring binary vector T-DNA *in trans* facilitating the selection of transgenic plants by their 'hairy roots' (Christey, 2001). When *Agrobacterium rhizogenes* strains harbouring the binary vector pBIN19-YFPPTS1 (Nowak *et al.*, 2004) were used, only *A. rhizogenes* K599 induced formation of fluorescent roots. Similar results were also observed with other plant species (Porter & Flores, 1991). One explanation for this phenomenon might be that only K599 allowed integration of a high copy number of bi-TDNA into the plant genome. Collier and colleagues showed that transformation with K599 leads to integration of up to eight copies of binary TDNA into the *N. tabacum* genome (Collier *et al.*, 2005). No obvious chimera roots, revealing fluorescent and non-fluorescent branches, were ever observed in the composite poplars, which was confirmed by closely observing the YFP fluorescence in all cell types (rhizodermis, cortex, endodermis, stele) in the cross sections of transgenic roots (from tip to base). Formation of each root is the result of an independent transformation (Costantino *et al.*, 1984). Positional effects of genome integrated T-DNAs (Collier *et al.*, 2005) or a different number of integration events may thus result in varying gene expression levels in individual roots. Indeed, different fluorescence intensities were also observed from different transgenic roots of the same plant suggesting various numbers of the binary T-DNA integrated into the genome. The fluorescent roots were recognized as hairy roots indicating the co-transformation of Ri-plasmid with T-DNA from binary vector. Co-transformation efficiency was in the similar

range as in other plants (Porter & Flores, 1991) revealing between 14-20% of roots where no YFP fluorescence could be observed.

The ability of *A. rhizogenes* strain K599 to induce transgenic root was also tested with other poplar species, *Populus trichocarpa* and *Populus tremula* × *tremuloides*. Although binary vector-based plant transformation was detected in the hybrid poplar *P. tremula* × *tremuloides* with a similar co-transformation efficiency seen in *P. tremula* × *alba*, transgenic root formation was not observed in *Populus trichocarpa* cuttings. In literature, many other *A. tumefaciens*-mediated *P. trichocarpa* transformations were also found to be unsuccessful (Böhlenius *et al.*, 2006).

4.1.2 Root cell wall autofluorescence may interfere with detection of targeted signal

A general problem of studying poplar roots especially when mycorrhized is its cell wall autofluorescence that covers a wide emission range. Hence interference with the signal emission of a target fluorophore can be problematic. This autofluorescence is mainly from phenolic compounds (Vierheiling *et al.*, 1999; Vierheilig *et al.*, 2001) embedded into the cell wall matrix. As root rhizodermal and cortical cells contain large central vacuoles resulting in only a thin cytoplasmic layer with similar thickness of the cell wall, signal detection of cytoplasm located fluorescent protein is rather difficult e.g. (Neb *et al.*, 2017). Although this autofluorescence could be avoided in poplar fine roots (but not in ectomycorrhiza) when argon laser intensities lower than 2 % were used, a strong target specific fluorescence signal is required for clear detection. Therefore, peroxisome targeted fluorescent proteins were used in this study, that allowed easy *in planta* detection of the fluorescent proteins without the interference of cell wall autofluorescence. Reasons are enrichment in a small cellular compartment and presence of peroxisomes away from the cell wall (e.g. in cytoplasmic tubes that cross the central vacuole).

Use of sucrose in growth medium

Sucrose is long known as a potent plant root morphogen (Freixes *et al.*, 2002; Lee-Ho *et al.*, 2007) and was used in the agar based medium for growing the poplar cuttings. *A. rhizogenes*-based transformation often results in modulation of root phytohormone response. They also carry several genes involved in the regulation of auxins and

cytokinin sensibility of the transformed plant tissue. As these plant hormones are involved in several stages of plant growth and development, presence of sucrose in the growth medium may enhance the hairy root phenotype in composite plants. It has been shown that the cells containing high contents of auxin and sucrose are ideal targets for hairy root induction (Nilsson & Olsson, 1997). The presence of sugar in the agar medium indeed increased the number of roots and branching. Omitting sucrose from the growth medium however also eases the sterile handling as sugar rich medium is susceptible to contamination during the incubation period.

4.1.3 Composite poplar system and its limitations

Compared to the conventional poplar transformation, which takes up to several months, composite poplars can be generated only within a few weeks. Nevertheless, there are several limitations depending on its respective use in various fields. The change in hormone balance in 'hairy root' might interfere with certain studies related to phytohormone regulations (Christey, 2001; Kiselev *et al.*, 2007). As the composite plants retain their non-transformed shoot from which the transformed roots are induced, this method is suitable for analysis of root related questions, such as ectomycorrhiza induced gene regulation study. It has already been proven that every root formation could be result of an independent transformation event (Costantino *et al.*, 1984) and different copy numbers or positional effects of bi-TDNAs genome integration would result in varied gene expression levels in individual roots (Collier *et al.*, 2005; Neb *et al.*, 2017). In such cases, examination of individual transformed roots is required. The limited amount of root material can be problematic for biochemical analysis. Transferring the plants into pot cultures may help, which however is barely feasible under sterile conditions (Neb *et al.*, 2017). Pooling of root material of different plants could be an alternative strategy, which however, will not allow the detection of differences in individual roots (Neb *et al.*, 2017). Composite poplars also formed a number of roots which showed no fluorescence signal; in such cases using a second constitutive visualization marker would be helpful.

4.2 Comparison of gene expression in mycorrhized and non-mycorrhized roots

One aim of the thesis was to compare transcript levels of selected genes in mycorrhized and non-mycorrhized *Populus tremula x tremuloides* fine roots. The primary selection of the target genes was based on a comparative microarray analysis of transcription levels in mycorrhized and non-mycorrhized poplar roots (Nehls unpublished).

Gene expression microarrays are the preferred method for large-scale (e.g., whole-genome) discovery experiments. A general problem with microarray based analysis is cross hybridization between different members of a gene family. In plants the chance of cross hybridization is rather high, due to the presence of large gene families. To confirm the microarray expression analysis, an alternative independent method was chosen. Real-time qPCR has developed to a robust alternative approach for studying gene expression for small to medium scale sample size due to its sensitivity and real time quantification (Pfaffl, 2001; Ren *et al.*, 2007). The measurement of gene expression for the targeted genes is normally done keeping housekeeping genes as references (Canales *et al.*, 2006; VanGuilder *et al.*, 2008).

Although DNA microarray provides an unprecedented capacity for whole genome profiling and qPCR is a common validation tool of microarray results, both types of analysis have inherent pitfalls (Freeman *et al.*, 1999; Bustin, 2002; Chuaqui *et al.*, 2002; Yang *et al.*, 2002; Wurmbach *et al.*, 2003). Non-specific and cross hybridisations of labelled targets to array probes (Chuaqui *et al.*, 2002) are frequently observed in microarray analysis. The exponential amplification of errors (Freeman *et al.*, 1999), mis-priming or the formation of primer dimers (Bustin, 2002), and the changing efficiency of qPCR at later cycles (Freeman *et al.*, 1999; Bustin *et al.*, 2005) are well known problems in qRT-PCR.

4.2.1 Search for a suitable *P. tremula x tremuloides* homolog

As the microarray was based on the annotated genome sequence of *P. trichocarpa* but the hybridization was performed with cDNA obtained from mycorrhized and non-mycorrhized *P. tremula x tremuloides* fine roots, cross hybridization could not be opted out. As RNA of the hybrid poplar was also source for further approaches, finding the

most likely homolog of the respective *P. trichocarpa* gene in the sequenced genomes of *P. tremula* \times *tremuloides* was necessary. *P. tremula*, *P. tremuloides* and *P. tremula* \times *tremuloides* genome sequences generated from assembled paired end-based Illumina HiSeq 2000 sequencing, are meanwhile available (UPSC draft genome release: <http://popgenie.org>). Sequences were expected to be different in both poplar species *P. tremula* and *P. tremuloides* and the qualitatively poorer sequence of the hybrid poplar.

In this project, a total of 50 ectomycorrhiza induced highly upregulated poplar genes were selected for revised gene expression analysis by qRT-PCR. Therefore, appropriate primer pairs had to be generated. To avoid simultaneous amplification of different members of a gene family that revealed a high degree of sequence identity, the reverse primer was always located in the non-translated 3' part of the mRNA. The genome sequences of *P. tremula*, *P. tremuloides* and the hybrid poplar were used as template for primer design. Appropriate 3' end of a given gene was identified by a BLAST search using *P. trichocarpa* cDNA as source. When appropriate 3' end of the coding regions were identified in the genome of one poplar species, this region was then aligned with the 3' coding region ends of the other poplar species and the genome sequence of the hybrid. Region identified in all the investigated poplar genomes were preferred for primer design. Out of the selected genes, only 21 genes revealed sufficient good homology between *P. trichocarpa* and other poplar species. Therefore, primers were designed for those genes. The absence of suitable homologs in the genome libraries of *P. tremula*, *P. tremuloides* and *P. tremula* \times *tremuloides* can be due to the fact that the databases used to search for the homologs represent only a draft assembly of the sequenced genomes. Future availability of more accurate assembled genomes and extended sequencing of missing or critical regions of these poplar species might increase the chances of finding respective homologs.

4.2.2 Designing the primer pairs for real time qPCR

The success of qPCR specificity depends considerably on the designed primers that bind to the desired target sequence. Apart from non-specific primer binding to the template (cDNA), formation of primer dimers is a general problem leading to wrong results in template quantification. Therefore special attention to parameters for primer designing

(Brownie *et al.*, 1997), such as length, GC content, the difference in melting temperatures between sense and antisense primers and concentration used, have been considered.

In this work, suitable primers were identified using Primer 3 as integral part of the Geneious software suite. The size of the primers was chosen to be between 18 and 27 bp, optimally 20 bp so that the nucleotide sequence is long enough to bind to specific sequences and short enough to avoid the probability of building secondary structures (e.g. hairpins). The maximum melting temperature (T_m) difference between the forward and reverse primer was chosen to be 2 °C as the T_m difference is generally recommended to be under 5 °C to avoid a poor amplification (SantaLucia, 2007). The T_m for individual primer was chosen to be between 57 and 63 °C, optimally 60 °C as primers with a melting temperature above 65 °C are known to have a tendency towards secondary annealing (SantaLucia, 2007). Furthermore, DNA fragment of a size between 110-200 bp were chosen for amplification, as such short sequences reveal optimal amplification kinetics.

The coding region is highly conserved between different poplar species. To increase species specific targeting of the desired genes, the untranslated regions were included in the primer identification. In this thesis the 3'-untranslated region (UTR) was chosen over 5'-untranslated region (UTR) to be included into the desired amplified product as it is rich in single-feature polymorphisms that can be used to distinguish even closely related transcripts (Bhatramakki *et al.*, 2002; Vroh Bi *et al.*, 2006). Furthermore, since oligodT primer targeting the poly-A site was used for the reverse transcription, presence of a long 3'-UTR region or intron etc. may also generate cDNAs containing incomplete or no 5'-UTR as the reverse transcriptase might not reach the 5'-UTR during cDNA synthesis at all. The average length of 3' UTRs is highly variable, ranging about 200 nucleotides in plants and fungi to 800 nucleotides in humans and other vertebrates (Mignone *et al.*, 2002). As most plant mRNAs contain at least 50 bp of 3'-UTR, a region of not more than 50 bp downstream from the 3' end of the coding region was chosen in this work.

4.2.3 Optimization of conditions for qRT-PCR based expression analysis

High Ta enhances amplification specificity; it on the other hand decreases the yield. The annealing temperature (Ta) for initial real time qPCR optimization was chosen to be about 2 °C lower than the Tm of the primers. However, for some primers the annealing temperature had to be lowered to around 10 °C below the primer melting temperature upon optimization of the PCR conditions for certain genes.

DNase treatment of RNA samples was crucial for expression analysis using poplar roots and mycorrhizas to eliminate chances of false positive results in the quantitative measurement. In order to test the designed primers and optimization of PCR condition a primary real time qPCR approach was performed with only two biological samples: cDNA of mycorrhized and non-mycorrhized roots for all the 21 targeted genes. PCR conditions were further optimized when poor or no reliable PCR products were generated. Thereafter, a second set of first strand cDNAs was tested.

Low copy number of the template present in the PCR reaction can favour primer dimer formation (Markoulatos *et al.*, 2002). Binding to a double stranded DNA but not to single stranded DNA, SYBR Green I fluorescence increases about >1000- fold (Dragan *et al.* 2012). Therefore, the intensity of SYBR Green I fluorescence will decrease strongly when the melting temperature of the product is reached as the two DNA strand will separate (Hunt 2010). As primer dimers frequently have a lower melting temperature than the specific PCR product, a temperature increase just below the melting temperature of the PCR specific product prior to fluorescence measurement would eliminate primer dimer fluorescence. This strategy was used for all the primer pairs, irrespective of the presence of primer dimers. As differences in transcript levels between mycorrhized and non-mycorrhized samples can reach several orders of magnitude, for some genes a specific PCR product was observed only for mycorrhized samples while fine root samples revealed only primer dimer formation.

4.2.4 Comparison of gene expression in mycorrhized and non-mycorrhized poplar fine roots

Out of the 21 *P. trichocarpa* genes showing good matching homologs in *P. tremula*, *P. tremuloides* and *P. tremula x tremuloides* database, after optimizing the PCR parameters only 12 genes could be used for further testing. Finally, for six of these

genes (homologs to the *P. trichocarpa* genes *Potri.002G188900*, *Potri.007G081200*, *Potri.006G009300*, *Potri.003G210600*, *Potri.007G095000* and *Potri.008G071100*) DNA microarray results were confirmed. All these six genes showed much higher gene expression in ectomycorrhized roots compared to non-mycorrhized fine roots. The differences in transcript levels obtained by real time qPCR were around 5 times higher compared to microarray data. Differences in gene expression may be underestimated in array analysis. One explanation for this can be that oligomers were designed based on *P. trichocarpa* genome sequence and do not fit perfectly to the *P. tremula* × *tremuloides* sequence. Unlike in array analysis, qPCR primers were designed according to *P. tremula*, *P. tremuloides* and *P. tremula* × *tremuloides* sequences. Another reason can be, data normalization that differs fundamentally between microarray and qPCR, while the former requires global normalization, the later utilizes reference genes (e.g. ubiquitin in this study) to calibrate targeted gene expression (Morey *et al.*, 2006).

For individual genes (*Potri.007G081200*, *Potri.003G210600*, *Potri.007G095000*, *Potri.008G071100*), relative expression ratio differed among the biological replicates by a factor of 3-5. This indicates that the expression of these genes is influenced by other factors than ectomycorrhiza formation. Based on the comparable expression pattern found both in microarray and qRT-PCR, two genes were chosen for further detailed investigation: *Potri.007G095000*, encoding a potential glycosyltransferase that revealed robust expression difference in all biological replicates with an overall 104 ($p = 4.44 \times 10^{-03}$) fold higher expression level in mycorrhized roots, and *Potri.008G071100*, encoding a potential transcription factor with a 33 ($p = 4.15 \times 10^{-04}$) fold higher transcript levels in mycorrhized roots.

4.3 Glycosyltransferase

4.3.1 Subcellular localization of *Potri.007G095000*

Heterologous expression of *Potri.007G095000*-YFP fusion protein in tobacco leaves indicated a cytoplasm localization of *Potri.007G095000*, a uridine diphosphate (UDP) - glycosyltransferase (UGT). The nuclear localization of *Potri.007G095000*-YFP (80 kDa) observed in some cells might be due to diffusion through the nuclear pore complex. Large GFP fusion proteins of 90-110 kDa are known to diffuse through the nuclear pore (Wang & Brattain, 2007). Presence of cryptic nuclear localization signal can be another

explanation. A cytoplasmic localization of Potri.007G095000 is in agreement with several studies on *A. thaliana* uridine diphosphate (UDP) –glycosyltransferases that were found frequently to be cytoplasm localized (Hart *et al.*, 1989; Ross *et al.*, 2001). However, few studies also suggested the presence of glycosyltransferases within both nucleoplasm and cytoplasmic compartments (Richard *et al.*, 1975; Berthillier *et al.*, 1980; Berthillier & Got, 1982; Berger & Hesford, 1985; Fayet *et al.*, 1988; Galland *et al.*, 1988; Shaper *et al.*, 1988; Srisomsap *et al.*, 1988).

Based on phylogenetic analysis, the UGTs are grouped into several classes. The *Arabidopsis* homolog of Potri.007G095000, AtUGT85A7 belongs to class 1 of UTGS, which are known to be involved in phytohormone glycosylation (Paquette *et al.*, 2003; Wang, 2009). It has been previously reported that altered expression of *AtUGT85A7* resulted in changes in life cycle, leaf morphology, auxin response and root development including loss of gravity sensing (Woo *et al.*, 2007). Microorganisms or fungi often manipulate the phytohormonal homeostasis in host plants to facilitate colonization (Sukumar *et al.*, 2013; Vayssières *et al.*, 2015). Auxin is known to play a central role in mycorrhizal developmental pathways (Charvet-Candela *et al.*, 2002; Reddy *et al.*, 2006; Felten *et al.*, 2009; Heller *et al.*, 2012; Flores-Monterroso *et al.*, 2013; Tarkka *et al.*, 2013). There are also reports that auxin is a key regulator of root morphogenesis during Hartig net formation (Gay *et al.*, 1994; Tranvan *et al.*, 2000) and also demonstrated lower IAA levels in ECM roots than in non-colonized roots of poplar (*Populus spp.*) and spruce (*Picea spp.*) (Wallander *et al.*, 1992; Luo *et al.*, 2009). Other studies suggested involvement of auxin in symbiotic organ formation (Reddy *et al.*, 2006; Luo *et al.*, 2009; Heller *et al.*, 2012). Increase in IAA conjugation and degradation results in low auxin levels during ECM formation (Vayssières *et al.*, 2015). Glycosyltransferases decrease the levels of active auxin by catalysing phytohormone ester synthesis and Class I UGTs are known to be frequently involved in the first stage of ester conjugate biosynthesis (Ostrowski & Jakubowska, 2014). Ectomycorrhiza induced upregulation of *Potri.007G095000* thus indicates elevated phytohormone glycosylation.

4.3.2 Promoter analysis of *Potri.007G095000*

Ectomycorrhiza induced gene upregulation of *Potri.007G095000* was observed, both in DNA microarray analysis (Nehls, unpublished) and real time qPCR (this thesis). For

investigation of the mycorrhiza specific gene induction, promoter analysis was carried out. Amplification of around 3 kb long promoter fragment was successfully carried from *Populus tremula x tremuloides* genomic DNA. The 3 kb promoter fragment and 5' truncated fragments (2656 bp and 1151 bp) were cloned in front of a peroxisome targeted YFP into a binary vector and composite poplars were generated by transgenic *Agrobacterium rhizogenes* (K599) followed by ectomycorrhiza formation. No visible YFP was detected by epifluorescence microscopy and only cell wall autofluorescence and non-specific background was observed over a broad range of detection window (510-650 nm) under the cLSM both in mycorrhized and non-mycorrhized roots. Such cell wall auto fluorescence and non-specific background is a general problem of poplar roots. One explanation can be that *cis*- acting elements are missing in the isolated promoter fragments; therefore, full length promoter fragment (around 6.5 kb) has to be analysed. Whether the analysed roots were a result of successful biT-DNA transformation cannot be confirmed in this work, as no visual screening for successful biT-DNA transformation was available and selection of roots for sectioning and cLSM analysis was random. Therefore, future trials with a constitutively expressed second marker cassette is helpful.

4.3 Ethylene responsive transcription factor

4.3.1 AP2/ERF domain and heterologous expression of *Potri.008G071100*

Potri.008G071100 was predicted as ethylene responsive transcription factor (ERF) (Nehls, unpublished), a member of the large AP2/ERF superfamily. Sequence alignment revealed *Arabidopsis AT2G40220* (known as *ABI4*) to be a homolog of *Potri.008G071100*. Although, *ABI4* is unique gene in *Arabidopsis* two potential homologs are found in *P. trichocarpa* (Chen *et al.*, 2013). Both of these two poplar genes show the conserved AP2/ERF DNA binding domain (about 60-70 amino acids) (Nakano *et al.*, 2006). Other homologs with sequence similarities have also been found in maize (*Zea mays*) and rice (*Oryza sativa*) genomes (Niu *et al.*, 2002; Yu *et al.*, 2002).

Maize-ABI4 binds to the DNA core sequence CACCG with similarity to the coupling element 1 (CE1), involved in ABA induced gene expression (Busk & Pagès, 1998; Niu *et al.*, 2002). A recent report also showed that a smaller CCAC motif might function as the core ABI4-binding element in genes down-regulated by *ABI4* (Rook *et al.*, 2006;

Koussevitzky *et al.*, 2007). Sequence similarities to the two potential ABI4-binding sites were found in the upstream sequences of several ABA- and sugar-regulated genes in maize, rice, barley, and *Arabidopsis* as well (Niu *et al.*, 2002; Acevedo-Hernández *et al.*, 2005; Koussevitzky *et al.*, 2007). It has been demonstrated that the induction of various genes upon sugar treatment is dependent on ABI4 (Arenas-Huertero *et al.*, 2000; Rook *et al.*, 2001; Arroyo *et al.*, 2003; Koussevitzky *et al.*, 2007). Studies on the *Arabidopsis* homolog of *Potri.008G071100* demonstrated ABI4 as a positive regulator of its own expression (Bossi *et al.*, 2009). On the other hand, studies on the ortholog from maize indicated that ABI4 acts as a transcriptional repressor (Niu *et al.*, 2002). Further studies suggested that whether ABI4 acts as an activator or repressor depends on the sequence context next to its binding site (Acevedo-Hernández *et al.*, 2005; Rook *et al.*, 2006).

To identify the *cis* elements in *Potri.008G071100* promoter, purification of the TF followed by EMSA analysis was planned. As isolation of protein in large amount was difficult from poplar mycorrhiza due to material limitation, heterologous expression in *E. coli* was targeted.

4.3.1.1 Choice of Strep-tag II as the affinity tag

The eight-amino-acid peptide (WSHPQFEK), Strep-tag II modified from Strep-tag (WSHPQFGG) that specifically binds to core streptavidin, a proteolytically truncated version of the natural bacterial protein (Pähler *et al.*, 1987) was chosen as the affinity tag for this study for several reasons – **a.** Strep-tag II provides greater flexibility in the choice of attachment sites compared to Strep-tag (Schmidt & Skerra, 1994; Korndörfer & Skerra, 2002; Laitinen *et al.*, 2006). Usually, Strep-tag II fusion proteins are eluted from the Strep-Tactin affinity column under physiological buffer conditions allowing isolation of sensitive proteins in native state (Skerra & Schmidt, 2000), which is crucial for *Potri.008G071100*; **b.** similar to other short-affinity tags (Terpe, 2003; Waugh, 2005), such as the His6tag (Skerra *et al.*, 1991; De Marco, 2006), the calmodulin-binding peptide (Stofko-Hahn *et al.*, 1992) or the Flag tag (Hopp *et al.*, 1988), Strep-tag II can easily be fused with a recombinant polypeptide during cloning and versatile expression vectors are available especially for *Escherichia coli* (Skerra, 1994; Han *et al.*, 2006) and for different hosts such as eukaryotic cells, including yeast (Boettner *et al.*, 2002; Prinz *et al.*, 2004; Lichty *et al.*, 2005), insect (Lichty *et al.*, 2005), mammalian (Cotten *et al.*, 2003; Junttila *et al.*, 2005; Lichty *et al.*, 2005) and plant cells (Witte *et al.*, 2004); **c.**

Another advantage of Strep-Tag II is it does not hamper protein folding or secretion and usually does not interfere with protein function; **d.** *Strep-tag* II is greatly resistant to cellular proteases and can be used in the presence of mild detergents and it is mostly biochemically inert, which gives an advantage over other short-tags (Korndörfer & Skerra, 2002; Lichty *et al.*, 2005), whose function is metal ion dependent (Korndörfer & Skerra, 2002).

4.3.1.2 Protein expression system, conditions and strategy

T7 based pET expression system (Studier *et al.*, 1990; Dubendorff & Studier, 1991) requires a host strain lysogenized by a DE3 phage fragment, encoding the T7 RNA polymerase under the control of IPTG inducible *lacUV5* promoter and is by far the most used recombinant protein preparation (Sørensen & Mortensen, 2005). *Escherichia coli*, the most extensively used prokaryotic heterologous expression system (Frommer & Ninnemann, 1995) was chosen due to its simplicity, rapid growth rate, low cost, simple process scale up and compatibility with almost all commercially available inducible cloning vectors (Yesilirmak & Sayers, 2009). Following two strains were tested - *E. coli* BL21 (Novagen, USA), deficient in Lon and OmpT proteases thus increases protein stability (Shaw & Ingraham, 1967) and strain Rosetta-gami with *trxB* and *gor* gene mutations helps in enhanced disulphide bond formation in their cytoplasm and an over expressing rare tRNA expression vector set. However, one major disadvantage of *E. coli* is that the heterologous recombinant protein is often over-expressed in cytoplasm as insoluble aggregates of folding intermediates, called inclusion bodies due to lack of eukaryotic post-transcriptional machinery (Ghosh *et al.*, 2004; Sahdev *et al.*, 2008; Yesilirmak & Sayers, 2009). It is known that the requirement of one or more rare tRNAs during over-expression of target gene in *E. coli* resulted in lower translation rate due to differences in codon usage (Goldman *et al.*, 1995; Kane, 1995; Kurland & Gallant, 1996). However, online analysis using ExPASy of SIB Swiss Institute of Bioinformatics revealed no such rare codon is present in the amino acid sequence of Potri.008G071100.

Expressed protein is known to account up to 30% of the total cell protein (Sahdev *et al.*, 2008), which is a tremendous metabolic load on the *E.coli* expression system and could thus become toxic to the host (Sahdev *et al.*, 2008). However, protein expression in *E. coli* growing at low temperature can overcome this problem and improve the solubility of many difficult proteins. At low temperature protein folding is more

accurate, as hydrophobic interactions of misfolded proteins leading to insoluble inclusion bodies is suppressed (Chesshyre & Hipkiss, 1989; Niiranen *et al.*, 2007; Vera *et al.*, 2007). Moreover, *E. coli* growth at lower temperature has been associated with increased expression of bacterial chaperones. Although growth at temperature in the range of 15-23 °C is helpful in reducing misfolding (Spiess *et al.*, 1999; Hunke & Betton, 2003), lower temperature strongly reduce the efficiency of traditional promoters used routinely in expression vectors leading to low yields (Vasina & Baneyx, 1997). Therefore, reduction in IPTG concentration for induction (Winograd *et al.*, 1993), protein over expression in a late log phase culture (Galloway *et al.*, 2003) or addition of glycerol (1-2 %) to the growth medium (Sahdev *et al.*, 2008) were tried to improve protein solubility and reduction of inclusion body formation. In this work all those attempts were however unsuccessful.

To recover the soluble protein from the inclusion bodies, the inclusion bodies can be solubilized in the presence of strong denaturants such as urea or guanidinium hydrochloride. Removal of the denaturants favour refolding under optimal conditions (Ghosh *et al.*, 2004). Although considerable development has been done for efficient protein refolding (Lilie *et al.*, 1998), specific folding conditions differ greatly from protein to protein. Studies on transcription factor *Arabidopsis thaliana* RGL3 have shown that under optimal condition 87 % of denatured protein of inclusion bodies was successfully renatured, when refolding was initiated from inclusion bodies solubilized in 8 M urea (Al-Samarrai *et al.*, 2007). Therefore, gradual change of denaturing (in 6 M urea) to native buffer conditions utilizing dialysis (Maeda *et al.*, 1995; Varnerin *et al.*, 1998) was also attempted without any success. It has been demonstrated that dialysis and diafiltration techniques can lead to aggregation during refolding compared to direct dilution (Sahdev *et al.*, 2008). Alternative strategies to remove denaturing agents are size exclusion chromatography (Werner *et al.*, 1994; Batas & Chaudhuri, 1996; Batas & Chaudhuri, 1999; Müller & Rinas, 1999), solid support assisted (Zouhar *et al.*, 1999; Bernd *et al.*, 2001; Cho *et al.*, 2001) and hydrophobic interaction mediated (Geng & Chang, 1992; Bai *et al.*, 2003; Gong *et al.*, 2004) protein refolding. However, these attempts were not followed in this work due to time limitation.

For future trials in expression of Potri.008G071100 there is room for additional improvements such as co-expression of molecular chaperons, IPTG induction in early log phase and use of longer fusion tags. Co-expression of molecular chaperons have

been demonstrated to assist *de novo* protein folding, facilitating expressed polypeptide's proper conformation attainment and/or cellular localization without becoming a part of the final structure (Nishihara *et al.*, 2000; Deuerling *et al.*, 2003). This co-expression strategy has been followed in several studies for preventing inclusion bodies which thereby increased recombinant protein solubility (Mogk *et al.*, 2002). Similarly, concomitant overexpression of thioredoxin (TrxA) is demonstrated to improve the solubility of expressed proteins (Yasukawa *et al.*, 1995). In recent literature, high yield of soluble proteins was obtained by combining early-log phase cultures and low temperatures for protein induction, where IPTG was added at OD600 = 0.1, which resulted in a 3 fold higher soluble protein yield than that obtained in the mid-log phase (OD600 = 0.6) (San-Miguel *et al.*, 2013). Another approach has gained considerable success in recent years is the use of gene fusion (LaVallie & McCoy, 1995). These fusion proteins in general include a partner or 'tag' that may or may not be linked to the protein of interest by a recognition site-specific protease (Sahdev *et al.*, 2008), utilized for the purpose of specific affinity purification and provides additional advantages by protecting the partner protein from intracellular proteolysis (Martinez *et al.*, 1995; Jacquet *et al.*, 1999) and enhances solubility (Davis *et al.*, 1999; Kapust & Waugh, 1999; Sørensen *et al.*, 2003). Fusion partners such as glutathione-S-transferase (GST), N-utilization substance A (NusA) and maltose binding protein (MBP) are of particular interest with regard to optimization of recombinant transcription factor expression. Bossi and co-workers used glutathione-S-transferase (GST) as fusion partner for ABI4 expression, purification and further *in vitro* assays (Bossi *et al.*, 2009). As ABI4 is the *Arabidopsis* ortholog of Potri.008G071100, a similar strategy might be successful. The authors, however also reported specific but weak binding of ABI4 to the regulatory sequences of *ABI4* and *ABI5* which was not observed by genetic studies. They discussed that it is a potential artefact due to the presence of the fusion partner which might affect ABI4 binding. In another study Hao and colleagues analysed three ERF domain-containing TF-maltose binding protein fusions originating from tobacco and *Arabidopsis* for their sequence specific binding properties (Hao *et al.*, 1998).

In addition to bacteria, eukaryotic expression system such as yeast (*Saccharomyces cerevisiae*, *Pichia Pastoris*) (Bertl *et al.*, 1995; Muchhal *et al.*, 1996; Fu & Luan, 1998; Dreyer *et al.*, 1999; Cereghino & Cregg, 2000; Macauley-Patrick *et al.*, 2005), insect cells (*Spodoptera frugiperda*, *Trichoplusia ni*) (Mizutani & Ohta, 1998; Fukuchi-Mizutani *et al.*,

1999; Hayashi *et al.*, 1999; Caldelari *et al.*, 2001; Hayashi *et al.*, 2002; Harashima *et al.*, 2007) and *Xenopus laevis* Oocytes (Boorer *et al.*, 1992; Miller & Zhou, 2000; Liu *et al.*, 2001; Mäser *et al.*, 2002; Sigel & Minier, 2005; Orsel *et al.*, 2006) have been successfully used to express various plant proteins. Proteins can be post-translationally modified in *Saccharomyces cerevisiae* and protein aggregation might be avoided (Yesilirmak & Sayers, 2009).

4.3.2 Sub-cellular localization of Potri.008G071100

Potri.008G071100-sYFP was clearly observed in nucleus surrounded by the DsRed illuminated nuclear membrane. DsRed was not directly excited at its maximal excitation wavelength at 558 nm. Strong sYFP expression fluorescence leads to sufficient emitted light (527 nm) to allow excitation of the red fluorescence protein. Even when epidermal cells were analysed, chlorophyll autofluorescence from chloroplasts of underlying mesophyll cells was frequently visualized due to the excitation light range used for YFP detection. Therefore, chlorophyll autofluorescence was separately monitored in order to interpret the correct location of the protein of interest as the autofluorescence was detected over a broad emission range including the sYFP channel (Haseloff *et al.*, 1997; Okada *et al.*, 2000; Corpas *et al.*, 2004).

A nuclear localization of Potri.008G071100 was expected from the Uniprot PROSITE (PRU00366) and SAAS annotation (SAAS00550310). Online prediction by ExPASy also indicated a nuclear target localization. The nuclear localization of other AP2/EREBP type transcription factors along with *Oryza sativa* (rice-ABI4) and *Arabidopsis* (ABI4) were also confirmed in literature (Park *et al.*, 2001; Chen *et al.*, 2003; Matsukura *et al.*, 2010; Lai *et al.*, 2014).

4.3.3 Promoter analysis of Potri.008G071100

To understand the mycorrhiza specific upregulation of *Potri.008G071100* upon ectomycorrhiza formation, a promoter analysis was started. Previous work has shown a 3 kb upstream sequence of the *Arabidopsis* homolog *ABI4* includes all necessary *cis*-acting motifs (Arroyo *et al.*, 2003). Therefore, a 3.4 kb fragment was amplified from *Populus tremula x tremuloides* genomic DNA. Full and 5' truncated fragments (1603 bp and 401 bp) were then cloned into a binary vector pPLV11-SharinII such a way that the transcription of a peroxisome targeted YFP gene was driven by the promoter

fragment of interest. The longest promoter-reporter constructs were used to produce composite poplars by *Agrobacterium rhizogenes* (K599) mediated strategy and rooted poplars were used for ectomycorrhiza synthesis with *Amanita muscaria*.

Surprisingly, the 3.4 kb long promoter fragment led to expression of the YFP reporter both in ectomycorrhized and non-mycorrhized fine roots, where no obvious differences in YFP signal were observed. This result, however, does not reflect the ectomycorrhiza specific upregulation of *Potri.008G071100* as in the DNA microarray (Nehls unpublished) and qPCR analysis. A reason for this discrepancy can be that the size of the isolated promoter fragment was too small and *cis*-acting elements responsible for mycorrhiza specific gene regulation are missing. The distance to the next upstream gene is approximately 8 kb which allows more room for the elongated promoter isolation.

5. Outlook

As not all roots formed by composite poplars revealed binary vector-based marker gene expression, the question whether newly formed roots harbour the T-DNA of a binary vector or only the T-DNA of the agrobacterial vector remained for the investigated mycorrhiza induced genes. A useful strategy for easy screening of roots containing bi-TDNA would be the presence of an additional fluorescent marker cassette driven by a constitutive promoter within the respective T-DNA region. The expression of the respective fluorescent protein would indicate transgenic root, harbouring the T-DNA of interest.

Many attempts were taken to optimize solubility of heterologously expressed Potri.008G071100 in *E. coli*. Problems of short tags leading to extensive inclusion body formation were also described in literature, a way worth trying would be fusion to a highly soluble protein like maltose binding protein. Whether such tag has to be removed proteolytically prior to further target protein analysis, has to be proven experimentally. Use of eukaryotic expression system is another possibility. If successful, the purified natively folded Potri.008G071100 can be examined for possible binding sites in the isolated promoter region and interaction with other possible transcription factors using an electrophoretic mobility shift assay (EMSA).

As mycorrhization was not achieved efficiently in every batch, further optimization is required. Therefore, additional ectomycorrhizal fungal species should be tested for their ability to form ectomycorrhiza in the sterile Petri dish system.

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9. Abbreviations

μ	Micro
35S-Promotor	Promoter of <i>Cauliflower mosaic virus</i>
A	Adenine
aa	Amino acid
ABA	Abscisic acid
ABI	Abscisic acid Insensitive
AM	Arbuscular mycorrhiza
AMP	Adenosine monophosphate
AP2	APETALA2
APG	Angiosperm Phylogeny Group
ATP	Adenosine triphosphate
biT-DNA	Binary T-DNA
bp	Base pair
BLAST	Basic Local Alignment Search Tool
CaMV	Cauliflower mosaic virus
cDNA	Copy DNA
Co. KG	Compagnie Kommanditgesellschaft (German)
Cp	Crossing point
CTAB	Cetyltrimethylammonium bromide
d	Day
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddH ₂ O	Double distilled water
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide
DPE	Downstream promoter element
DREB	Dehydration Responsive Element-Binding
<i>Dr. rer. nat.</i>	Doctor rerum naturalium
dsDNA	Double stranded DNA
dTTP	Deoxythymidine triphosphate
E	Efficiency
ECM	Ectomycorrhiza
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
EREBP	Ethylene-responsive element binding protein
ERF	Ethylene Responsive Factors
et al.	<i>Et alii</i> (and other)
EtBr	Ethidium bromide
e.g.	<i>Example gratia</i> (for example)
FDA	Food and Drug Administration

for	Forward
FP	Fluorescence protein
G	Guanine
g	Acceleration of gravity (approximately 9.81 m/s ²)
gDNA	Genomic DNA
GFP	Green fluorescent protein
GmbH	Gesellschaft mit begrenzter Haftung (German)
GT	Glycosyl transferase
GTP	Guanosine triphosphate
h	Hours
INR	Transcription initiation region
INRA	Institut national de la recherche agronomique (French)
IPTG	Isopropyl β -D-1-thiogalactopyranoside
K	Kilo
Kan	Kanamycine
Kb	Kilo base
KGaA	Kommanditgesellschaft auf Aktien (German)
l	Liter
LB	Lysogeny broth
m	Milli
M	Molar
max.	Maximum
MBP	Maltose binding protein
MCS	Multiple cloning site
MES	2-(N-morpholino)ethanesulfonic acid
Mg	Magnesium
min	Minute
MMN	Modified Melin Norkrans Medium
Mn	Manganise
mRNA	Messenger RNA
MS	Murashige-Skoog Medium
n	Nano
NEB	New England Biolabs
No.	Number
NLS	Nuclear Localization Signal
<i>nptII</i>	Kanamycine resistance gene
OD	Optical density
ORF	Open reading frame
Ori	Origin of replication
p	Plasmid
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	<i>potentia hydrogenii</i> - decimal logarithm of the reciprocal of the hydrogen ion activity
PMT	Photo multiplier
Prof.	Professor
Pt	<i>Populus trichocarpa</i>
PTS1	Peroxisomal targeting signal 1
Ptt	<i>Populus tremula x tremuloides</i>

qRT-PCR	Quantitative real-time PCR
RAV	Related to ABA Insensitive3 (ABI3)/ Viviparous1
rev	Reverse
RFP	Red fluorescent protein
Ri Plasmid	Root inducing plasmid
rpm	Revolutions per minute
RNA	Ribonucleic acid
RNAi	RNA Interference
RNase	Ribonuclease
<i>rol</i>	Root oncogenic locus
RT	Reverse transcriptase
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
ssDNA	single stranded DNA
ssT-strand	single stranded T-strand
Std.	Standard deviation
sYFP	Super yellow fluorescent protein
sYFPPTS1	sYFP with a peroxisomal targeting signal 1
T	Thymine
T4	Bacteriophage T4
T7	Bacteriophage T7
Ta	Annealing temperature
t-DNA	Transfer DNA
TAE	Tris-Acetate-EDTA (Buffer)
Taq	Thermus aquaticus
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
TF	Transcription factor
Tm	Melting temperature
TM	Registered trade mark
Ti-Plasmid	Tumour inducing plasmid
TiT-DNA	T-DNA of Ti-Plasmid
T-complex	Complex of T-DNA bound with proteins
TL-DNA	Left T-DNA of Agropin Ri-Plasmid
TR-DNA	Right T-DNA of Agropin Ri-Plasmid
Tris	2-Amino-2-hydroxymethyl-propane-1,3diol
U	Unit
UDP	Uridine diphosphate
UPE	Upstream promoter element
UTR	Untranslated region
UV	Ultra violett
V	volt
v/v	volume per volume
<i>vir</i>	Virulence gene of <i>Agrobacterium</i>
vol.	Volume
w/v	Weight per volume
YFP	Yellow fluorescent protein
YFPPTS1	YFP with a peroxisomal targeting signal 1

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11. Erklärung

Name: Arpita Das

Bremen, 13.04.18

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Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

Ectomycorrhiza Development: Investigation of Selected Ectomycorrhiza Induced Poplar Genes

selbständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

(Arpita Das)

12. Curriculum Vitae

Education

- 2013 – 2018 Doctorate at the University of Bremen,
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- 2010 - 2012 Master of Science in Applied Microbiology,
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Scholarships and Awards

- 2017 STIBET Promotionsabschlussstipendium, DAAD, Germany
- 2015 Travel grant, iMMM2015, University of Cambridge, UK
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12.1 List of publications

Journal

NEB D., **DAS A.**, HINTELMANN A., NEHLS U. **2017**. Composite poplar plants for investigation of ectomycorrhiza formation and function. *Plant Cell Reports*. 36(12): 1959–1970.

DAS A., VENKATARAMANA M., CHANDRANAYAKA S., MURALI H.S. and H. V. BATRA. **2012**. Molecular characterization of *Fusarium oxysporum f. sp. cubense* isolates from banana. *Pest Management in Horticultural Ecosystems*. 18 (2): 171-178.

Book Chapter

NEHLS U., **DAS A.**, NEB D. **2016**. Carbohydrate metabolism in ectomycorrhizal symbiosis. *Molecular Mycorrhizal Symbiosis*. Martin F. ed.: Wiley & Sons. 161-177.

Oral presentations

DAS A., HINTELMANN A., NEHLS U. "Ectomycorrhiza dependent gene regulation in poplar "- 2nd International Molecular Mycorrhiza Meeting (IMMM). University of Cambridge, UK. **2015**

DAS A., NEHLS U. "Investigation of transcript levels and subcellular localisation of ectomycorrhiza regulated genes"- Doctoral student's meeting. University of Bremen. **2015**

DAS A., VENKATARAMANA M. and MURALI H.S. "Molecular Characterization of South Indian Isolates of *Fusarium oxysprum f. sp. cubense* of Banana" - IV National Symposium on Plant Protection in Horticulture Crops: Emerging Challenges and Sustainable Pest Management. IIHR, Bangalore. **2012**

DAS A., VASUDEVAN L., JACOB T. AND JAYARAMAN G. "Biodegradation of Tannery Effluent collected from Ranipet region using Halotolerant Bacterial strains" - 3rd International Science, Engineering and Technology Conference. Vellore Institute of Technology, Vellore. **2011**

DAS A., VASUDEVAN L., JACOB T. AND JAYARAMAN G. "Study of Application of Halotolerant Bacillus, VITP9 in the Bioremediation of Industrial Effluents" - 2nd International science, Engineering and technology Conference. Vellore Institute of Technology, Vellore. **2011**

Poster Presentations

DAS A., VENKATARAMANA M. and MURALI H.S. "Morphological and Molecular Characterization of Fumonisin Producing *Fusarium proliferatum* Strains Isolated from Paddy Samples Originated from Southern India" - National seminar on Challenges, opportunities and Emerging Trends in Microbial Technology. Kakatiya University, Warangal. **2012**

DAS A., VENKATARAMANA M. and MURALI H.S. "Molecular Detection of Fumonisin Producing *Fusarium verticilloides* Recovered from Freshly Harvested Maize Kernels Collected from Southern India" - VITbiosummit. Vellore Institute of Technology, Vellore. **2012**

13. Appendix

13.1 Technical equipment

Table 9: Laboratory instruments

Centrifuges	Eppendorf Centrifuge 5804R with rotor A-2-DWP and F-34-6-38 (Eppendorf AG, Hamburg, Germany)
	Eppendorf Centrifuge 5417 R with rotor F 45-30-11 (Eppendorf AG, Hamburg, Germany)
	Heraeus Biofuge Fresco with rotor 3325 (Fisher Scientific GmbH, Schwerte, Germany)
PCR Thermocycler	Biometra PC Personal Cycler (Biometra GmbH, Göttingen, Germany)
	Biometra TGradient Thermoblock (Biometra GmbH, Göttingen, Germany)
	Bio-rad MyiQ™ Real-Time PCR machine (Bio-Rad Laboratories GmbH, Munich, Germany)
	peqlab primus 25 advanced (PEQLAB Biotechnologie GmbH, Erlangen, Germany)
	Roche LightCycler® 480II Real-Time PCR System (Roche Deutschland Holding GmbH, Grenzach-Wyhlen, Germany)
Incubators	Certomat®H, type 886342/3 with shaker 886302/4 (B. Braun Biotech International GmbH, Melsungen, Germany)
Thermo shaker	Eppendorf Thermomixer 5436 (Eppendorf AG, Hamburg, Germany)
Sterile bench	Scanlaf Clean Bench Fortuna 1500 (Labogene APS, Lyngø, Denmark)
	Clean Air CA/RS4 (Clean Air Supplies Deutschland GmbH, Haan, Germany)
Gel documentation	UV desk 312 nm (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in combination with Camera system of Doc Print II (PEQLAB Biotechnologie GmbH, Erlangen, Germany)
Spectrophotometer	Nano Drop™ DN1000 (PEQLAB Biotechnologie GmbH, Erlangen, Germany)
Cell density meter	Biochrom WPA C08000 (Biochrom Ltd., Cambridge, United Kingdom)
Vibratom	VT1000S (Leica Microsystems, Wetzlar, Germany)

Freeze-drying	Vacuum pump RZ5 with DCP3000 vacuum gauge (Vacuubrand GmbH + Co., Wertheim, Germany) connected to KF-2-60 (Bachofer GmbH, Reutlingen, Germany)
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Table 10 Microscopes

Microscope	Mz10F (Leica Microsystems, Wetzlar, Germany) combined with an external light source LEj LQ-HXP 120 ((Leistungselektronik JENA GmbH, Jena, Germany) and a Leica DFC425C (Leica Microsystems) camera
Stereo-microscope	Leica DMRB (Leica Microsystems, Wetzlar, Germany) combined with light source LEj LQ-HXP 120 ((Leistungselektronik JENA GmbH, Jena, Germany) and a Leica DFC425C (Leica Microsystems) camera
Confocal microscope	LSM 780 / ELYRA PS.1 (Carl Zeiss, Göttingen, Germany)

13.2 Kits

Table 11 Kits used in this thesis

Name	Purpose	Manufacturer
ABsolute™ QPCR SYBR® Green reaction mixture	Real time qualitative PCR	Fermentas GmbH, St.Leon-Rot, Germany
CloneJET™ PCR Cloning Kit	Blunt end DNA fragment cloning	
NucleoSpin® gel and PCR clean up	Purification of PCR products and DNA fragments from gel electrophoresis	MACHEREY-NAGEL GmbH & Co. KG , Düren, Germany
NucleoSpin® Plasmid	Isolation of total RNA from roots	

13.3 Enzymes

Table 12 Enzymes used for several assays

Enzyme	Function	Manufacturer
Ribonuclease A (RNase A)	RNA degradation	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
FastAP™ (thermosensitive alkaline phosphatase)	Dephosphorylation of linearized vector DNA for preventing re-circularisation	Thermo Fisher Scientific GmbH, St.

Reverse Transcriptase Revert Aid™ Premium RT	cDNA synthesis	Leon Rot, Germany
RNase Inhibitor RiboLock™	Inhibition of RNase (A, B, and C)	
Phusion® DNA polymerase	DNA polymerisation in PCR	
Several restriction enzymes	Directed hydrolysis of DNA	
T4 Polynucleotide kinase	Phosphorylation of DNA containing a 5' hydroxyl terminus	New England Biolabs (NEB) GmbH, Frankfurt, Deutschland
T4 DNA ligase	Ligation of DNA fragments	
T4 DNA polymerase	Catalysis of DNA synthesis in the 5'→3' direction	
Several restriction enzymes	Directed hydrolysis of DNA	

13.4 Chemical list

Table 13 List of chemicals used for different assays

Chemical Name	Manufacturer
2-mercaptoethanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
3, 5-dimethoxy-4-hydroxyacetophenone (Acetosyringone)	Sigma-Aldrich Corporation, St. Louis, USA
Acetic acid	VWR International GmbH, Darmstadt, Germany
Acryl/bisacrylamide	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Agar agar	DUCHEFA Biochemie B.V., Haarlem, Netherlands
	Merck KGaA, Darmstadt, Germany
Agarose	Biozym Scientific Hessisch Oldendorf, Germany
Ammonium persulfate (APS)	Serva Electrophoresis GmbH, Heidelber, Germany
Ampicillin sodium salt	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Betaine	Sigma-Aldrich Corporation, St. Louis, USA
Bromophenol blue	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
CaCl ₂ x 2H ₂ O	Merck KGaA , Darmstadt, Germany
Carbenicillin Disodium	DUCHEFA Biochemie B.V., Haarlem, Netherlands
Cefotaxim	DUCHEFA Biochemie B.V., Haarlem, Netherlands
Chloroform	Sigma-Aldrich Corporation, St. Louis, USA
CuSO ₄ x 5H ₂ O	Merck KGaA, Darmstadt, Germany

Cetyltrimethylammonium bromide (CTAB)	Sigma-Aldrich Corporation, St. Louis, USA
Diethyl pyrocarbonate (DEPC)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
D-glucose	DUCHEFA Biochemie B.V., Haarlem, Netherlands
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
dNTPS (dATP, dCTP, dGTP, dTTP)	Thermo Fisher Scientific GmbH, St. Leon-Rot, Germany
Ethylene-diamine-tetra-acetic acid (EDTA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol	Sigma-Aldrich Corporation, St. Louis, USA
Ethidium bromide	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
FeCl ₃ x 6 H ₂ O	Sigma-Aldrich Corporation, St. Louis, USA
Glycerol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
HCl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Isopropanol	VWR International GmbH, Darmstadt, Germany
K ₂ HPO ₄ x 3H ₂ O	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Kanamycin sulphate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
KCl	Sigma-Aldrich Corporation, St. Louis, USA
KH ₂ PO ₄	Sigma-Aldrich Corporation, St. Louis, USA
KOH	Sigma-Aldrich Corporation, St. Louis, USA
MES (2-N-morpholino ethanesulfonic acid)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
MgCl ₂ x 6H ₂ O	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
MgSO ₄ x 7H ₂ O	Merck KGaA, Darmstadt, Germany
MnCl ₂ x 4 H ₂ O	Honeywell Riedel-de Haën™ Seelze, Germany
MOPS	Sigma-Aldrich Corporation, St. Louis, USA
LiCl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Melin Norkrans medium	DUCHEFA Biochemie B.V., Haarlem, Netherlands
Murashige and Skoog medium	DUCHEFA Biochemie B.V., Haarlem, Netherlands
NaCl	Sigma-Aldrich Corporation, St. Louis, USA
NaOH	Merck KGaA, Darmstadt, Germany
(NH ₄) ₂ HPO ₄	Sigma-Aldrich Corporation, St. Louis, USA
(NH ₄) ₆ Mo ₇ O ₂₄ x 4H ₂ O	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Polyethylenglycol (PEG)	Sigma-Aldrich Corporation, St. Louis, USA
Peptone	DUCHEFA Biochemie B.V., Haarlem, Netherlands
Phenol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Potassium acetate	Sigma-Aldrich Corporation, St. Louis, USA
Primer	Eurofins MWG GmbH, Ebersberg, Germany
Pyridoxine HCL	DUCHEFA Biochemie B.V., Haarlem, Netherlands
RbCl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Rifampicin	DUCHEFA Biochemie B.V., Haarlem, Netherlands
RNase	Sigma-Aldrich Corporation, St. Louis, USA
SDS (Sodium dodecyl sulphate)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sephadex G-50	Sigma-Aldrich Corporation, St. Louis, USA
Sodium acetate	Merck KGaA, Darmstadt, Germany
Streptomycin sulphate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sucrose	Acros Organics N.V, Geel, Belgium
Tetramethylethylenediamine (TEMED)	Serva Electrophoresis GmbH, Heidelber, Germany
Tetracycline	Sigma-Aldrich Corporation, St. Louis, USA
Thiamine HCL	DUCHEFA Biochemie B.V., Haarlem, Netherlands
Tris (hydroxymethyl) aminomethane	Sigma-Aldrich Corporation, St. Louis, USA
Triton X 100	Serva Electrophoresis GmbH, Heidelber, Germany
Yeast extract	DUCHEFA Biochemie B.V., Haarlem, Netherlands

13.5 Primers

Table 14 List of forward and reverse primers designed for selected poplar genes.

Shown are the *P. trichocarpa* gene ids, the respective designed forward and reverse primers for real time qPCR, together with their expected size of the PCR product.

Gene id of <i>P. trichocarpa</i> CDS	Forward primer	Reverse primer	Expected length (bp)
<i>Potri.007G095000</i>	GCTTTGCAATGGAAGAAGAAAGC	GGATGGATTCCACAGAAATGTGAG	158
<i>Potri.011G132100</i>	TCTGATTTTCTGTGTTGTACAGTG	AGAGCAATCTTTCTTGATTCAA	150
<i>Potri.009G028600</i> ³	GGGAAAACCCCTCRAAACACA*	GCTTTTGCATTAATAATTGTAAATTGGT	105
<i>Potri.009G028600</i> ³	TGGGAAAACCCCTCAAACACAT	GCTTTTGCATTAATAATTGTAAATTGGT	106
<i>Potri.001G085900</i>	AGCTGAATTCAAGGGTGAAGY*	TGTTTCCTTTTCCACTGATACTGAT	154
<i>Potri.004G045600</i>	TGGAGTGAGGTGGCAGTTAG	TCTCATAGCTGTCGAGTCAAGA	160
<i>Potri.014G106600</i> ⁴	CAGGAGGGAAAGAACGGACC	TCCTTCAATTTCCACAAACGGA	157
<i>Potri.003G210600</i>	GCCATGCCTGGTGTAGAGT	AGATAAGTTGGGCAGCTGCT	135

<i>Potri.014G106600*</i>	GGGAAAGAACGGACCGAAGA	TCCTTCAATTTCCACAAACGGA	152
<i>Potri.002G180600</i>	GGAGGGAAAGAACGGACTGA	TGCATCCTCCAATTTCTGCA	159
<i>Potri.009G127900</i>	TCTTTGGGATGGTCATTGGGT	ACCATCCTCTTCCATATCCTTGA	156
<i>Potri.004G068800</i>	TAGGGAGAGATCAGWGGGGC*	TACAGCTCAAACCACCCAC	122
<i>Potri.011G046600</i>	ACATGCTGTGCCACTCTTCA	TGAGGAACAAGATGGCTGGT	124
<i>Potri.006G009300</i>	GGAAGCAACGGACCAGTTCA	GGCTGCAAGATTTTTCTCTGAGG	142
<i>Potri.012G117100</i>	TCGGGRAAAAGCTCAGCTCT*	GCAGCATGGCARACATAAGC*	154
<i>Potri.002G188900</i>	TGTGGCAATTGTTCTTCTTGCT	TTAGTGGCTGGCAACCATCT	108
<i>Potri.007G012400</i>	TTCTCCACCTTCAAGCAGCT	GGCCCTCGAGACTGGTTTTTA	160
<i>Potri.007G081200</i>	GAGCACCCAGTGGATCTCAG	AAGTCCGCAAACCCTACAGG	127
<i>Potri.016G104300</i>	TACCATCCCCAAACGTTGCA	TGCATGGTCGCTAGCTATGG	122
<i>Potri.010G244400</i>	GGCCTGGCTGATGCTAAAGA	TTCACGATCGGTTCTTGCT	137
<i>Potri.004G019300</i>	ACGGACTTTACCACGTTGATT	GGGAGCAGTCCTCATCACAG	131
<i>Potri.008G071100</i>	CGGGTCATCTTCAGGATCCG	TCATGAATATCCCCACCAGCTC	144
<i>Potri.010G196900</i>	TGCACTCACAATTGAAGGAAC	AGTACTAGTATTATAGCTGCGCT	155

^{3,4} *P. trichocarpa* CDS for which different homologs were found, two sets of primer pairs were designed on each homolog.

* Due to single base pair mismatches, especially in the 3' end of the primers between different poplar homologs for respective *P. trichocarpa* CDS, mixed bases were used for the primer synthesis. R, Y and W represents mixed bases of A and G, C and T, A and T respectively.

Table 15 List of primers used for heterologous expression of Potri.008G071100.

Shown are the primers used for stop codon depleted *Potri.008G071100* CDS amplification for cloning into expression vector pET28a and sequencing for further verification.

Primer number	Primer name	Sequence 5'-3'	Description
1	Pt8G0711CDSf	GCAGCGACTCTCTGTCTCTC	Amplification of <i>Potri.008G071100</i> CDS (8G-CDS- 1275bp)
2	Pt8G0711CDSr	CGTACCGGCCTTTGATGGAT	
3	8G0711-CDSfATG	CCATGGCAATGCAGTTATGGTCGAGAC	Amplification of stop codon depleted <i>Potri.008G071100</i> CDS. Contains <i>NcoI</i> and <i>EcoRI</i> restriction sites as overhang (8GCDS-pET28a)
4	Pt8G0711CDSrL	GAATTCAAATCAAACAAGAAAGGATC	
5	ADPet28a_8G07f or	TTCTGTTACGGCAGCTTCGT	<i>Potri.008G071100</i> CDS specific forward sequencing primer

Table 16 List of primers used for analysing subcellular localization of Potri.008G071100.

Shown are the primers used for amplification of stop codon depleted *Potri.008G071100* CDS for blunt end cloning into pCXUN-sYFP and pPLV11-Sharinal.

Primer number	Primer name	Sequence 5'-3'	Description
6	8GCDS_BluntEndF	ATGCAGTTATGGTCGAGAC	Amplification of stop codon depleted <i>Potri.008G071100</i> CDS (8GCDS-SL - 1146bp), for conventional blunt end cloning
7	8GCDS_BluntEndR	ACAAAATCAAACAAGAAAGGATC	

Table 17 Primers used for amplification and cloning of 3.5 kb *Potri.008G071100* upstream promoter fragments into pPLV11-SharinalII and pPLV04.

Primer number	Primer Name	Sequence 5'-3'	Description	
8	Ptt8G071100Pr3f	TCGTCGATCCCAACAAGC	Amplification of 3498bp upstream promoter fragment	
9	Ptt8G071100Pr3r	GCCCCAGCTTCTTTGCCTA		Amplification of 3635bp upstream promoter fragment
10	Ptt8G071100Pr4f	CGCCTAGGTGCAAGCATCT		
11	8Gpro3KbPLV11sRe	CCCAAGCTTGGGCTATTTATATTTTTATATTCGGAG	Cloning into pPLV11-SharinalII with <i>Hind</i> III (reverse) recognition site overhang (8G11Pr3.4Kb)	
12	8Gpro3KbPLV11sFo	CGGGGTACCCCGACCCTGACTTCAATTTCC	Cloning into pPLV11-SharinalII and pPLV04 with <i>Kpn</i> I (forward) recognition site overhang (8G11Pr3.4Kb and 8G04Pr3.4Kb)	

Table 18 List of primers used for analysing subcellular localization of Potri.007G095000.

Shown are the primers used for amplification of stop codon depleted *Potri.007G095000* CDS for LIC (ligase independent cloning) and blunt end cloning into pCXUN-sYFP and pPLV11-Sharinal respectively.

Primer number	Primer name	Sequence 5'-3'	Description
13	Pt7G095000v1F	CAAGCATGGGTTCTTTCAACAAC	CDS amplification (7GCDS - 1465bp)
14	Pt7G095000v1R	GAGTTAATTCCTTAAGATCAGTG	
15	7G095000LIC-CDSf	TAGTTGGAATGGGTTCGAAGATGGGTTCTTTCAACAACAAAC	with LIC overhang for stop codon depleted CDS amplification (7GCDSLIC - 1483bp)
16	Pt7G095000LICr2	TTATGGAGTTGGGTTCGAACGTGAATATTTCTTTCAAATGCAGC	
17	7GCDS_bluntendF	ATGGGTTCTTTCAACAACAAAC	stop codon depleted CDS (7GCDSblunt - 1445bp) amplification for blunt end cloning
18	7GCDS_bluntendR	ACGTGAATATTTCTTTCAAATGC	

Table 19 List of primers for amplification and cloning of *Potri.007G095000* promoter region into pPLV11-Sharinall.

Primer number	Primer name	Sequence 5'-3'	Description	
19	7GpromoPti_4kbF	CTTGGACATCATTACGGTGCC	Forward primers designed on <i>P. tremuloides</i> genome	4kb promoter
20	7GpromoPti_3kbF	TAGCTCTATTCAAACGTATAACGG		3kb (7GPr3Kb) promoter
21	7GpromoPtre_4kbF	ATCAACTCAATGCTAACCTAGTTGG	Forward primer designed on <i>P. tremula</i> genome	4kb promoter
22	7GpromoPtre_3kbF	TAACTTTGACGTTAATACATGTGTAGC		3kb promoter
23	7G09_promo2rev	TGGCCATTAGACCGGACTA	Reverse primer for amplification of promoter fragments	
24	7Gpr3Kb_PLV11sF	CCGCTCGAGCGGACGTTAATACATGTGTAGC	Forward primer containing <i>XhoI</i> recognition site for amplifying 3kb promoter fragment (7GPr3KbpLV)	
25	7Gpr3Kb_PLV11sR	CCGGAATTCGGCTTGTATAACTATGGTTTTTG	Reverse primer containing <i>EcoRI</i> recognition site amplifying 3kb promoter fragment (7GPr3KbpLV)	

13.6 PCR conditions

Table 20 Selected *P.trichocarpa* genes for which PCR conditions were optimized using additional biological and technical replicates in real time qPCR.

Shown are the genes of *P.trichocarpa* CDS, optimized annealing and fluorescence detection temperature.

Gene ID	Annealing Temp. (°C)	Fluorescence Detection Temp. (°C)
<i>Potri.007G081200</i>	54	81
<i>Potri.007G012400</i>	54	
<i>Potri.002G188900</i>	54	
<i>Potri.012G117100</i>	54	
<i>Potri.006G009300</i>	54	80
<i>Potri.011G046600</i>	54	79
<i>Potri.001G085900</i>	54	
<i>Potri.003G210600</i>	54	
<i>Potri.004G019300</i>	54	
<i>Potri.007G095000</i>	54	78
<i>Potri.008G071100</i>	54	

Table 21 Optimized PCR conditions for amplification of *Potri.008G071100* and *Potri.007G095000* coding sequences for different cloning purposes.

Steps	Temperature (°C)	Time (sec)	Cycles
Initial denaturation	98	30	1
Denaturation	98	10	35
Annealing	62.5 (8GCDS), 65 (8GCDS-pET28a), 58.9 (8GCDS-SL), 58(7GCDS), 64 (7GCDSLIC) and 58.9 (7GCDSblunt)	20	
Elongation	72	30	
Final elongation	72	300	1

Table 22 Optimized PCR conditions for amplification of *Potri.008G071100* and *Potri.007G095000* upstream promoter regions for cloning.

Steps	Temperature (°C)	Time (sec)	Cycle s
Initial denaturation	98	180	1
Denaturation	98	20	35
Annealing	64.9 (8GPr3.4/3.6Kb), 58.2 (8G11Pr3.4Kb) 60.7 (8G04Pr3.4Kb), 61.3 (7GPr3Kb) and 62 (7GPr3KbpLV)	35	
Elongation	72	180	
Final elongation	72	600	1

13.7 Vector map

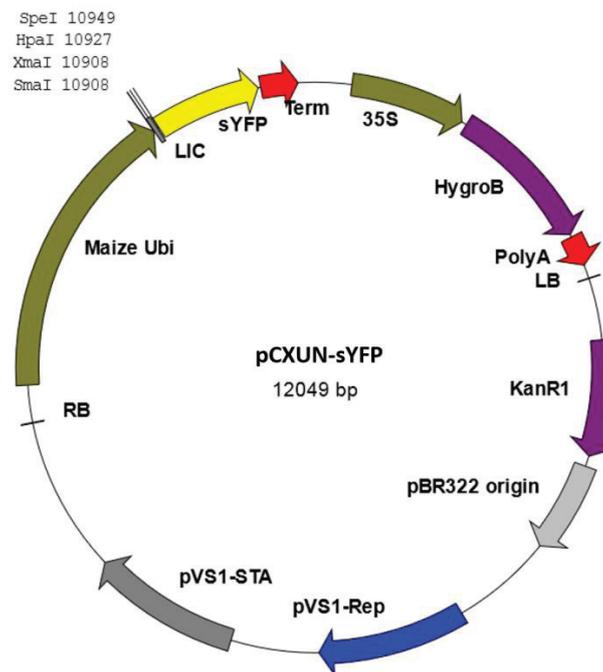


Figure 39: pCXUN-sYFP vector map. Drawn using <http://www.rf-cloning.org/savvy.php>: accessed on 21.10.2016

Shown are the functional elements and selected recognition digestion sites: T-DNA contains a maize ubiquitin-1 promoter (Maize Ubi), multiple cloning site harbouring ligase independent cloning site (LIC), super yellow fluorescent protein (sYFP) and a single 35S promoter (p35S) driven Hygromycin B coding region (HygroB) for plant hygromycin B resistance. The pCambia backbone contains a Kanamycin resistance coding region (KanR1), pBR322 and pVS1 origin of replication for stability both in *E. coli* and *Agrobacterium*.

13.8 Supplemented data

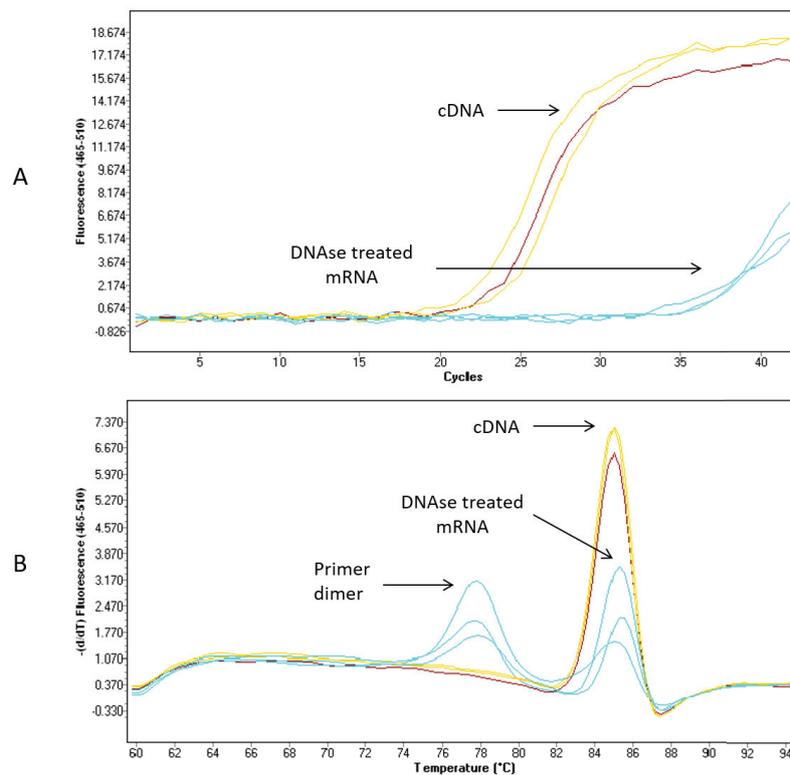


Figure 39: Amplification and melting curves of cDNA and DNase treated mRNA samples of two mycorrhized and non-mycorrhized fine roots.

The amplification curves of the cDNA and DNase treated samples (A) demonstrating the development of the fluorescence over the PCR cycles; the cDNA samples shows lower C_p (crossing point) values than the corresponding DNase treated mRNA samples. Melting curves of the final qPCR products (B), the melting peaks are higher where the cDNA was used as templates, compared to those from DNase treated mRNAs. Melting peaks at a lower temperature, representing the primer dimer was observed for the DNase treated samples.

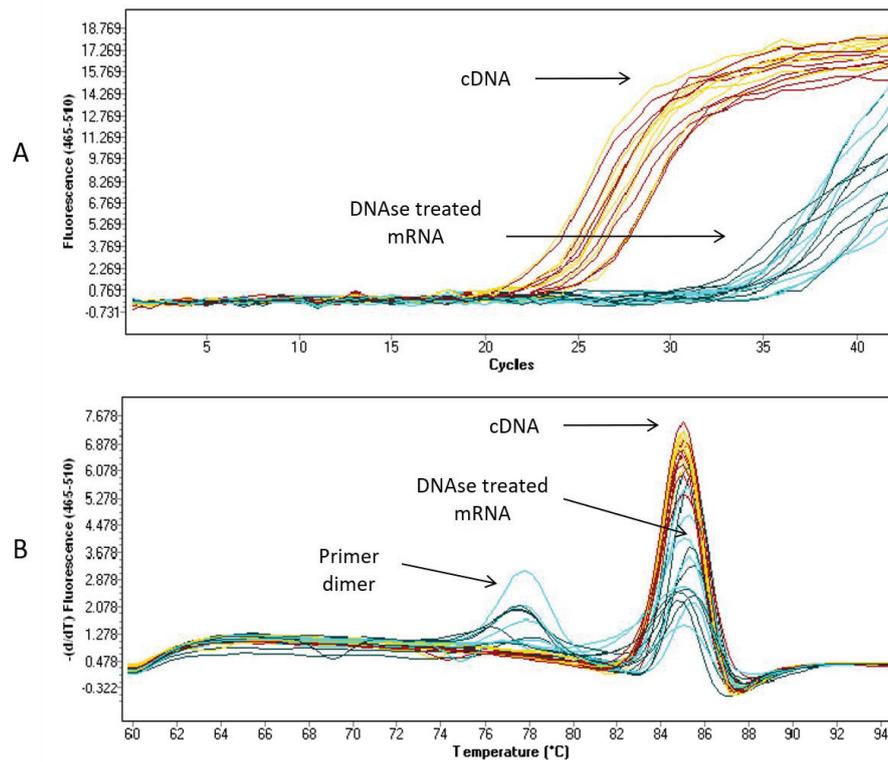


Figure 40: Amplification and melting curves of different biological replicates used for the gene expression analysis

Shown is the fluorescence development over increasing qPCR cycle numbers for different biological replicates using first strand cDNA and DNase treated mRNA (A). The cDNA revealed lower Cp values than DNase treated mRNAs. The melting curves of the final PCR products (B) demonstrate generation of longer specific products with a higher melting peak for cDNA than DNase treated mRNAs. The shorter melting peaks represent primer-dimer formation.

Table 23: Slope and determination of PCR efficiency

Shown are the gene ids, slope value, standard error, efficiency and the percentage of efficiency of the selected genes. The amplification efficiency was evaluated from the calculated slopes of linear regressions using the formula $E_{\text{efficiency}} = 10^{(-1/\text{slope})}$. Slopes between -3.2 to -3.5 indicate optimal PCR conditions giving nearly 100% amplification efficiency (Wittwer et al. 2001; Pfaffl2001; Hahn et al. 2004; Hands et al. 2006).

Gene id	Slope (m)	Standard error	Efficiency	Percentage (%)
<i>Potri.007G081200</i>	-3.484	0.0891	1.937	96.85
<i>Potri.003G210600</i>	-3.335	0.0963	1.995	99.75
<i>Potri.002G188900</i>	-3.526	0.0426	1.921	96.05
<i>Potri.006G009300</i>	-3.190	0.103	2.058	102.9
<i>Potri.007G095000</i>	-3.531	0.0341	1.919	95.95
<i>Potri.008G071100</i>	-3.488	0.0887	1.921	96.05

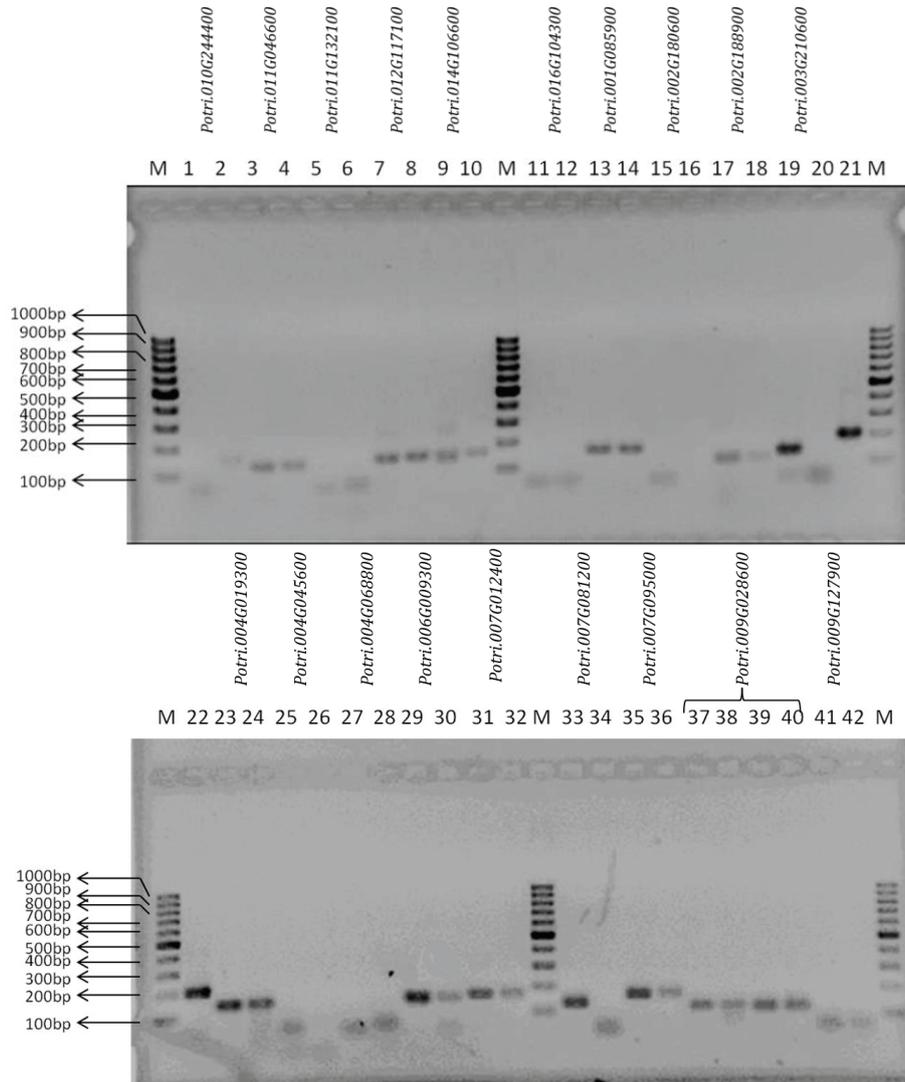


Figure 41: Agarose gel electrophoresis of the initial qRT-PCR products.

Shown is the gel electrophoresis separation of PCR products of the selected genes. Gene specific primers were used for the amplification from *Populus tremula* × *tremuloides* cDNA. Odd and even numbers represents the DNA fragments obtained from mycorrhized and non-mycorrhized sample respectively. Primer-dimers can be recognized from their smaller size (<100bp) compared to gene specific product. A 100bp DNA ladder was used for comparison of product size.