Impact of sucrose transporters on fungal carbon nutrition in ectomycorrhiza

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Impact of sucrose transporters on fungal carbon nutrition in ectomycorrhiza

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Contents

1 Introduction

1.1 *Populus* as model plant

Forests cover about 30 % of the Earth's terrestrial surface emphasizing the outstanding role they play in ecology but also in provision of fibers and fuels (Tuskan et al., 2006).

In modern plant science, only a few species are generally used as model organisms for exploring the basic biochemical, physiological as well as ecological processes defining the biological features plants have. Concentration on only a few model plants is a productive and promising way in pushing plant research forward. Although some mechanisms and processes are conserved among the plant kingdom some have diverged during evolution, requiring model plants either representing different clades of this kingdom (Flavell, 2009) or beeing established in diverse ecosystems with specialized demand. Perennial woody plants are different compared to annual herbaceous plants in many aspects and are therefore expected to have developed an own repertoire of functional genes with unique expression profiles (Park et al., 2004).

The genus *Populus* belongs to the Salicaceae family within the Eurosid clade of the Eudicotyledonous, comprises about 30 species and is rich in genetic variation. It is widely spread in the temperate climates of the northern hemisphere (Park et al., 2004). *Populus* is closely related to the most commonly used model species in plant science, *Arabidopsis thaliana*, belonging to the Brassicaceae family. Therefore, functional analyses of *Arabidopsis* genes/proteins can be useful for investigation in *Populus* (Jansson and Douglas, 2007). However, certain tree-specific features cannot be deduced from *Arabidopsis thaliana* like dormancy (e. g. during the winter season), long-term perennial growth, or seasonality. Moreover, there is a major feature making *Populus* attractive for research: unlike *Arabidopsis*, *Populus* is able to form biotic interactions with mycorrhiza-forming fungi, even under laboratory conditions. This type of symbiosis is characteristic for most forest trees living in the northern hemisphere (Jansson and Douglas, 2007).

Members of the genus *Populus* are usually fast growing, easily cultivable and allow simple vegetative propagation. Furthermore, stable *Populus* transformation by *Agrobacterium* was proven successfully (Taylor, 2002; Jansson and Douglas, 2007). In 2006, Tuskan et al. published the genome sequence of *Populus trichocarpa*

(female genotype Nisqually 1) that reveals a relatively small size of about 485 Mb. In 2012 annotations were completely revised resulting in version v2.2 gene model of the *Populus* genome (Grigoriev et al., 2012; Wullschleger et al., 2013, www.phytozome.net).

Taken all together *Populus* is suitable as a representative organism for woody perennials.

1.2 Mycorrhiza

Mycorrhizal symbiosis is a mutualistic interaction between plants and fungi. In this symbiosis the fungal partner retrieves inorganic nutrients and water from the soil and delivers them in part to the plant partner, where it receives carbohydrates in exchange.

There are different types of mycorrhiza that are either described by morphological features of the fungus or by features of the plant partner (Brundrett, 2004). The endomycorrhizal morphotype can be subdivided into ericoid, orchid and arbuscular mycorrhiza (Parniske, 2008). The latter one is the most ancient form of mycorrhiza that has developed presumably 400 million years ago (Fitter, 2005). Arbuscular mycorrhiza is supposed to be the most widespread mycorrhiza type, which is formed by 70 – 90 % of terrestrial plant species (Parniske, 2008) and is expected to play a key role during colonization of terrestrial habitats by plants, which was necessary to overcome limited access to nutrients (Fitter, 2005).

Ectomycorrhiza and endomycorrhiza differ in the way the fungal hyphae enter the root. While hyphae of most ectomycorrhizal fungi are only able to grow along the middle lamella into the root cortex, hyphae of endomycorrhizal fungi can grow straight through plant cells, penetrating the entire cell wall. Even though these hyphae enter plant cells in the endomycorrhizal symbiosis, they do not penetrate the plasma membrane (Smith and Read, 2008).

Ectomycorrhiza is the dominant symbiosis form of middle European forests. Forests soils are rich in complex carbon sources like cellulose and lignin that are only degradable by specialized microorganisms. Degradation is a slow process and hence the amount of simple usable carbohydrates in forest soils is low. In contrast plant root exudates are rich in simple carbohydrates making an association to them attractive to microbes including mycorrhizal fungi (Nehls, 2008).

Major nutrients like nitrogen and phosphate are fixed in the organic layer of forests or are comprised in organisms. Trees have only limited access to these nutrients (Smith and Read, 2008). However, mycorrhizal fungi are able to mobilize nutrients in the organic matter making an association to ectomycorrhizal fungi attractive for forest trees.

Ectomycorrhiza

About 7,000 – 10,000 fungal species are capable to form ectomycorrhiza (ECM) and about 8,000 phytobionts can join ECM symbiosis (Taylor and Alexander, 2005). 95 % of ECM fungi belong to homobasidiomycetes, 4.8 % to ascomycetes, and also a few zygomycetes can participate in ectomycorrhizal symbiosis. The majority of plant participants are woody perennials. ECM host trees include families like Fagaceae, Betulaceae, Myrtaceae, Pinaceae, and Salicaceae, to which the genus *Populus* belongs to (Taylor and Alexander, 2005).

1.2.1 The fungal associate

Ectomycorrhizal fungi can form large colonies, composed by soil growing hyphae, and mycorrhizas. Within an ectomycorrhiza two fungal networks are distinguished: the fungal mantle and the Hartig net (Kottke and Oberwinkler, 1986; see Fig. 1). Soil growing hyphae explore the litter for nutrients and interconnect different parts of the colony by fungal cords or rhizomorphs, which enable long-distance transport of nutrients and plant derived carbohydrates. The fungal mantle is a multi-layer structure that develops outside of the root and surrounds it. Hence, an isolating effect of the infected fine root from the soil environment is assumed (Taylor and Alexander, 2005). In the fungal mantle nutrients delivered by soil growing hyphae and carbohydrates delivered by the plant can be intermediately stored. Within the apoplast of the root cortex a highly branched hyphal network, called Hartig net, is formed that provides a large surface area for an intensive nutrient and metabolite exchange (Kottke and Oberwinkler, 1986; Nehls et al., 2007).

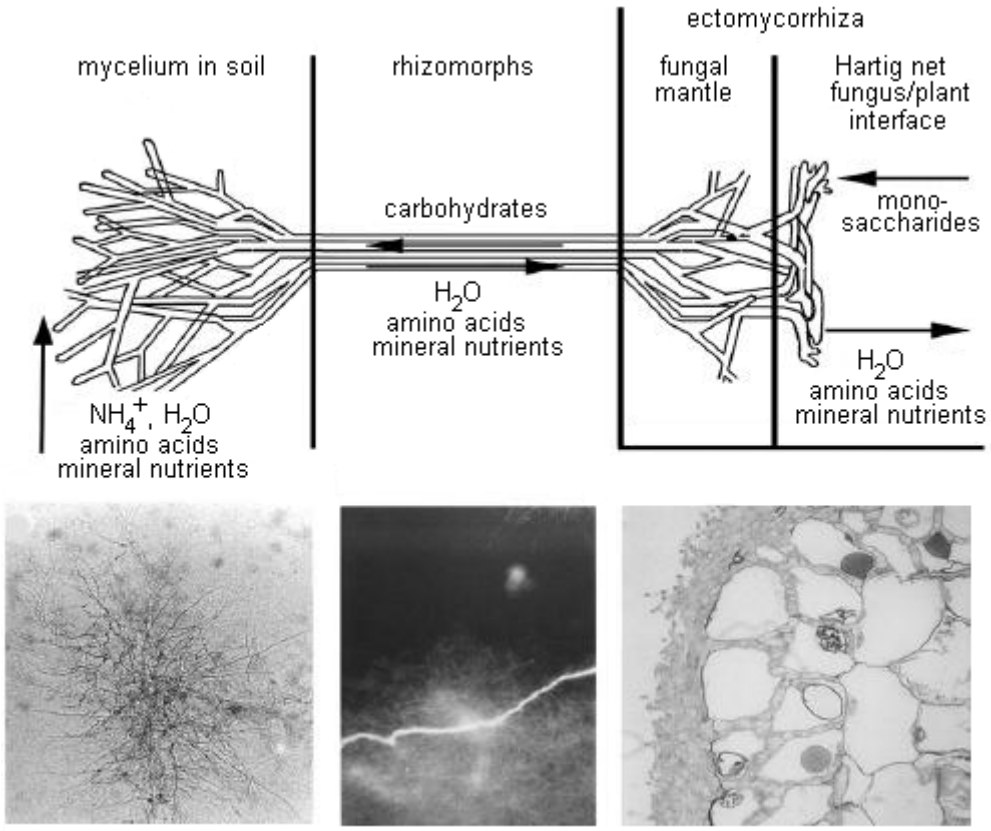

Fig. 1: Illustration of an ectomycorrhizal fungal colony without fruiting bodies (taken from Nehls, 2008): A scheme of an ectomycorrhizal colony (upper part) and matching photographs of the respective fungal structure are shown (lower part).

1.2.2 The plant associate

In order to meet the fungal carbon demand, which is supposed to count for up to 15 % of the net photosynthesis rate (for reviews: Nehls et al., 2007; Nehls, 2008), the plant partner increases its photosynthetic efficiency. Investigations of *in vitro* mycorrhized *Castanea sativa* plants (with *Pisolithus tinctorius* as fungal partner) revealed elevated photosynthetic and decreased respiratory rates, a lower CO_2 compensation point, and an increased amount of ribulose-1.5-bisphosphate carboxylase/oxygenase (Martins et al., 1997). Moreover, Loewe et al. (2000) discovered elevated net assimilation rates in gas exchange experiments for *Picea abie*s and *Populus tremula* × *tremuloides* plants mycorrhized with *Amanita muscaria* or *Paxillus involutus*.

Mycorrhizal plants may also control the carbon drain towards the fungus to avoid fungal parasitism. Such carbohydrate drain control is supposed to occur at different levels: control of sucrose export into the common apoplast, which is still a poorly understood process; control of sucrose hydrolysis by cell wall located acid invertases and/or competition for hexoses that are present in the common apoplast. Nehls et al.

(2007) gave evidence that in poplar plants hexose importer gene expression as well as the activity of cell wall localized acid invertase is increased upon mycorrhiza formation at the functional interface.

Photosynthetic carbon dioxide fixation is the basis for carbohydrate production in higher plants. Assimilated carbon is often locally stored before it is transported from sources, which are autotrophic tissues, to sinks, which are heterotrophic tissues. Apart from energy production, carbohydrates are needed for the generation of primary or secondary metabolites like amino acids and phytohormones and hence they play an important role for development and growth. Carbohydrate-demanding processes include flowering, seed formation, leaf formation, vascular strand formation, fine root formation, carbon storage, or mycorrhiza formation and function (Williams et al., 2000).

1.3 Sugar transport in plants

Carbohydrates are mainly transported as sugars and therefore sugars and their transport are of special interest for understanding carbohydrate allocation processes. In many plant species sucrose is the major carbohydrate used for long-distance transport in the phloem (Büttner, 2007). However, sucrose together with polyols, or oligosaccharides such as raffinose, stachyose, and/or verbascose as dominant form, which are synthesized from sucrose, are used as well in certain plants (Williams et al., 2000; Sauer, 2007). Usually sugars are loaded into the sieve elements of the phloem where it is transported towards sink-tissues by differences in hydraulic pressure as driving force (Lalonde et al., 1999). Unloading the phloem at sink-tissues results in maintenance of an osmotic gradient between source and sink tissues (Williams et al., 2000). Dependant on the plant species, its growth rate, and the developmental state of organs or tissues different pathways of phloem loading/unloading are used (Williams et al., 2000; Fu et al., 2011). Generally sugar transport between cells can either be symplastic or apoplastic. Symplastic transport happens by diffusion through plasmodesmata, enabling neighboring cells an energy-independent passive exchange of soluble compounds. In contrast, an active process, e. g. proton-coupled proton co-transport, is involved in apoplastic transport across the plasma membrane. However, apoplastic transport can also include passive mechanisms if facilitators and an appropriate chemical gradient are involved (Sauer,

2007; Slewinski, 2011). Specialized sugar transport proteins play therefore a key role in source-to-sink interactions, which makes them attractive for manipulation to investigate principles of source sink relationships.

1.3.1 Fungal carbohydrate support

Sucrose was long time favored to be the carbon source for fungal support in ectomycorrhiza. However, not only sucrose but also its monomers glucose as well as fructose were considered. Since the mycobiont as part of the mutualistic interaction has the ability to degrade cellulose and lignin, which are components of the phytobiont's cell wall, these two compounds could be also taken into consideration. A few articles dealing with the question which carbohydrate is exuded by the host plant and utilized by the fungal partner represent different models that evolved for fungal carbohydrate support.

In 1969 cellulose was seen as carbon source for orchid endophyte fungi (Hadley, 1969). In the orchid mycorrhiza the fungus is essential for protocorm growth (cells produced in the first stage of the germination process of orchid seeds). At that time prove was given that growth and development of protocorms are improved in cultures in which cellulose compared to glucose was used as carbon source and hence cellulose might play a role in a wide range of orchid-fungus systems (Hadley, 1969).

In 1993, Harley and Smith speculated that trehalose, a fungal storage compound, consisting of two linked glucose monomers, was synthesized from glucose in fungal tissues in the ECM symbiosis thereby maintaining a glucose concentration gradient that consequently enables the transfer of glucose from the plant to the fungus. In 1995, Shachar et al. identified glucose to be the carbon source for fungal support in vesicular-arbuscular mycorrhiza that is found for a large variety of crop plants. The course of carbon metabolism was traced by nuclear magnetic resonance spectroscopy using 13-C labeled glucose to examine the roots of *Allium porrum* that were colonized by *Glomus etunicatum*. In the time course the fungal metabolites trehalose and glycogen got labeled and hence glucose was seen as presumable substrate for vesicular-arbuscular mycorrhizal fungi under symbiotic conditions (Shachar et al., 1995).

Moreover, Hampp et al. (1995) suggested that apoplastic invertases hydrolyse sucrose that is exuded by host roots and thereby enhance glucose uptake by the

fungus. They have shown an increased capacity for sucrose formation in seedlings of spruce under mycorrhized conditions. Further examinations using protoplasts of the ectomycorrhizal fungus *Amanita muscaria* revealed uptake properties for glucose but not sucrose. This fact supports the idea of invertase activity and the associated hydrolysis of sucrose, which is restricted to the host. Hence, sucrose transporters as possible mediator for sucrose export of the host roots were long time of special interest regarding the fungal carbon support.

However, in 2012 the identification of a novel transporter gene family called SWEETs came into consideration for fungal carbon support in mycorrhizal symbiosis (Chen et al., 2012; Nintemann, 2012). Some of them were already shown to be capable of transporting glucose, which is now the favored carbon source for fungal carbohydrate nutrition in ectomycorrhiza.

1.3.2 Sucrose transporters as potential carbohydrate exporters in ECM symbiosis

Based upon different observations sucrose is expected to be the major plant-derived apolastic carbohydrate source (for a recent review see Nehls et al., 2010) in ECM symbiosis. Accordingly, sucrose must be exported by cortical cells of plant's fine roots into the common apoplast (Nehls, 2008). But what are the potential efflux carriers? Most proton-coupled sucrose transporters have been demonstrated to function as sucrose importers. Nevertheless, Carpaneto et al. (2005) could show that under certain conditions these transporters can also work as sucrose efflux carriers. They could prove that the proton-coupled sucrose transporter ZmSUT1 of *Zea mays*, which is expressed in source- as well as in sink-organs, is capable of mediating both sucrose uptake into the phloem in mature source-leaves and sucrose export from phloem vessels into heterotrophic sink-tissues.

The source site of the sieve element/companion cell-complex is determined by an outward-directed sucrose and an inward-directed proton gradient over the plasma membrane. The typical plant plasma membrane potential of about -150 mV results in a proton-coupled sucrose import into the phloem cells. Under this condition sucrose can accumulate to cytosolic concentrations of up to 1 M (Carpaneto et al., 2010). However, at the sink site of the sieve element/companion cell-complex the plasma membrane potential can be much lower (-60 mV) (Carpaneto et al., 2005). Together

with a steep sucrose gradient over the plasma membrane (with high cytosolic and low apoplastic sucrose concentrations) proton-coupled sucrose transporters should be capable of releasing sucrose into the apoplast under these conditions. This hypothesis was proven by heterologous expression of *ZmSUT1* in *Xenopus laevis* oocytes. Carpaneto et al. (2005) could demonstrate that under conditions of a low membrane potential and high cytosolic sucrose concentrations *ZmSUT1* expressing oocytes released sucrose (in contrast to a vector control).

In addition, Zhou et al. (2007) discovered members of the *SUT* gene family in seeds of *Pisum sativum*, (PsSUF1 and PsSUF4) and *Phaseolus vulgaris* (PvSUF1) that show high homology to known proton-coupled sucrose transporters. However, these proteins revealed a bi-directional, pH-, and energy-independent sucrose transport and were therefore described as sucrose facilitators.

Both mechanisms, depolarization of the plasma membrane and the presence of sucrose facilitators could enable sucrose efflux from plant cells and would explain fungal sucrose support in ectomycorrhizal symbiosis.

Recently, a novel gene family of sugar facilitators, called *SWEETs,* (Chen et al., 2010) was discovered as completion of the SUT gene family. SWEET proteins consist of 7 transmembrane helices and are supposed to form a membrane pore. Chen et al. (2012) could prove that six out of 17 members of the *Arabidopsis thaliana SWEET* gene family (AtSWEET10 to AtSWEET15) and two out of about 21 members of the *Oryza sativa SWEET* family (OsSWEET11, OsSWEET14) can transport sucrose. As this sucrose transport occurred in a pH-independent manner in both directions over the plasma membrane, a uniporting/facilitating mode of action was supposed (Chen et al., 2010; Slewinski, 2011; Chen et al., 2012).

Independent on the mechanism of sugar efflux, a steep sucrose gradient over the plasma membrane is necessary. Continuous carbohydrate uptake by fungal hyphae at the plant/fungus interface is thought to be the driving force of the plant based fungal carbon support in mycorrhizal symbiosis. Conversion of carbohydrates into fungal metabolites by enhanced glycolysis or production of fungal storage compounds is supposed to be essential for maintaining the observed strong carbon sink in symbiosis.

1.3.3 Sucrose transporters of the *SUT* gene family in *Populus trichocarpa*

Tuskan et al. (2006) initially identified six sucrose transporter genes *SUT1*, *SUT2*, *SUT3*, *SUT4*, *SUT5*, and *SUT6* in the genome of *Populus trichocarpa* (Nisqually 1). However, in the updated genome assembly *SUT1* and *SUT2* were mapped to a single locus resulting in only five members of the *sucrose transporter* gene family in *Populus* (www.phytozome.net; Table 1). The couples *PtaSUT1* and *PtaSUT3* as well as *PtaSUT5* and *PtaSUT6* share high degree of similarity regarding their nucleotide and amino acid sequence (≥ 90 %), while *PtaSUT4* is part of a third group sharing only about 64 % amino acid sequence similarity to *PtSUT1* and *PtSUT3*. *PtaSUT5* and *PtaSUT6* comprise fourteen exons while the other *PtSUT*s only contain three to four exons. Furthermore, the predicted polypeptide size of PtSUT5 and PtSUT6 about 64 kDa is larger than that of the other PtSUTs (55 – 57 kDA) (Payyavula et al., 2011).

Table 1: Properties of primary sequences of *sucrose transporter*s of *Populus*. In the updated *Populus* genome assembly, *SUT4* is mapped to three potential gene models. Modified according to Payyavula et al. (2011). Abbreviations: n.t. – nucleotide, a.a. – amino acid.

	Size			% Similarity (n.t./a.a.) to				Locus in updated *Populus* genome assembly
Gene	**a.a.**	**kDa**	**Exon no.**	***SUT3***	***SUT4***	***SUT5***	***SUT6***	
SUT1	535	56.9	4	90/93	56/64	46/57	46/56	POPTR_0013s11950
SUT3	532	56.7	4	-	56/64	46/57	46/56	POPTR_0019s11560
SUT4	510	55.4	5	-	-	46/55	45/54	POPTR_0002s10710 POPTR_0002s10730 POPTR_0002s10750
SUT5	597	64.2	14	-	-	-	91/92	POPTR_0008s14760
SUT6	601	64.6	14	-	-	-	-	POPTR_0010s10370

According to phylogenetic analysis Sauer (2007) arranged SUT proteins into four distinct groups, a classification that was mainly confirmed by Payyavula et al. (2011), (Fig. 2). According to Payyavula et al. (2011) group I represents proton-coupled SUTs of monocots whereas group II contains proton-coupled SUTs of dicots. PtaSUT1 and PtaSUT3 belong to the latter group and cluster together with orthologs of other perennial species like RcSUT2 from *Ricinus* and VvSUT27 from *Vitis*. Group II SUTs have been characterized as plasma membrane-localized proton-coupled transporters mediating apoplastic phloem loading (Chandran et al., 2003; Hackel et al., 2006). Group III and group IV are each divided into a mono- and

dicot-specific subclade. PtaSUT5 and PtaSUT6 belong to group III and cluster with orthologs of other perennial species. However, the function of group III SUTs is less clear. The dicot subgroup of group IV is further divided into a legume-specific and a non-legume branch to which PtaSUT4 is associated to. Group IV SUTs are tonoplast-localized transporters as shown for AtSUC4 from *Arabidopsis* (Endler et al., 2006), LjSUT4 from *Lotus* (Reinders et al., 2008) and PtSUT4 from *Populus* (Payyavula et al., 2011).

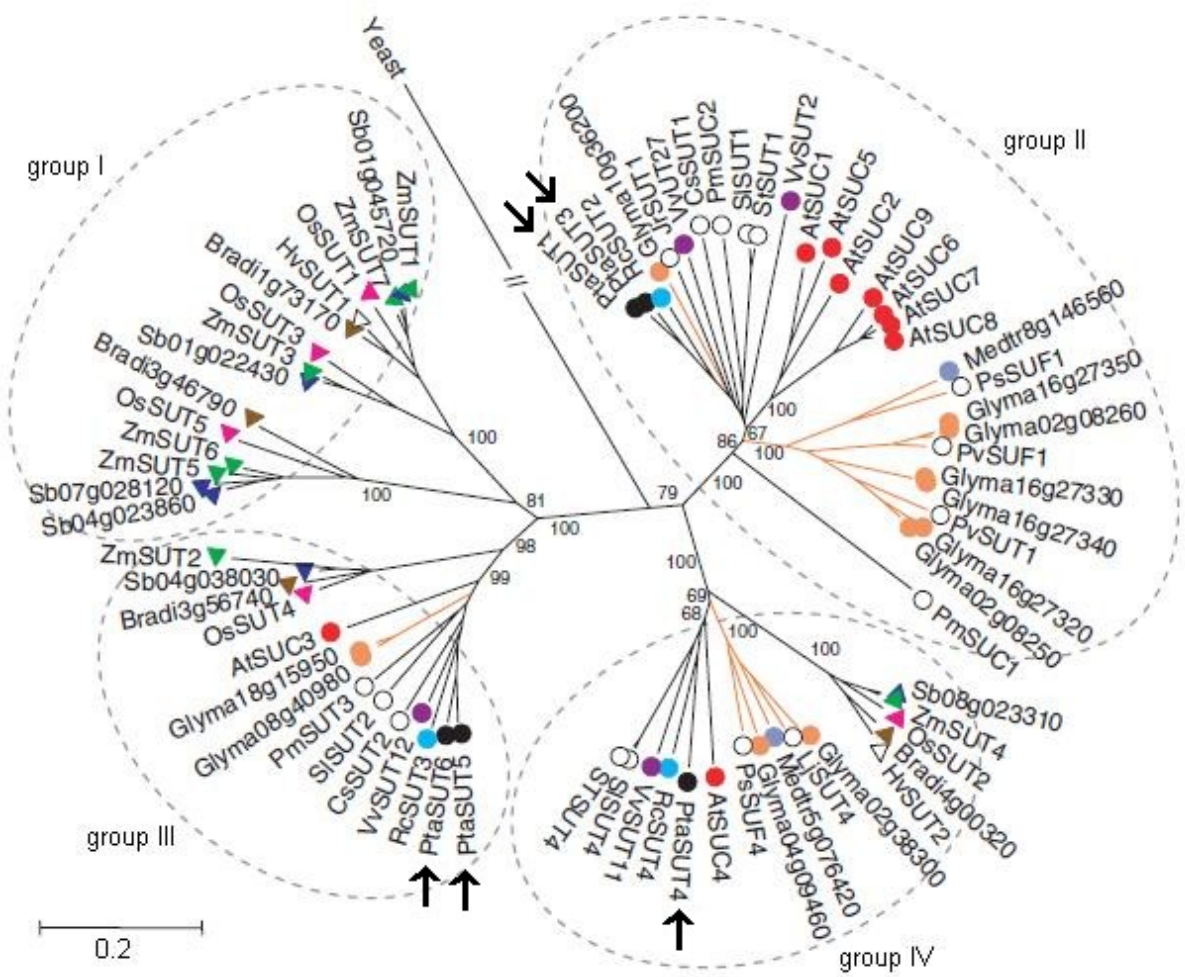

Fig. 2: Neighbor-joining tree of putative sucrose transporter proteins taken from Payyavula et al. (2011). Dicot sequences are indicated by circles (light orange represents legume branches) and monocot sequences by triangles. Bootstrap values for the major nodes are shown. The sucrose transporter of yeast was used as outgroup. Black arrows point at SUTs from *Populus tremula* × *P. alba*.

Gene expression analyses revealed the highest transcript levels of *PtaSUT1* and *PtaSUT3* in roots and stems whereas *PtaSUT5* and *PtaSUT6* were expressed in all investigated tissues (Payyavula et al., 2011). *PtaSUT4* revealed its highest expression in mature poplar leaves (Fig. 3).

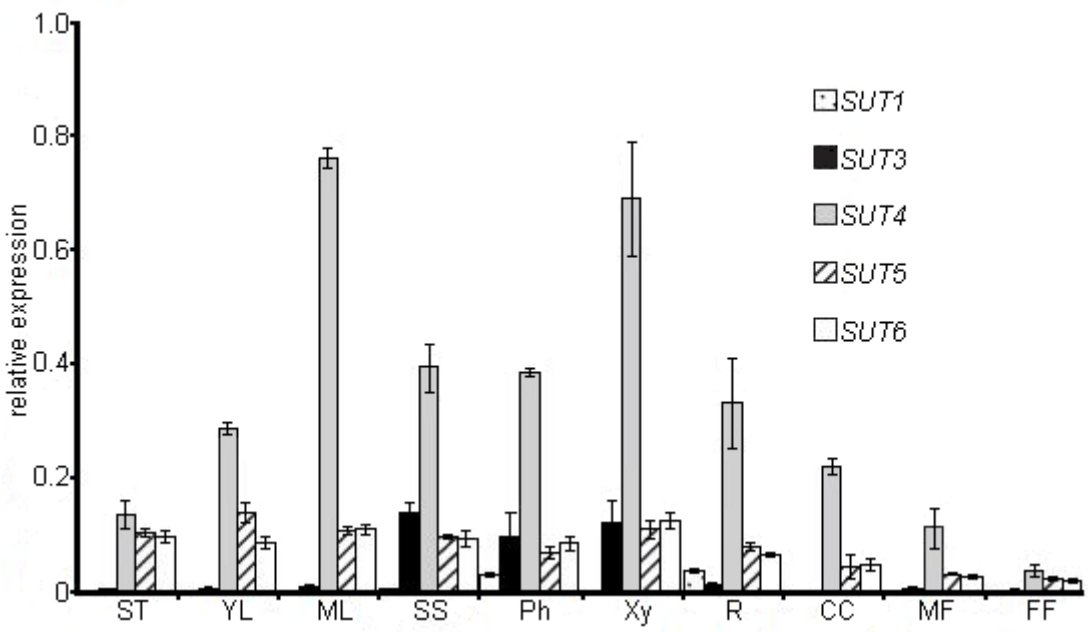

Fig. 3: *SUT* gene expression in several *Populus* tissues taken from Payyavula et al. (2011). Data of three biological replicates are shown. ST – shoot tip, YL – young leaves, ML – mature leaves, SS – secondary stem, Ph – phloem, Xy – xylem, R – root, CC – cell culture, MF – male flower, FF – female flower.

Transcript localization by *in situ* hybridization revealed *PtaSUT3* to be mainly expressed in shoots and in minor veins of leaves. In the shoot *PtaSUT3* expression signals were strong in xylem parenchyma compared to the adjacent phloem (Payyavula et al., 2011). *PtaSUT4* transcripts could be detected in epidermal cells, the lower palisade layer, spongy mesophyll cells, conducting phloem cells, and in parts of the secondary xylem (ray parenchyma, vessels and fibres). *PtaSUT4* was also expressed in the vascular cambium, dividing xylem cells, and cells of primary stem internodes. Due to their high sequence homology, signals for *PtaSUT5* and *PtaSUT6* could not be distinguished by *in situ* hybridization and were thus analyzed as group. *PtaSUT5*/*PtaSUT6* transcripts were detected in all cell types. Weak signals were detected in phloem tissues (including phloem fibers) but only where transcripts of *PtaSUT3* and *PtaSUT4* were absent.

Functional analysis of PtaSUT3, PtaSUT4, and PtaSUT5 was performed by heterologous expression in the yeast strain Susy7/ura3 (Payyavula et al., 2011), which cannot utilize external applied sucrose unless a functional foreign sucrose importer is expressed. As all three investigated poplar SUTs were able to complement the yeast mutant's phenotype and were thus indicated as functional sucrose transporters (Payyavula et al., 2011).

When expressed in *Nicotiana tabacum* PtaSUT4 was targeted to the tonoplast (Payyavula et al., 2011). To further determine the biological function of PtaSUT4, RNA interference (RNAi)-mediated gene silencing was done. Compared to wild-type plants elevated sucrose and starch contents were detected in source-leaves of transgenic *Populus tremula* × *P. alba* plants (Payyavula et al., 2011). Due to the detected elevated sucrose and starch content in transgenic *Populus* source leaves and the additional reduction of the hexose content the authors hypothesized that either sucrose was built up or the sucrose hydrolysis was reduced leading to the increase of sucrose content in transgenic source leaves.

As *PtaSUT4* was expressed in source- as well as sink-tissues it was thus supposed by the authors that an important function of PtaSUT4 was the coordination of subcellular compartmentation in source-leaves and long-distant transport of sucrose in *Populus* plants.

Frost et al. (2012) gave further evidence that down-regulating *PtaSUT4* expression by RNAi affects water uptake. They could show that uptake of water by RNAi plants was slower compared to that of wild-type plants and thus postulated that vacuolar secretion of sucrose is an essential part of natural adaptive response of plants to shifts in the water status. Hence, Frost et al. (2012) indicate a direct link between the expression of *PtaSUT4* (and therewith sucrose export by the belonging proteins) and the adaption to shifts in the water status.

1.4 Demand for root-specific promoters as tool for investigations in poplar

Frequently, gene expression is not restricted to a single plant organ or tissue. When the expression of such genes is manipulated to investigate the biological function of the respective protein, multiple effects are frequently observed. To understand the function of certain sugar transporters in ectomycorrhizal symbiosis, gene manipulation has to be at least root-specific. One strategy to ensure root-specific manipulation of gene expression would be the utilization of a root-specific promoter. Requested features of a promoter that is helpful for ectomycorrhizal research are a) a strong expression in roots that is b) ectomycorrhizal-independent. To identify such a promoter Krützmann (2010) made use of a genome wide comparison of gene expression profiles in vegetative poplar organs (Quesada et al., 2008). Out of 42,364

potential poplar genes, 17 turned out to be root-specifically expressed (Nehls et al. unpublished). Five of these genes were chosen by Krützmann (2010) because of their high expression levels and were reinvestigated by quantitative RT-PCR in vegetative organs of six-month old poplar plants. One gene, coding for a putative peroxidase was finally chosen because of an ectomycorrhiza-independent expression pattern, together with high transcript levels especially at the symbiotic interface of infected fine roots (Krützmann, 2010). However, with regard to the peroxidase promoter the question raised under which conditions and how the root-specific gene expression and regulation is mediated.

1.5 Structure of eukaryotic promoters

Understanding the mechanism, which leads to a temporal or tissue-specific expression pattern, is often the basis to understand the function of certain genes in a broader context. The expression pattern of a certain gene is determined by its promoter. A eukaryotic promoter can be divided into three regions. The core promoter region characterizes the region around the transcription start site (TSS). The core promoter ranges from about 40 bp downstream to 60 bp upstream of the TSS. The core promoter is followed upstream by the 200 to 300 bp long proximal promoter region. The promoter region that is positioned upstream of the proximal promoter is called distal promoter region (Herpich, 2012).

The control of the transcription initiation is a crucial mechanism to determine if and to which extend a gene is expressed. The promoter sequence of a certain gene is crucial for its transcription and regulation (Cartharius et al., 2005). For gene regulation in eukaryotic cells *cis*-acting DNA sequences are required. These sequences are ten to twelve base pairs in length and known as transcription factor binding sites or regulatory elements. From TSS to about -100 bp upstream promoter elements are located (CAAT-box, GC-box). The proteins that interact with these sequences are known as transcription factors (Maniatis et al., 1987) and are very important for differential expression of genes. For example the TATA-box binding protein binds to the TATA-box (about -25 bp) and provokes the initiation of the transcription initiation complex and therewith the production of the corresponding protein. The importance of transcription factor binding proteins was highlighted by Riechmann et al. (2000), who stated that *Arabidopsis thaliana* dedicated 5 % of its genome to encode more than 1,500 transcription factors. Interactions of transcription

factors and regulatory elements, as well as interactions among the transcription factors themselves, regulate gene expression. The proximal and distal promoter contain binding sites for transcription factors like enhancers and/or silencers that induce or repress the attachment of the RNA-polymerase to the promoter region resulting in enhanced or repressed expression of a certain gene (Maniatis et al., 1987).

1.6 Root transformation by *Agrobacterium rhizogenes*

A different way to manipulate root-specific gene expression is to only transform the organ of interest - the root. Since Chilton et al. (1982) gave evidence that infection with *Agrobacterium rhizogenes* leads to stable root transformation, the usefulness of so called composite plants became more and more attractive for plant research. The name “composite plant”- was chosen because apart from transformed roots the other plant organs remain untransformed. This approach might be a shortcut for scientists investigating transgenic roots and their interaction with soil micro-organisms and mycorrhiza-forming fungi (Whitham et al., 2008).

Agrobacterium as pathogen

The first publications about diseases causing crown galls and hairy roots can be traced back to the early 20th century (Smith and Townsend, 1907; Riker et al., 1928). At that time, it became clear that both infections are only two types of the same disease. Today, several *Agrobacterium* strains causing a variety of neoplasms are known. Gall inducing strains are *A. tumefaciens* (crown gall disease)*, A. rubi* (cane gall disease)*,* and *A. vitis* (galls on grape). Moreover, there is *A. rhizogenes* causing hairy root disease, and *A. radiobacter*, a strain belonging to a disarmed group of *Agrobacterium*, which is non-tumor inducing.

The genus *Agrobacterium* has a wide host range. Next to plants, human HeLa cells (Kunik et al., 2001) and sea urchin embryos (Bulgakov et al., 2006) were successfully transformed by *Agrobacterium*. But also fungi, including ascomycetes and basidiomycetes are suitable as hosts (Gelvin, 2003). Delimiting to herbal hosts angiosperms and gymnosperms can be transformed by *Agrobacterium.* However, plants differ in their susceptibility towards *Agrobacterium* mediated transformation not only among species. Nam et al. (1997) gave evidence that susceptibility even differs

among cultivars. Some plants are resistant and some are hypersusceptible to transformation by *Agrobacterium*.

Ri and Ti plasmid of *Agrobacterium*

Virulent *Agrobacterium* strains contain tumor inducing (Ti) or root inducing (Ri) plasmids (Gelvin, 2009; Fig. 4). During transformation, part of this plasmid, referred to as T-DNA, which is terminated by the left and right borders, is transferred into the host cell. After transfer and integration into the host plant's genome, genes encoded by the T-DNA, are expressed. The genes, which are encoded in the T-DNA, are of bacterial origin but have eukaryotic regulatory sequences allowing their expression in infected plant cells (Mishra and Ranjan, 2008). These are, next to genes involved in opine (*Agrobacterium tumefaciens*) or mannopine and agropine (*Agrobacterium rhizogenes*) biosynthesis, genes responsible for tumor or root induction. Opines are excreted by transformed host cells and used by *Agrobacterium tumefaciens* as carbon and/or nitrogen source (Oger et al., 1997). This nutritional function is taken over by *rol A*, *rol B*, *rol C*, and *rol D*, genes for mannopine and agropine synthesis in *Agrobacterium rhizogenes.* Genes for the biosynthesis of auxin and cytokine are affecting phytohormone levels in host plants (Spanier et al., 1989). Changes in the phytohormone balance leads to uncontrolled cell division and organ malformation. In case of *Agrobacterium rhizogenes iaaM* and *iaaH* genes are responsible for auxin biosynthesis.

Apart from the T-DNA, the backbone of the Ti or Ri plasmids includes the *vir* region, an origin of replication, ori, and an opine catabolism region. The *vir* region encodes several *vir* genes, which play an important role for the generation of a single stranded T-DNA molecule and its transport to the plant cell and further into its nucleus. The opine region encodes genes for utilizing opines as nutrient, which the *Agrobacterium* incited before. Transformation of *Agrobacterium* with a vector possessing a target DNA between its left and right borders enables a T-DNA transfer and the expression of genes located in the T-DNA region in the plant host.

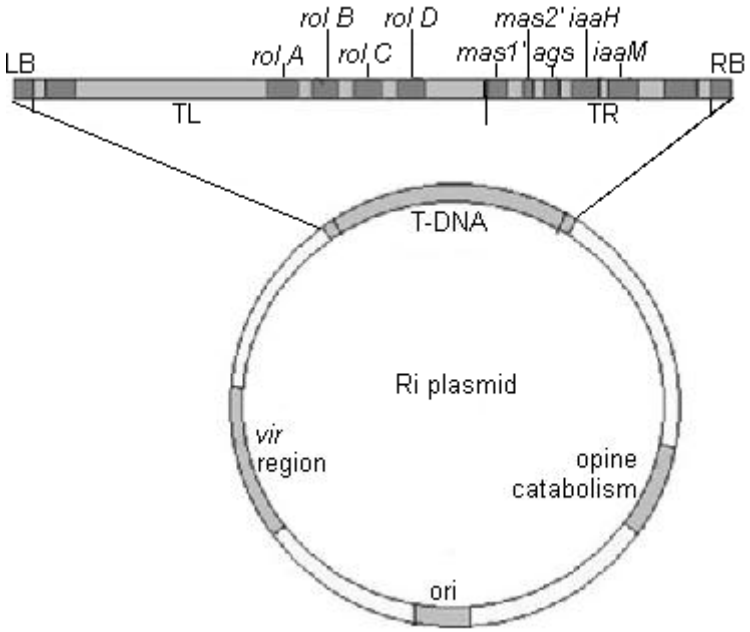

Fig. 4: Schematic map of a Ri plasmid of *A. rhizogenes*. The root inducing plasmid contains next to an origin of replication, ori, genes for the opine catabolism, the *vir* gene region, and the T-DNA region. The latter is determined by the left and right border (LB and RB). The T-DNA of the Ri plasmid consists of the *rol* genes, necessary for root induction, *mas1'*, *mas2*' and *ags* gene, necessary for opine synthesis, and *iaaH* and *iaaM* genes, which are responsible for the auxin biosynthesis. The T-DNA is separated into two parts named T-DNA left, TL, and T-DNA right, TR, by a 15 kb long fragment of non-transferred T-DNA. Taken from Chandra (2012).

1.7 Aim of the thesis

Despite of the literature summarized above, the way how the fungal carbohydrate support by the plant partner is accomplished in ectomycorrhizal symbiosis is still not completely understood. In literature it is commonly accepted that sucrose is exported by root cells of plant hosts at the functional interface and that it is hydrolyzed in the common apoplast by plant derived acid invertases into glucose and fructose. However, the way how sucrose efflux into the common apoplast of plant and fungus is mediated, is still a black box. The questioned efflux carriers are most likely sucrose transporting proteins. To fill this research gap, this thesis focuses on the characterization of putative sucrose transporters of *Populus trichocarpa.*

The functional characterization of SUTs gives hints on their transport properties like substrate specificity but also on enzyme kinetics, and pH-dependence. And this in turn will probably explain their role in supporting the fungal partner with carbohydrates in ECM. Heterologous expression of the putative *SUT*s in *Saccharomyces cerevisiae* is an appropriate approach for their functional characterization.

For the investigation of root-specific processes root-specific promoters are helpful tools, which can be used to suppress the gene expression rate of target genes only in roots using the RNAi approach. To check if such a promoter is a promising tool a root-specific promoter that was identified before will be analyzed. The *peroxidase* promoter provokes a strong root-specific and mycorrhiza-independent expression pattern especially at the symbiotic interface of infected fine roots. With the help of an *in silico* analysis regulatory elements of the peroxidase promoter will be predicted. Using peroxidase promoter fragments differing in length driving the expression of a marker gene will localize regulatory regions in the promoter sequence since different marker gene expression patterns can be attributed to the varying promoter length.

A different way to analyze the function of a certain protein in a root-specific manner is to transform only the relevant plant organ. Since prove was given that *Agrobacterium rhizogenes* is an appropriate tool for stable root transformation, a composite plant approach will be established for *Populus* using *Agrobacterium rhizogenes*.

Summing up, this thesis focuses mainly on three projects: Firstly, it deals with the functional analyses of *PtSUT*s by heterologous expression in *Saccharomyces cerevisiae*. Secondly, it focuses on the identification of putative regulatory regions of the root-specific peroxidase promoter mediating root-specific gene expression and regulation. Thirdly, a composite plant approach for stable *Populus* root transformation by *Agrobacterium rhizogenes*, allowing transformation of the root without affecting other organs, will be established.

2 Material and methods

2.1 Organisms

Escherichia coli

The strain TOP 10 F' (Invitrogen, Groningen, Netherlands) F-mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG) was used for cloning.

Agrobacterium tumefaciens and *Agrobacterium rhizogenes*

Poplar transformation was performed with two different strains of *Agrobacterium tumefaciens* C58 (Goodner et al., 2001; Wood et al., 2001) and AGL1 (Lazo et al., 1991) and one *Agrobacterium rhizogenes* (Riker et al., 1928; Hildebrand, 1931) strain K599 (Mankin et al., 2007).

Saccharomyces cerevisiae

The yeast strain SuSy7/ura3 MATa, ura3-52, leu2-3, 112, trp1, mal0, suc2Δ::URA3, ura3Δ, Leu2::128A2-SuSy (Riesmeier et al., 1992) was used for heterologous expression of putative *sucrose transporters* of *Populus trichocarpa* × *P. deltoides* and *SWEET*s of *Populus trichocarpa*.

Populus species

Two different poplar hybrids, *Populus tremula* L. × *P. tremuloides* Michx., T89, (Tuominen et al., 1995), *Populus tremula* × *P. alba* (No. 7171-B4, Institut de la Recherche Agronomique, INRA) were used for transformation. For the amplification of the coding sequences of *sucrose transporters*, RNA from *Populus trichocarpa* × *P. deltoides* (Bassman and Zwier, 1991) was used for first strand c-DNA synthesis.

2.2 Culture conditions

Escherichia coli

For liquid cultures 3 ml of LB medium (1 % peptone, 0.5 % yeast extract, 85.5 mM sodium chloride) were inoculated with a single colony and grown over night at 37°C under agitation (140 rpm, Certomat R, Braun, Melsungen, Germany). Alternatively, bacteria were grown on LB plates (LB medium supplemented with 1.8 % agar) over night at 37°C.

Agrobacterium tumefaciens and *Agrobacterium rhizogenes*

For liquid cultures of *Agrobacterium tumefaciens* 3 ml of YEB (0.5 % tryptone, 0.1 % yeast extract, 0.5 % peptone, 14.6 mM sucrose, 2 mM magnesium sulfate) and for *Agrobacterium rhizogenes* 3 ml of CPY (0.5 % peptone, 1 % yeast extract, 14.6 mM sucrose, 2 mM magnesium sulfate, pH 5.8) were inoculated with a single bacteria colony and incubated over night at 28°C under agitation (140 rpm, Certomat R). Alternatively, the respective *Agrobacterium* cells were incubated on plates containing either YEB or CPY supplemented with 1.8 % agar over night at 28°C. Due to genomic encoded antibiotic resistance of *Agrobacterium*, strain-dependent antibiotics were added to the growth medium, which are listed in Table 2.

Table 2: End concentration [mM] of antibiotics in the respective media for *Agrobacterium* wild-type strains. The end concentration of gentamicin could not be exactly determined, due to an undefined molecular weight between 694.75 and 723.75 g/mol, 25 mg gentamicin per l medium were used.

	***Agrobacterium tumefaciens* strains**		***Agrobacterium rhizogenes* strain**
Antibiotic	AGL1	C58	K599
Ampicillin	0.27	-	0.27
Gentamicin	-	$3.45 – 3.6 \times 10^{-2}$	-

Saccharomyces cerevisiae

For liquid cultures either 3 ml YPD medium (1 % yeast extract, 2 % casein hydrolysate, 100.9 mM glucose) or YNB medium (100 mM MES, 0.67 % Yeast Nitrogen Base including amino acids and ammonium sulfate, 2 % carbon source, pH 6.0) were inoculated with single colonies and incubated at 28°C under agitation (140 rpm, Certomat R) for two days.

Populus species

Populus plants were cultured on solid MS6 medium (0.22 % Murashige & Skoog medium including vitamins, 29.2 mM sucrose, 0,7 % plant agar, pH 5.6) according to Murashige and Skoog (1962). Plants were exposed to 17°C and a 12 h light/dark cycle with a light intensity of about 100 µmol photons m^{-2} s^{-1}.

2.3 Transformation

Escherichia coli

For the preparation of chemically competent TOP 10' cells 300 ml of LB medium were inoculated with 3 ml overnight culture and grown under agitation (140 rpm, Certomat R) at 37°C for about 4 h to reach an OD_{600} of 0.8. The flask was swirled in an ice bath for 2 min followed by a centrifugation step at 660 × g for 5 min at 4°C in ice cold centrifuge bottles. The pellet was resuspended in 30 ml of ice cold TSS (10 % polyethylene glycol 3,350, 5 % dimethylsulfoxid, 20 mM magnesium sulfate, in LB medium). Aliquots of 200 µl were frozen in liquid nitrogen and stored at -80°C until use.

For the transformation one 200 µl aliquot of competent cells was slowly thawed on ice. 100 µl of the cell suspension were added to an other tube containing 20 µl KCM (0.5 M potassium chloride, 0.15 M calcium chloride, 0.25 M magnesium chloride) and the ligation mixture in a total volume of 100 µl. The cell suspension was kept on ice for 20 min and afterwards exposed to 42°C for 2 min. 600 µl of LB medium were added and cells were allowed to recover for 40 to 60 min at 37°. Then, the cell suspension was centrifuged at 660 × g for 5 min. About 600 µl of the supernatant were removed, the pellet was resuspended in the remaining LB medium, and cells were plated onto LB agar plates containing the plasmid-dependent antibiotic. The plates were incubated at 37°C over night.

Agrobacterium tumefaciens and *Agrobacterium rhizogenes*

For the preparation of chemically competent *Agrobacterium* cells, 10 ml of strain-dependent medium (for details see chapter 2.2) containing the respective antibiotic (Table 2) were inoculated with a bacterial colony and were incubated overnight at 28°C under agitation (140 rpm, Certomat R). The following day 200 ml of the respective medium supplemented with the antibiotic were inoculated with the overnight culture and incubated at 28°C under agitation (140 rpm, Certomat R) until an OD_{600} of 0.5 was reached. The cells were harvested by centrifugation at 3,210 × g for 5 min and the pellet was resuspended in 100 ml of 0.15 M sodium chloride and centrifuged as described before. Then, the pellet was resuspended in 10 ml of 20 mM ice cold calcium chloride. Aliquots of 200 µl were frozen in liquid nitrogen and stored at -80°C until use.

For transformation one aliquot of competent cells was slowly thawed on ice for 1 to 1.5 h. 100 to 200 µg plasmid DNA were added, carefully mixed and kept on ice for a further 10 min. After freezing in liquid nitrogen, the mixture was exposed to a heat shock for 5 min at 37°C. 800 µl of strain-dependent medium were added and cells were allowed to recover for 3 to 4 h at 28°C under agitation (140 rpm, Certomat R). The cells were pelleted at 2,650 × g for 5 min and about 900 µl of the supernatant were removed, the pellet was resuspended in the remaining volume of medium for plating onto an agar plate containing the strain-dependent medium and antibiotic (Table 2) and the plasmid-dependent antibiotic. The plate was incubated at 28°C for three days.

Saccharomyces cerevisiae

Transformation of yeast cell was done according to Gietz and Woods (2002). For the transformation of yeast cells, 30 ml of YPD medium were inoculated with 100 µl of a stationary culture and incubated at 28°C under agitation (140 rpm, Certomat R) until a final OD_{600} of 0.6 to 0.8 was reached. The yeast suspension was cooled down on ice and centrifuged for 10 min at 1,550 × g and 4°C. The pellet was washed with water twice and centrifuged as described before. Then, the pellet was resuspended in 20 ml of TE/LiAc buffer (Tris(hydroxymethyl)aminomethane pH 7.5, 100 mM lithium acetate, 1 mM ethylenediaminetetraacetic acid pH 7.5) and centrifuged again. The pellet was resuspended in 150 to 300 µl of TE/LiAc buffer and kept on ice. 3.3 µl sheared and for 2 min at 99°C denatured fish sperm DNA (5 mg/ml) together with 1 µg plasmid DNA were added to 16.7 µl competent yeast cells. Moreover, 100 µl PEG/lithium acetate solution (10 mM Tris(hydroxymethyl)-aminomethane pH 7.5, 100 mM lithium acetate, 1 mM ethylenediaminetetraacetic acid, 100 mM polyethylene glycol 4,000) were added and the cell suspension was mixed thoroughly. After 20 min of incubation at 28°C a heat shock was given for 15 s at 42°C. The yeast suspension was plated onto YNB agar plates containing 100.9 mM glucose. Plates were incubated for three days at 28°C.

***Populus* species**

Stable transformation

About 7 mm long young stem and leaf stalks of two- to three-month-old *Populus* plants were partly cut lengthwise at their ends. At most 30 stalks were placed onto

one M1 plate (MS: 0.22 % Murashige & Skoog medium including vitamins, 58.4 mM sucrose, 1.4 mM L-glutamine, 2.1 µM D-panthothenate calcium, 0.4 µM biotin, 5.7 µM L-cysteine pH 5.9; 10 µM α-naphtalene acetic acid, 5 µM zeatin, 0.7 % plant agar). Plates were kept in the dark for up to 6 days at 26°C. For co-culture, an overnight culture of transformed *Agrobacterium tumefaciens* cells (strain C58 or AGL1) was prepared (for details see chapter 2.2). Poplar stalks were transferred into 100 ml Erlenmeyer flasks containing 20 ml liquid MS6 medium. The *Agrobacterium* suspension was added until a final OD_{600} of 0.3 was reached. The flasks were kept in the dark for 16 h at 26°C under agitation (130 rpm in circular motion, Edmund Bühler KL-2, Hechingen, Germany). After incubation all poplar stalks were retrieved, dried on paper and put back onto the M1 plate, which was then incubated in the dark for one day at 26°C. The following day, all poplar stalks were transferred into Erlenmeyer flasks and washed shortly three times with 10 ml water. For decontamination, they were agitated (120 rpm, RS-300 electronic, Born Gerätebau, Gladenbach, Germany) in 20 ml of a 41.6 µM tetracycline solution for 15 min, followed by one 10 min washing step and two short ones with bidestilled water (20 ml each). The poplar stalks were dried on paper and put onto M2 plates (MS supplemented with 1.18 mM carbenicillin, 0.52 mM cefotaxime, and 0.7 % plant agar). For callus generation the plates were incubated for ten days in the dark at 26°C. After ten days the poplar stalks were transferred onto new M2 plates for a further ten days and incubated as described before. For leaf and shoot generation the poplar stalks were put onto M3 plates and incubated at 22°C with a 12 h light/dark cycle. Illumination never exceeded 100 µmol photons m^{-2} s^{-1}. From that point on, growing calli were monthly transferred onto new M3 plates (MS supplemented with 1.18 mM carbenicillin, 0.52 mM cefotaxime, 0.1 µM thidiazuron, 0.08 mM kanamycin, and 0.7 % plant agar) until an adequate number of shoot tips were obtained. For root development the shoots were cut and placed onto MS6 medium containing 0.05 mM kanamycin. The plants were kept at 17°C with a 12 h light/dark cycle with a light intensity of about 100 µmol photons m^{-2} s^{-1}.

Composite plants

9 ml of the strain-dependent medium containing the corresponding antibiotics (for details see chapter 2.2) were inoculated with a single colony of transformed *Agrobacterium rhizogenes* K599 cells. After 24 h of incubation under agitation

(140 rpm, Certomat R) at 28°C cells were harvested by centrifugation at 3,000 × g for 20 min. The pellet was resuspended in the appropriate volume of activation medium according to Llave et al. (2000) (10 mM MES, 10 mM magnesium chloride, 1.5 mM acetosyringone dissolved in dimethyl sulfoxide, pH 5.6) for receiving an OD_{600} of one. After resuspension the cells were incubated for 2 h at 28°C under agitation (140 rpm, Certomat R). Shoots of *Populus tremula* × *P. tremuloides* were cut and dipped for 5 s in the activated bacterial solution. Then, the shoots were placed in standing petri dishes containing MS6 medium. The medium was changed into MS6 medium supplemented with 1.18 mM carbenicillin and 0.52 mM cefotaxime after three and six days after the dip. Since the *Populus* shoots generated small roots, they were placed onto a cellophane membrane on sugar free MMN plates (0.42 mM sodium chloride, 3.67 mM potassium dihydrogen phosphate, 1.89 mM di-ammonium hydrogen phosphate, 0.45 mM calcium chloride, 0.61 mM magnesium sulfate, 3.7 µM iron chloride, 65.93 mM potassium chloride, 100 mM boric acid, 20 mM manganese sulfate, 8 mM zinc sulfate, 3.13 mM copper sulfate, 0.58 mM ammonium heptamolybdate, 0.3 µM thiamine hydrochloride, 4.86 µM pyridoxine hydrochloride, 8.12 µM nicotinic acid, 0.55 mM myo-inositol, 1,8 % plant agar) according to Hampp et al. (1996) supplemented with 0.52 mM cefotaxime.

Antibiotics for selection

For selection of transformed bacteria and *Populus* plants, antibiotics were used. Transgenic bacteria were selected either by application of 0.27 mM ampicillin or 0.08 mM kanamycin to the growth medium whereas 0.05 mM kanamycin was added to growth media for selection of transgenic *Populus* plants.

2.4 Molecular biological techniques

2.4.1 Expression analyses by quantitative real-time polymerase chain reaction

2.4.1.1 RNA isolation using cesium chloride precipitation

At least 200 mg (fresh weight) roots of composite plants were ground in a mortar with pestle under liquid nitrogen until a fine tissue powder was obtained. 3 ml of RNA extraction buffer (4 M guanidinium isothiocyanate, 1 % polyvinylpyrrolidone, 25 mM sodium acetate pH 6.8, 86 mM β-mercaptoethanol (addition directly before use)) were added. During slowly thawing powder and extraction buffer were homogenized

thoroughly. The extract was filled into a 1.5 ml reaction tube, which was then centrifuged at 20,810 × g for 20 min. The RNA containing supernatant was transferred into an ultra centrifuge tube filled with 3 ml of caesium chloride solution (5.7 M caesium chloride, 25 mM sodium acetate pH 6.8). After centrifugation for at least 20 h at 166,000 × g (Beckman Coulter Optima™ LE-80K Ultracentrifuge; Global Medical Instrumentation Inc., Minnesota, USA) the pellet was dissolved in 150 µl diethylpyrocarbonate treated autoclaved water (addition of 6.91 mM diethylpyro-carbonate to water, incubation over night) and transferred into a 1.5 ml reaction tube. Afterwards, 15 µl of 3 M sodium acetate pH 6, diethylpyrocarbonate treated, were added. The solution was mixed thoroughly before 300 µl of 100 % ethanol were added. After incubation at room temperature for 20 min, the reaction tube was centrifuged for 60 min at 20,810 × g. The pellet was washed with 1 ml 80 % ethanol (diluted with diethylpyrocarbonate treated water). The tube was centrifuged for 10 min as described before. After the pellet was air-dried, it was dissolved in 30-50 µl diethylpyrocarbonate treated water and incubated for 10 min at 50°C. The RNA solution containing tubes were frozen in liquid nitrogen and stored at -80°C.

2.4.1.2 First-strand cDNA synthesis

First strand c-DNA synthesis for amplification of the coding sequence of *sucrose transporter* genes of *Populus trichocarpa* × *P. deltoides* and for expression analyses

For the amplification of the coding sequences of *sucrose transporters* RNA from main and fine roots of *Populus trichocarpa* × *P. deltoides* were used for first strand c-DNA synthesis. For expression analysis of *sucrose transporter* genes RNA from dissected *Populus tremula* × *P. tremuloides* mycorrhizal fine roots were used. The fungal partner was *Amanita muscaria*, strain ME2. RNA from dissected mycorrhizal *Populus tremula* × *P. tremuloides* fine roots (0.2 to 0.3 mm) was extracted.

First strand c-DNA synthesis for expression analysis of the β-glucuronidase encoding *GUS* gene, and *nptII* gene in *Populus tremula* L. × *P. tremuloides* Michx. roots

RNA, isolated by caesium chloride precipitation, was treated with DNase for the destruction of remained DNA. Therefore, 1 µg RNA, 1 µl of 10 × reaction buffer

containing magnesium chloride, 1 µl RiboLock RNase Inhibitor (Thermo Scientific, St. Leon-Rot, Germany) and 1 u peqGOLD DNase I were pipetted into a 1.5 ml reaction tube. The total volume was adjusted to 10 µl with diethylpyrocarbonate treated water and all was mixed carefully. A 15 min incubation step at 20°C followed. Before another incubation of 10 min at 65°C, 1 µl of 50 mM ethylenediaminetetraacetic acid pH 8 was added. The reaction tube was placed on ice. After cooling down 100 ng oligo(dT) primer, 4.75 µl DNase treated RNA, and diethylpyrocarbonate treated water for receiving a final volume of 20 µl were added. The sample was incubated for 5 min at 75°C before the tube was placed again on ice. DNTP mix (final concentration 0.5 mM), 1 µl of RiboLock RNase Inhibitor, 4 µl of 5 × reverse transcriptase buffer, and 1 µl of RevertAid Premium Reverse Transcriptase (Thermo Scientific) were added to the sample. After incubation steps for 10 min at 25°C and 30 min at 50°C, 30 µl 5 mM Tris(hydroxymethyl)aminomethane pH 8 were added. Aliquots of 10 µl were frozen in liquid nitrogen and stored at -80°C.

2.4.1.3 Quantitative real-time polymerase chain reaction (RT-PCR)

Gene expression analyses of the putative sucrose transporters (*PtSUT1*, *PtSUT3*, *PtSUT4*, *PtSUT5*, *PtSUT6*) and *ubiquitin of Populus trichocarpa* × *P. deltoides* mycorrhizal fine roots were performed by RT-PCR using a MyiQ System (Version 1.0.410). *GUS* gene expression under control of the peroxidase promoter or the respective fragments was analyzed using the LightCycler® 480 RT-PCR System (Version 1.5.0). RT-PCRs were carried out using 0.5 µl or 5 µl first strand cDNA, 0.1 µl of each gene-specific primer (100 µM), 10 µl SYBR Green Mix (Thermo Scientific) according to the manufacturer's instructions and water (final volume 20 µl). The quantitative RT-PCR protocols are listed in Table 3 and Table 4. Primer names, their sequences and their annealing temperatures are listed in Table 19 of the appendix.

Table 3: Temperature [°C], time [s], number of cycles, and ramp rates [°C/s] of RT-PCR steps for expression analyses of the putative sucrose transporter genes *PtSUT1*, *PtSUT3*, *PtSUT4*, *PtSUT5*, *PtSUT6*, and *ubiquitin* of *Populus trichocarpa* × *P. deltoides* are listed.

Step	Temperature [°C]	Time [s]	Cycles	Ramp rate [°C/s]
Initial denaturation	95	900	1	3.3
Amplification	95	10	42	3.3
	50	30		2.0
	72	25		3.3
	80	10		3.3
Melting curve	95	60	1	3.3
	60	60	1	2.0
	60	10	80	0.5

Table 4: Temperature [°C], time [s], number of cycles, and ramp rates [°C/s] of RT-PCR steps for expression analyses of the *GUS* gene (driven by the peroxidase promoter or the respective fragments), *nptII* gene, and *ubiquitin* gene of *Populus trichocarpa* × *P. deltoides* composite plant roots are listed.

Step	Temperature [°C]	Time [s]	Cycles	Ramp rate [°C/s]
Initial denaturation	95	900	1	4.4
Amplification	95	10	42	4.4
	54	5		2.2
	72	30		4.4
	82	2		4.4
Melting curve	95	5	1	4.4
	60	30	1	2.2
	95	-	1	0.11

2.4.2 Isolation of plasmid DNA from *E. coli* cells

2 ml of overnight culture were taken for plasmid preparation. The cell suspension was centrifuged for 5 min at 10,620 × g. The pellet was resuspended in 200 µl of solution one (50 mM Tris(hydroxymethyl)aminomethane pH 7.5, 10 mM ethylenediamine-tetraacetic acid, 0.1 % RNAse A). 200 µl of solution two (200 mM sodium hydroxide, 35 mM sodium dodecyl sulfate) were added and the reaction tube was inverted eight times. After an incubation of a maximum of 5 min, 200 µl of solution three (1.5 M potassium acetate pH 4.8) were added and the tube was inverted again eight times. Then, the tube was centrifuged for 10 min at 17,950 × g and 750 µl ice cold 100 % isopropanol were added to the supernatant. The tube was stored at -20°C for 10 min and a centrifugation step at 17,950 × g and 4°C for 20 min followed. The pellet was washed with 500 µl ethanol (70 %). After another centrifugation step

of 5 min at 8,600 × g the pellet was air-dried and dissolved in 35 µl of 5 mM Tris(hydroxymethyl)aminomethane pH 8 and stored at -20°C.

2.4.3 Restriction analyses

All restriction endonucleases, which were used for the construction and analysis of inserts and vectors, were provided either by Thermo Scientific or New England Biolabs (Ipswich, Massachusetts, USA).

Ethanol precipitation

If DNA had to be digested with several endonucleases, requiring different buffer properties, the DNA was precipitated between the restriction digestion treatments. 1/10 volume of 3 M sodium acetate pH 5.2 and two volumes of 100 % ethanol were added to the sample, followed by a thorough mixture. After an incubation of at least 20 min at -20°C a centrifugation step followed at 20,810 × g for 15 min at 4°C. The pellet was washed with 100 µl of 70 % ethanol. Another centrifugation step for 5 min followed as described before. The pellet was air-dried and dissolved in the respective volume of water needed for the following restriction digestion.

Analytical restriction digestion

Analyses of isolated plasmid DNA of *E. coli* cells were carried out by the use of restriction endonucleases. Presence and orientation of inserts were determined by this method. For analytical restriction digestion 1 µl of the plasmid DNA preparation sample, at least 5 units of the appointed restriction endonuclease, 2 µl of the corresponding endonuclease buffer, and 16.5 µl water were mixed, followed by at least 3 h of incubation at 37°C, if the endonuclease did not require a different temperature. If a double digestion by two restriction endonucleases was performed, 4 units of each endonuclease were used and the volume of water was reduced accordingly. DNA fragments were separated by gel electrophoresis.

Preparative restriction digestion

For a preparative restriction digestion, 1.5 µg plasmid DNA was used in a mixture with 1.5 µl of the appointed restriction endonuclease, 4 µl of the corresponding endonuclease buffer, and water for a total volume of 40 µl. After incubation of at least 3 h at 37°C, if the endonuclease did not require a different temperature.

2.4.4 Gel electrophoresis, excision and isolation of DNA fragments out of the gel

The separation of DNA fragments was done by gel electrophoresis. Depending on the fragment length 1 % (longer than 500 bp) or 2 % (shorter than 500 bp) agarose (SeaKem agarose for analytic gels; Topvision agarose for preparative gels) were dissolved in 0.5 × TAE buffer (40 mM Tris(hydroxymethyl)aminomethane, 20 mM acetic acid, 1 mM ethylenediaminetetraacetic acid). For determination of DNA fragment length the following marker were used. Lambda DNA digested with the restriction endonuclease *Eco*32I (Thermo Scientific) and the GeneRuler 100 bp DNA Ladder (Thermo Scientific) served as markers. Visualization of the DNA fragments was performed after incubation in a 0.2 ‰ ethidium bromide solution for 15 min by a UV light illuminator (312 nm). Referring to preparative gels DNA fragments of the desired length were excised out of the agarose gel with a clean scalpel under UV light. For elution of DNA the NucleoSpin II gel extraction kit (Macherey & Nagel, Düren, Germany) was used following the manufacturer's instructions.

2.4.5 Ligation

Ligation of digested DNA backbones and inserts was performed in a ratio 1:3.

Sticky end ligation

For sticky end ligation 1 µl T4 DNA ligase (Thermo Scientific), 2 µl of the corresponding ligation buffer, variable volumes of vector backbone and insert, and water (final volume 20 µl) were mixed. Sticky end ligation mixture was incubated at 15°C over night.

Blunt end ligation

Blunt end PCR products, which were generated by the Phusion® High Fidelity DNA Polymerase were ligated into the pJET1.2/blunt entry vector (50 ng/µl) of the CloneJET™ PCR Cloning Kit (Thermo Scientific). For blunt end ligation 1 µl T4 DNA ligase (Thermo Scientific). 2 µl of the appropriate ligation buffer and variable volumes of vector backbone and insert were mixed with water (final volume 20 µl). Blunt end ligation mixture was incubated at 22°C for 4 h.

2.4.6 Sequencing analysis

A PCR reaction was carried out using the Big Dye polymerase (Invitrogen, Groningen, Netherlands) and the chain-terminating nucleotides ddNTPs (Sanger et al., 1977). The composition of the PCR mixture and the PCR protocol are listed in Table 5 and Table 6. Vector dependent primers were used, which are listed in Table 7. PCR products and 10 µl of water were refined by Sephadex G-50 (swollen for 3 h in 300 µl water, centrifuged for 5 min at 910 × g, washed with 150 µl water, centrifuged for 5 min at 910 × g) in a centrifugation step (5 min at 910 × g). Fragment analysis was performed by a capillary sequencer (Applied Biosystems 3130xl Genetic Analyzer, Darmstadt, Germany).

Table 5: Composition of the 5 µl PCR mixture for one sequencing reaction.

Component	Volume
2.5 × Big Dye buffer	1.2 µl
Big Dye polymerase	0.8 µl
Primer (10 µM)	1.0 µl
Template	200 - 500 ng
Nuclease free water	variable

Table 6: Protocol for the sequencing PCR. The annealing temperatures (T_A) are listed in Table 7.

Step	Temperature [°C]	Time [s]	Cycles
Initial denaturation	96	20	1
Amplification	96	10	59
	T_A (Table 7)	5	
	60	240	

Table 7: Primer names, combinations, their sequences, and annealing temperatures (T_A), used for the sequencing reaction. Sequences are given in 5`→3` direction.

Vector name	Primer name	Primer sequence 5‘→3‘	Annealing temperature [°C]
pJET1.2	pJET1.2_fwd pJET1.2_rev	CGACTCACTATAGGGAGAGCGGC AAGAACATCGATTTTCCATGGCAG	55.0
pBI121	pBI121_for pBI121s_rev	CGGATATTTTCGTGGAGTTCC GCATCGAAACGCAGCACG	58.0
pDR196	M13-seq	GTAAAACGACGGCCAGTG	54.5

2.4.7 Polymerase chain reaction (PCR)

All PCRs were carried out in the Biometra TGradient Thermocycler (Biometra GmbH, Göttingen, Germany) using template DNA, target-DNA-specific primers, and the proof reading Phusion® High Fidelity DNA Polymerase (Thermo Scientific) according to the manufacturer's instructions. For each PCR reaction 4 µl of 5 × Phusion® reaction buffer, 0.16 µl dNTP mix (10 mM each), 0.1 µl of each primer (100 µM), 0.2 µl of Phusion® DNA polymerase, 1 µl cDNA as template, and 14.4 µl water were mixed thoroughly. The PCR protocol is listed in Table 8. Primer names, their sequences and their annealing temperatures are listed in Table 18 of the appendix.

Table 8: PCR protocol for the amplification of target DNA. Primer properties including the annealing temperatures (T_A) are listed in Table 18 of the appendix.

Step	Temperature [°C]	Time [s]	Cycles
Initial denaturation	98	30	1
Amplification	98	10	35
	T_A	30	
	72	15-30/kb	
Final elongation	72	600	1

2.5 Fluorometric GUS assay

2.5.1 Isolation of a protein containing extract

Isolation of a protein extract from *E. coli* cells

25 ml of LB-medium were inoculated with a single colony of *E. coli* cells harboring the vector pUCgusA2 containing a *GUS* gene, which were kindly provided by Dr. Andrea Krause (University of Bremen, Germany). The *GUS* gene expression was under control of the lac promoter. A *GUS* expression had to be induced by the addition of isopropyl β-D-thiogalactoside with an end concentration of 2 mM 6 h before cell harvest. The cell suspension was centrifuged for 5 min at 10,620 × g and the pellet was resuspended in 10 ml of GUS extraction buffer (100 mM sodium dihydrogen phosphate, 1 mM dithiothreitol). Afterwards, the cell suspension was applied to the 4°C pre-cooled 10 ml French Press cell (American Instrument Company, Silver Springs, USA). A pressure of 8,000 psi (about 55 MPa) was applied. After decompression non-disrupted cells and cell debris were pelletized by centrifugation for 20 min at 20,810 × g. The supernatant was stored at -80°C.

Isolation of a protein extract from composite plant roots

At least 150 mg of composite plant root tissue were taken for grinding with mortar and pestle under liquid nitrogen. After receiving a fine tissue powder 500 µl of GUS extraction buffer were added. The thawed extract was transferred into a 2.0 ml reaction tube. For cell disruption the extract was treated with an ultrasonic probe (Branson Sonifier, microtip, output control four) twice for 5 s each. Between the treatments the tube was cooled down on ice. After a centrifugation step at 20,810 × g for 20 min the supernatant was frozen in liquid nitrogen and stored at -80°C.

2.5.2 Protein determination by a modified Bradford protein assay

Determination of the protein content was done using a micro-approach according to Mauer (2010). Defined volumes of a 1 µg/ml albumin solution from bovine serum (standard curve for calibration) and defined volumes of protein extract were added to the wells of a microtitre plate separately. 80 µl of Bradford Reagent (60.5 µM Coomassie Brilliant Blue G-250 dissolved in 10 ml 100% ethanol, 175 ml 85 % phosphoric acid, 815 ml water) and water (final total volume 200 µl) were added to each well. Without any prior incubation the absorbance was measured at 595 nm in the BioTek Ultra Microplate Reader (Bad Friedrichshall, Germany).

2.5.3 Fluorometric determination of the β-glucuronidase activity

To determine the peroxidase promoter strength quantitatively the activity of the β-glucuronidase was measured by the fluorescence of into 4-methylumbelliferon transferred 4-methylumbelliferyl-β-d-glucuronide dihydrate in a Thermo Scientific Fluoroskan Ascent FL using a black microtitre plate (Greiner Bio-One, Frickenhausen, Germany). A defined extract volume was filled up with the corresponding amount of GUS extraction buffer (final total volume 100 µl). The reaction was started by addition of 200 µl GUS reaction buffer (50 mM sodium phosphate buffer pH 7.4, 10 mM ethylenediaminetetraacetic acid, 0.1 % Triton X-100, 0.4 % *N*-Lauroylsarcosine, 0.85 mM-methylumbelliferyl-β-d-glucuronide dihydrate, 100 mM β-mercaptoethanol). The program used for measuring the kinetics is listed in Table 9. Measurements were done with an excitation of 355 nm and emission of 460 nm at 30.1°C.

Table 9: The program used for kinetic determination of the ß-glucuronidase in the fluorometer (excitation 355 nm; emission 460 nm).

Step	Cycle description
1	Incubation at 30,1°C for 5 min
2	Background measurement
3	Addition of 200 µl GUS reaction buffer/well
4	Shaking at 240 rpm for 10 s
5	120 measurements: kinetic interval of 30 s

2.5.6 Statistical Analyses

Statistical calculations were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software (La Jolla California, USA). To determine a normal distribution of a dataset the Shapiro-Wilk normality test, *P*-value < 0.05, was performed (Sachs and Hedderich, 2009). If a normal distribution could be determined an unpaired two-tailed parametric t-test with Welch's correction, *P*-value < 0.05, was performed for significance analysis (Sheskin, 2004). If the datasets were not normally distributed the unpaired non-parametric Mann-Whitney test, *P*-value < 0.05, was accomplished (Sheskin, 2004). The significance can be subdivided dependent on the degree of significance: *P*-values ranging from < 0.05 to 0.01 are described as significant. *P*-values ranging from 0.01 to 0.001 are described as very significant. *P*-values ranging from 0.001 to < 0.0001 are described as extremely significant.

3 Results

3.1 Analyses of putative *sucrose transporters* in the *Populus trichocarpa* genome

3.1.1 Identification and amplification of putative *SUT* open reading frames

This part of the work was aimed to characterize sucrose transporters as putative sugar efflux carriers in the ectomycorrhizal symbiosis. Therefore, the *Populus trichocarpa* genome (Grigoriev et al., 2012) was screened for genes encoding proteins with a respective function. The investigation of the *SUT* gene family in this work started before Payyavula et al. (2011) had published their results on the characterization of the *SUT* gene family. To avoid any confusion, the nomenclature of Payyavula et al. (2011) was taken in this work. Five *SUT* genes could be identified in the *Populus trichocarpa* genome on the basis of homology search using BlastX (Altschul et al., 1990) within the JGI *Populus* genome database v2.2 (Nehls, unpublished): *PtSUT1*, *PtSUT3*, *PtSUT4*, *PtSUT5*, and *PtSUT6*. Primers designed for the amplification of the open reading frames (ORFs) of all five *SUT* genes are listed in Table 18 of the appendix. PCR amplification of the coding regions was performed from first strand cDNA of *Populus trichocarpa* × *P. deltoides* RNA obtained from roots using a proof-reading *Taq* polymerase. DNA fragments were obtained for all *SUT* genes except for *PtSUT3*. The DNA fragments were cloned into the pJET1.2 entry vector and checked for proper amplification by sequencing analysis. When comparing the *PtSUT* sequences obtained from *Populus trichocarpa* × *P. deltoides* to those predicted in the *Populus trichocarpa* genome, a number of nucleotide exchanges were observed. Since the observed changes in the nucleotide sequences of *SUT* ORFs occurred in the DNA sequence of several independent clones, they were assumed as species-dependent differences between *Populus trichocarpa* and *Populus deltoides*. However, only some of them led to amino acid changes (Table 10). The obtained sequence of *PtSUT1* and *PtSUT4* revealed 11 and 13 nucleotide exchanges compared to the *Populus trichocarpa* genome resulting in four and five amino acid changes, respectively. Furthermore, the deduced protein sequences of *PtSUT5* and *PtSUT6* were eight and two amino acids, respectively, shorter than those predicted according to the *Populus trichocarpa* genome. However, neither the gap in the *PtSUT5* sequence nor the missing nucleotides in the *PtSUT6* sequence led to a frame shift.

Table 10: Properties of the obtained *SUT* sequences of *Populus trichocarpa* × *P. deltoides*. Comparison was done to putative *SUT* sequences derived from the JGI *Populus* genome v2.2 gene model (Grigoriev et al., 2012). Abbreviations: n.t. – nucleotide, a.a. – amino acid.

	% Similarity		Number of changes		Amino acid change	
Gene	**n.t.**	**a.a.**	**n.t.**	**a.a.**	**position**	**replacement**
SUT1	99.3	99.3	11	4	101	R → S
					344	H → R
					527	P → A
					531	S → T
SUT4	99.2	99.0	13	5	11	A → P
					26	Q → H
					94	M → I
					215	V → I
					388	S → Y
SUT5	98.6	98.7	2	8	88 - 95	truncation
SUT6	99.0	98.7	18	7	92	truncation
					94	P → L
					95	S → G
					96	S → I
					97	R → G
					241	truncation
					481	A → E

3.1.2 Construction of yeast expression vectors harboring *SUT*s of *Populus*

For heterologous expression in yeast, the open reading frames of all four obtained *SUT* genes were cloned into the yeast expression vector pDR196 (Rentsch et al., 1995; Fig. 5).

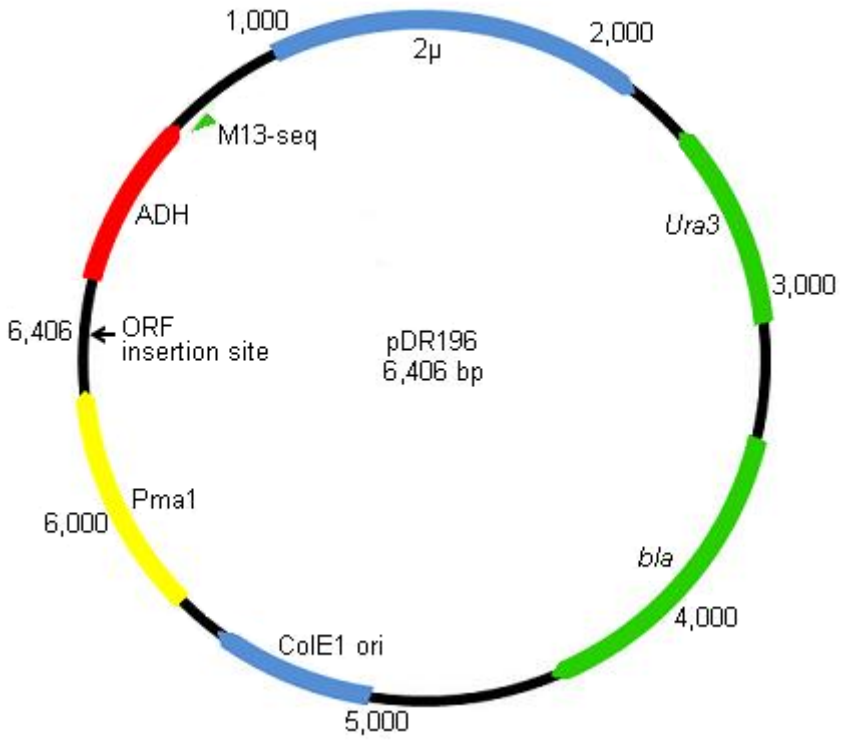

Fig. 5: Map of the yeast expression vector pDR196 (Rentsch et al., 1995). The vector possesses two replicons: 2µ and ColE1 ori (blue). It features an *Ura3* cassette for complementation of the uracil biosynthesis in *Saccharomyces cerevisiae*, and the ampicillin resistance gene *bla* (each in green). Insertion site of the ORFs (arrow) was between the strong constitutive plasma membrane ATPase promoter Pma1 (yellow) and the alcohol dehydrogenase terminator ADH (red). The primer M13-seq, which was used for sequencing, is indicated.

For the insertion of *PtSUT1*, the pDR196 vector was linearized by *Bam*HI digestion. The coding region of *PtSUT1* was released from entry vector pJET1.2 by *Bgl*II digestion. *Bam*HI and *Bgl*II generate compatible overhangs, enabling the integration of *PtSUT1* into the pDR196 by sticky end ligation (Fig. 6, right side).

Due to restriction recognition sites within the coding regions of the remaining *SUT*s, a different strategy was followed. Their open reading frames were again PCR amplified (Fig. 6, left side) using primers integrating restriction enzyme digestion sites (forward primer: *Xma*I, reverse primer: *Xho*I). Their sequences are listed in Table 18 of the appendix. The PCR products were inserted into the pJET1.2 entry vector. The *SUT* coding regions were released by an *Xma*I and *Xho*I double digestion and were ligated into the pDR196 that was digested with the same restriction enzymes before. The successful insertion of *SUT* coding regions into pDR196 was checked by sequencing analysis.

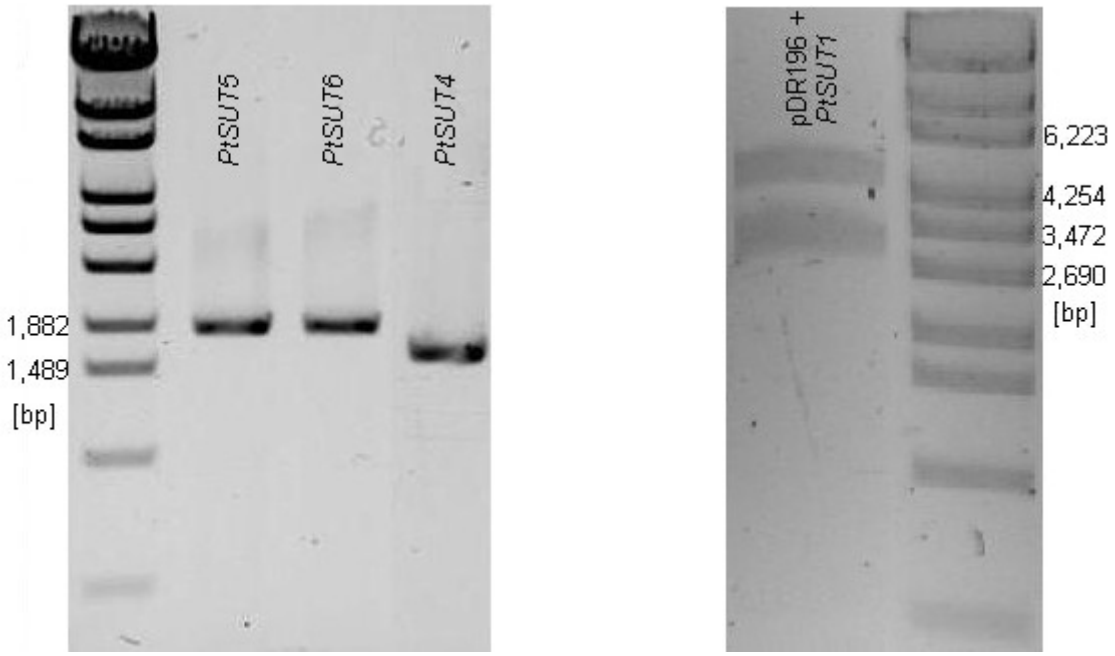

Fig. 6: Gel images of left side) PCR-amplification of *PtSUT4*, *PtSUT5*, and *PtSUT6* using restriction site inserting primers and a proof reading *Taq* polymerase and right side) restriction digestion of the yeast expression vector pDR196 harboring *PtSUT1* with *Bsu*15I. Desired fragment lengths [bp] after amplification: *PtSUT4* – 1,536, *PtSUT5* – 1,814, *PtSUT6* – 1,823; and after restriction digestion of pJET1.2 + *PtSUT1*: 3,652 + 4,312.

3.1.3 Heterologous expression of *SUT*s and *SWEET*s in *Saccharomyces cerevisiae*

The yeast strain Susy7/ura3 (Riesmeier et al., 1992) cannot utilize external applied sucrose unless the expression of a functional foreign sucrose importer. This strain is furthermore deficient in uptake of maltose. Therefore, Susy7/ura3 is a good tool to analyze the capability of transport proteins to import any of these disaccharides.

In 2011 growth test with Susy7/ura3 cells incorporating a yeast expression vector that harbored the ORFs of *SUT3*, *SUT4*, and *SUT5* were published (Payyavula et al., 2011). Due to this publication only *PtSUT1* and the empty vector pDR196 (negative control) was used for *Saccharomyces* transformation in this work. Viability was proven by yeast growth on medium supplemented with glucose. However, heterologous expression of the investigated *PtSUT1* ORF did not enable Susy7/ura3 cells to grow on medium supplemented with sucrose as sole carbon source (Fig. 7).

Fig. 7: Growth of *Saccharomyces cerevisiae* Susy7/ura3 cells transformed with the yeast expression vector pDR196 harboring the *PtSUT1* ORF on selected carbon sources. Transgenic yeast cultures were pre-grown overnight in liquid YNB medium containing glucose as carbon source. Cultures were washed with water twice to remove remaining sugar, calibrated to an OD_{600} of one and streaked onto YNB agar plates supplemented with either 101 mM glucose or 58 mM sucrose. The plates were incubated at 28°C for five days.

In his Master thesis Nintemann (University of Bremen, Germany) investigated the role of a novel class of putative *Populus* sugar facilitators, called SWEETs, in glucose transport. The *SWEET* gene family has been initially discovered in *Arabidopsis* (Chen et al., 2010; Chen et al., 2012). To examine if these transporters were capable in using sucrose and/or maltose in addition to glucose, four pDR196-SWEET expression constructs (SWEET7350, SWEET7360, SWEET13410, and SWEET 36550; Nintemann, 2012), were used for Susy7/ura3 transformation (this work). The resulting transformants were investigated for complementation of sucrose and maltose import deficiency by growth tests on agar plates (Fig. 8). Yeast growth on plates supplemented with glucose was used as viability control (Fig. 8 a). Yeast cells transformed with the empty pDR196 vector served as negative control. Since VW4000 yeast cells (Wieczorke et al., 1999) contain a functional maltose importer they served as positive control (Fig. 8 c).

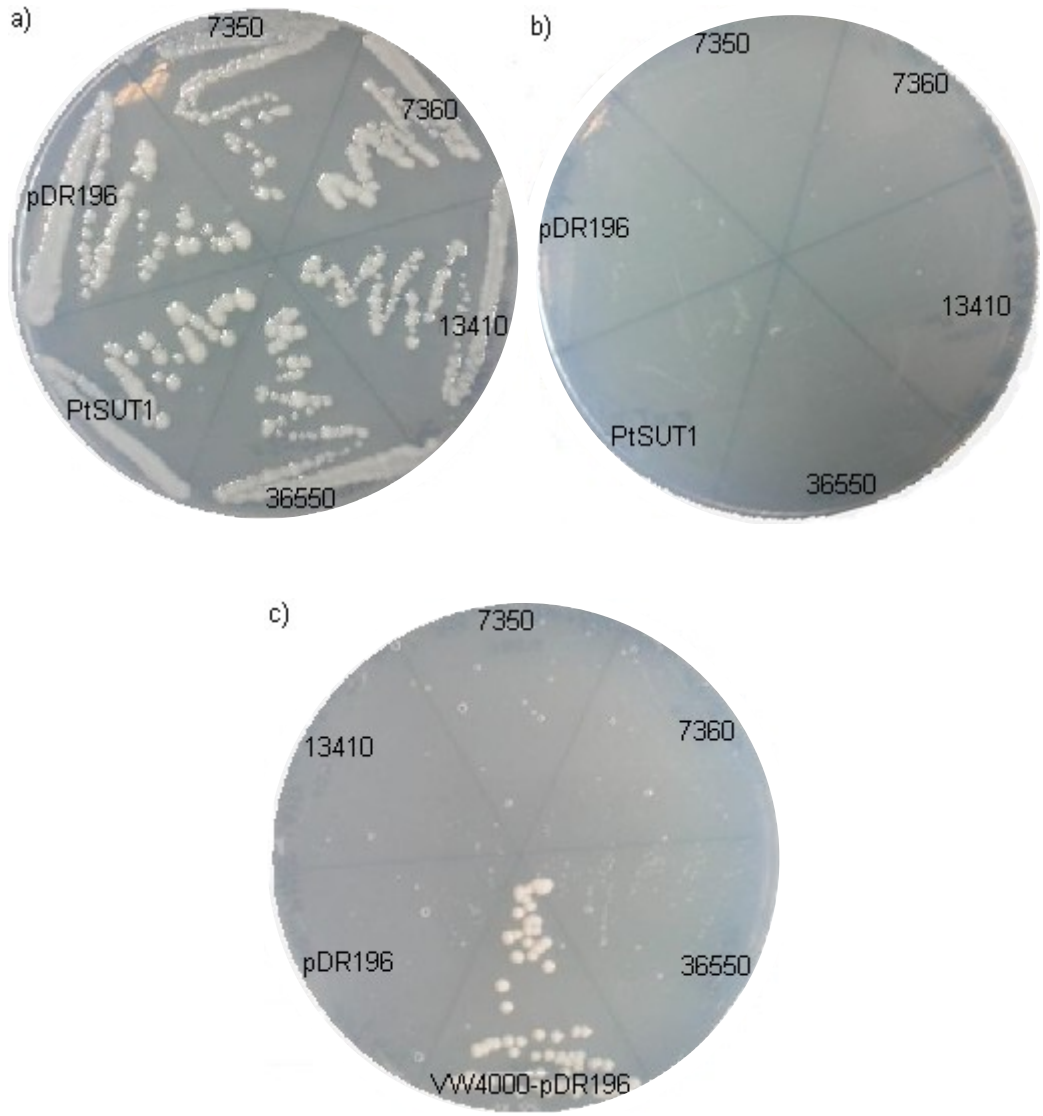

Fig. 8: Growth of transgenic *Saccharomyces cerevisiae* Susy7/ura3 cells on selected carbon sources. Transgenic yeast cultures were pre-grown overnight in liquid YNB medium containing glucose as carbon source. Cultures were washed with water twice to remove remaining sugar, calibrated to an OD_{600} of one and streaked onto YNB agar plates supplemented with a) 101 mM glucose, b) 58 mM sucrose, and c) 55.5 mM maltose. The plates were incubated at 28°C for five days. 7350, 7360, 13410, 36550 represent Susy/ura3 cells transformed with pDR196-SWEET expression constructs, pDR196 represents Susy7/ura3 cells transformed with the empty vector, VW4000-pDR196 represents VW4000 cells transformed with the empty vector pDR196.

All transgenic Susy7/ura3 cells were able to grow on glucose confirming viability (Fig. 8 a). However, heterologous expression of none of the investigated *SWEET* ORFs enabled yeast growth on either sucrose (Fig. 8 b) or maltose (Fig. 8 c) as sole carbon source.

3.1.4 Expression analysis of *SUT* genes

Previous expression analysis revealed reduced transcript levels of all *SUT* genes except of *PtSUT4* upon ectomycorrhiza formation (Nehls, unpublished). As the vacuolar localization of PtSUT4 (Payyavula et al., 2011) hadn't been discovered yet, PtSUT4 was a good candidate for the questioned plant root sucrose efflux carrier in the ectomycorrhizal symbiosis. Sucrose efflux is postulated only for the plant/fungal interface of functional ectomycorrhizas (Nehls et al., 2010). As mycorrhizas had been dissected from the tip towards the base of infected fine roots and total RNA was available (Nehls, unpublished), first strand cDNA of the different sections, kindly provided by Annette Hintelmann (University of Bremen, Germany), was used for quantitative RT-PCR based expression analyses of the different *SUT* genes. The used primers (provided by Prof. Nehls) are listed in Table 19 of the appendix.

mRNA content of the *SUT* genes was calculated relatively to that of *ubiquitin* according to Göhringer (2007) and was calibrated to the lowest ratio resulting in an expression rate of 1. This way of calculation enabled the comparison of gene expression rates in different samples obtained by one primer pair.

Gene expression analyses revealed presence of PtSUT3 and PtSUT4 transcripts in all investigated sections of the ectomycorrhizal fine root while transcript levels of the other investigated *SUT* genes were below the detection limit. The highest transcript abundance of *PtSUT3* (Fig. 9 a) was detected in the root tip and was at least three fold as high as in all other sections. The highest transcript abundance of *PtSUT4* (Fig. 9 b) was also detected in the root tip and section 1 (expansion zone) and declined progressively towards the root base.

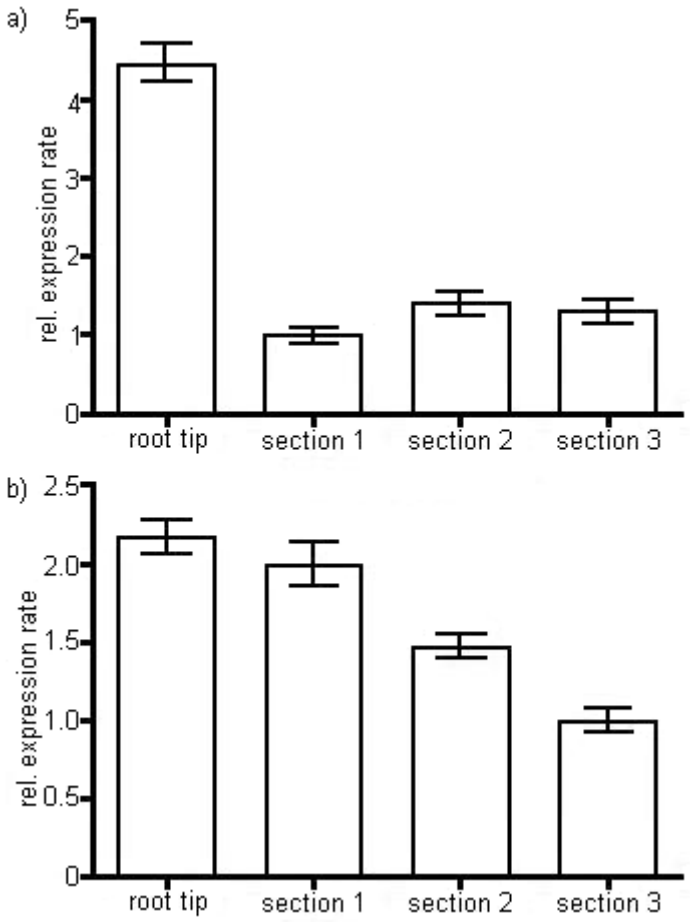

Fig. 9: Expression rates of a) *PtSUT3* and b) *PtSUT4* in sections of a dissected ectomycorrhizal fine root. Mycorrhizas were dissected from the root tip towards the base in 0.2 to 0.3 mm long sections and total RNA was isolated from pooled material of distinct sections (Nehls, unpublished). First strand cDNA (provided by Annette Hintelmann) was used for gene expression analysis. Expression rates were calculated relatively to *ubiquitin* expression according to Göhringer (2007). The number of technical replicates for each gene was n = 7.

Since a non-parametric test has less power compared to parametric tests, it is always advisable to check the distribution of the datasets. The Shapiro-Wilk normality test (Sachs and Hedderich, 2009) was used to determine a normal distribution of the datasets. For this test the expression rates of each section regarding *PtSUT3* and *PtSUT4* were separately examined. Normal distribution was accepted when $P > 0.05$. The Shapiro-Wilk test revealed P-values > 0.05 in all tests. This proved the normal distribution of all datasets. The P-values of the Shapiro-Wilk test are listed in Table 11.

Table 11: *P*-values of the performed Shapiro-Wilk normality test. The *P*-values resulted from expression rates of *PtSUT3* and *PtSUT4* in sections of a dissected ectomycorrhizal fine root. The number of technical replicates for each gene was n = 7.

	SUT3	***SUT4***
Root tip	0.285	0.678
Section 1	0.418	0.578
Section 2	0.594	0.528
Section 3	0.558	0.674

To check the expression rate differences statistically, an unpaired two-tailed t-test with Welch's correction was chosen due to normal distributions of the datasets and unequal standard deviations (Sheskin, 2004). A significant difference was accepted when $P < 0.05$.

A very significant difference concerning *PtSUT3* was revealed between the expression rates of the root tip and section 1 ($P = 0.002$) as well as between the root tip and section 3 ($P = 0.009$). In relation to *PtSUT4* statistical significant differences occurred between the root tip and section 3 ($P = 0.023$) as well as between section 1 and section 3 ($P = 0.04$). The *P*-values of the t-tests are listed in Table 12.

Table 12: Table of *P*-values of the performed unpaired two-tailed t-test with Welch's correction for a) *PtSUT3* and b) *PtSUT4*. The *P*-values resulted from expression rates of *PtSUT3* and *PtSUT4* in sections of a dissected ectomycorrhizal fine root. The number of technical replicates for each gene was n = 7. Green colored boxes indicate *P*-values < 0.05 whereas red ones indicate *P*-values > 0.05.

a)

	Root tip	**Section 1**	**Section 2**	**Section 3**
Root tip		0.002	0.080	0.009
Section 1	0.002		0.243	0.600
Section 2	0.080	0.243		0.488
Section 3	0.009	0.600	0.488	

b)

	Root tip	**Section 1**	**Section 2**	**Section 3**
Root tip		0.975	0.210	0.023
Section 1	0.975		0.360	0.040
Section 2	0.210	0.360		0.074
Section 3	0.023	0.040	0.074	

3.2 Promoter analysis of a *Populus trichocarpa* gene (*Potri.003G214500*), coding for a putative peroxidase

3.2.1 *In silico* analysis of the peroxidase promoter

The poplar gene *Potri.003G214500* encodes a putative peroxidase. A promoter fragment with a length of 1622 bp was amplified using a proof reading *Taq* polymerase and was ligated into the pJET1.2 entry vector. It was kindly provided by Jennifer Krützmann (for details see Krützmann (2010)). An *in silico* analysis of the promoter region of *Potri.003G214500* was performed using NSITE-PL (http://linux1.softberry.com/berry.phtml, functional motifs of plants were selected from the RegSite Database, developed by Softberry) and the MatInspector tool (http://www.genomatix.de/, Catharius et al., 2005) The NSITE-PL analysis indicated eight putative regulatory *cis* elements that are listed in Table 13 and outlined in Fig. 10. All transcription factors that are known to bind to these predicted regulatory elements are encoded in the *Populus trichocarpa* genome. Their DNA sequences can be found using the poplar genome database (www.phytozome.net). One of the predicted *cis* elements was recognized as putative Alfin1 binding site. In *Medicago sativa L*. Alfin1 enables root-specific gene expression (Winicov, 2000), putatively explaining the root-specific expression of *Potri.003G214500*. Additionally, both, the NSITE-PL and MatInspector analyses predicted the TATA-box from base -491 to -477. To this box the TATA-box binding protein attaches as requirement for the formation of the transcription initiation complex. Furthermore, the MatInspector tool predicts two CAAT-boxes from -1,380 to -1,372 and -1,228 to -1,220. Neither the NSIT-PL software nor the MatInspector tool predicted a putative TSS in the peroxidase promoter region.

Table 13: Results of the *in silico* analysis of the peroxidase promoter using NSITE-PL (http://linux1.softberry.com/berry.phtml). Small letters in the sequence indicate mismatches.

Accession number	Organism	Regulatory element	Binding factor	Position	Sequence 5'→3'	Number of mismatches
RSP00472	*Medicago sativa*	Alfin1 BS2	Alfin1	-308 to -319	AAAGTGGGtGCA	1
RSP00617	*Lycopersicon esculentum*	AT-rich II	unknown nuclear factor	-420 to -438	AaTTAgAATTCTtCTTAAT	3
RSP00861	*Arabidopsis thaliana*	GA-2	BPC1	-1,312 to -1,320	AGAAAGAGA	0
RSP00297	*Arabidopsis thaliana*	Box II/telo-box (motifs 2 & 3)	At-TCP20	-1,450 to -1,454 -1,563 to -1,569	GGCCCc ACCCTAA	1 0
RSP01011	*Arabidopsis thaliana*	AtEBP	AtEBP	-898 to -909	TCCAACCATtCA	1
RSP01209	*Lepidium africanum*	EM1 (CArG box 1)	MADS-box proteins	-747 to -756	CTTTTTTTGG	0
RSP02200	*Lycopersicon esculentum*	CArG (073F)	RIN	-747 to -756	CTTTTTTTGG	0

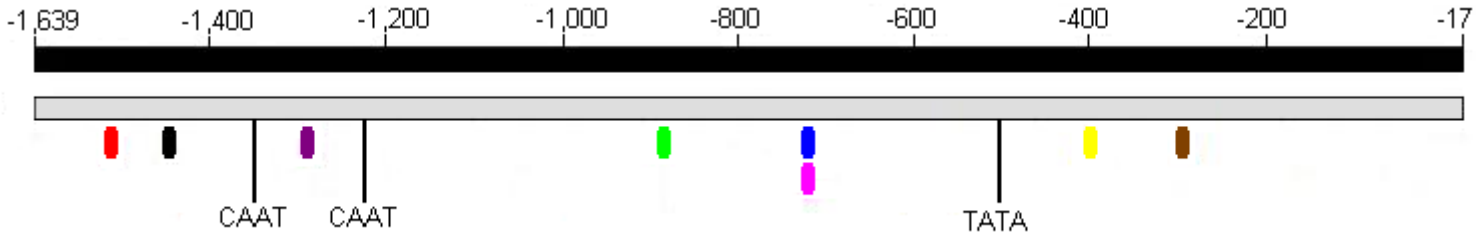

Fig. 10: The peroxidase promoter region that was analyzed was from the 17th to the 1,639th base in front of the translation start site. This scheme shows the location of predicted potential *cis* elements in the investigated peroxidase promoter region. The putative regulatory elements (Table 13) are color indicated: red - Box II/telo-box (motif 3), black - Box II/telo-box (motif 2), purple - GA-2, green - AtEBP, blue - EM1 (CArG box 1), pink - CArG (073F), yellow - AT-rich II, brown - Alfin1 BS2. The predicted TATA-box (-491 to -477) and CAAT-boxes (-1,380 to -1,372, -1,228 to -1,220) are outlined.

3.2.2 Construction of plant transformation vectors containing peroxidase promoter fragments driving marker gene expression

To localize regulatory elements responsible for the root-specific gene expression of *Potri.003G214500*, a series of truncated peroxidase promoter fragments (401 bp to 1,622 bp in length) driving bacterial *ß-glucuronidase* (*GUS*) gene expression were generated by a PCR based strategy. The initially obtained promoter fragment served as template. The primers used for the amplification of the different peroxidase promoter fragments are listed in Table 18 of the appendix. Forward primers, all including an additional *Xma*I restriction site, were combined with backward primers to generate the respective promoter fragments using a proof reading *Taq* polymerase. The initially amplified promoter fragment served as template. A total number of four truncated promoter fragments were generated in addition to the initial fragment as outlined in Fig. 11. The amplified DNA fragments were inserted into the pJET1.2 entry vector and checked by restriction digestions (Fig. 12).

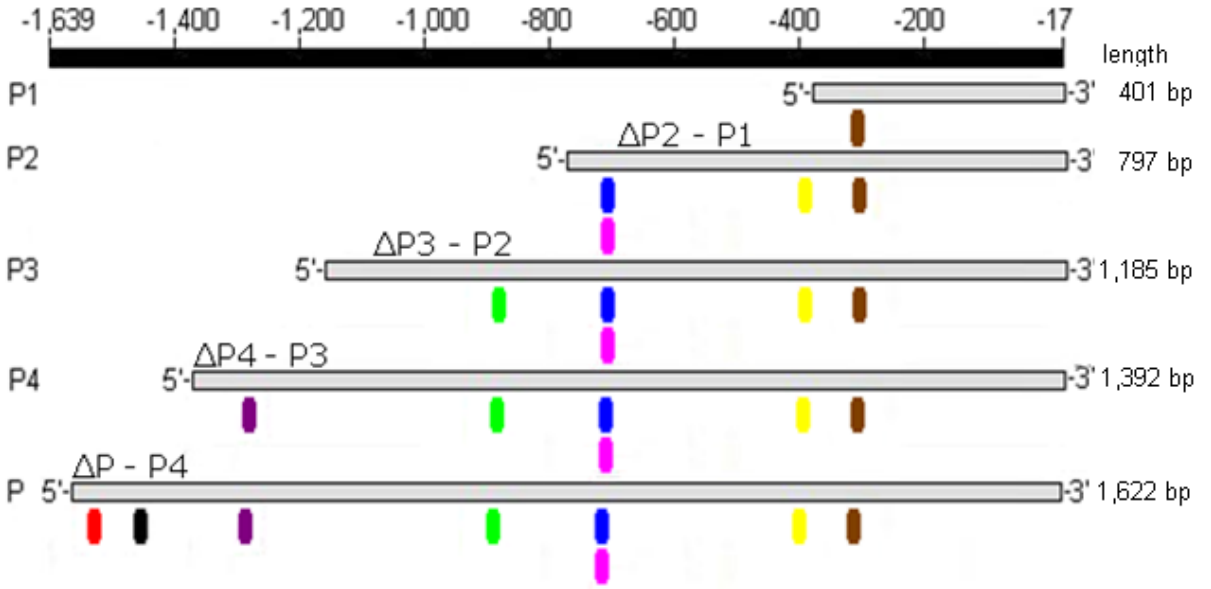

Fig. 11: Alignments of DNA sequences of the truncated peroxidase promoter fragments together with the initial peroxidase promoter fragment (P) as reference. The promoter fragments (P4, P3, P2, and P1) with progressively truncated 5'-ends were PCR amplified. Locations of putative regulatory elements (Table 13), obtained from *in silico* analysis are color indicated: red - Box II/telo-box (motif 3), black - Box II/telo-box (motif 2), purple - GA-2, green - AtEBP, blue - EM1 (CArG box 1), pink - CArG (073F), yellow - AT-rich II, brown - Alfin1 BS2.
For the purpose of simplification, the part of the sequence that made the difference in length between the particular peroxidase promoter fragments is in the following described as Δ followed by the peroxidase promoter fragments abbreviation, for example ΔP-P4.

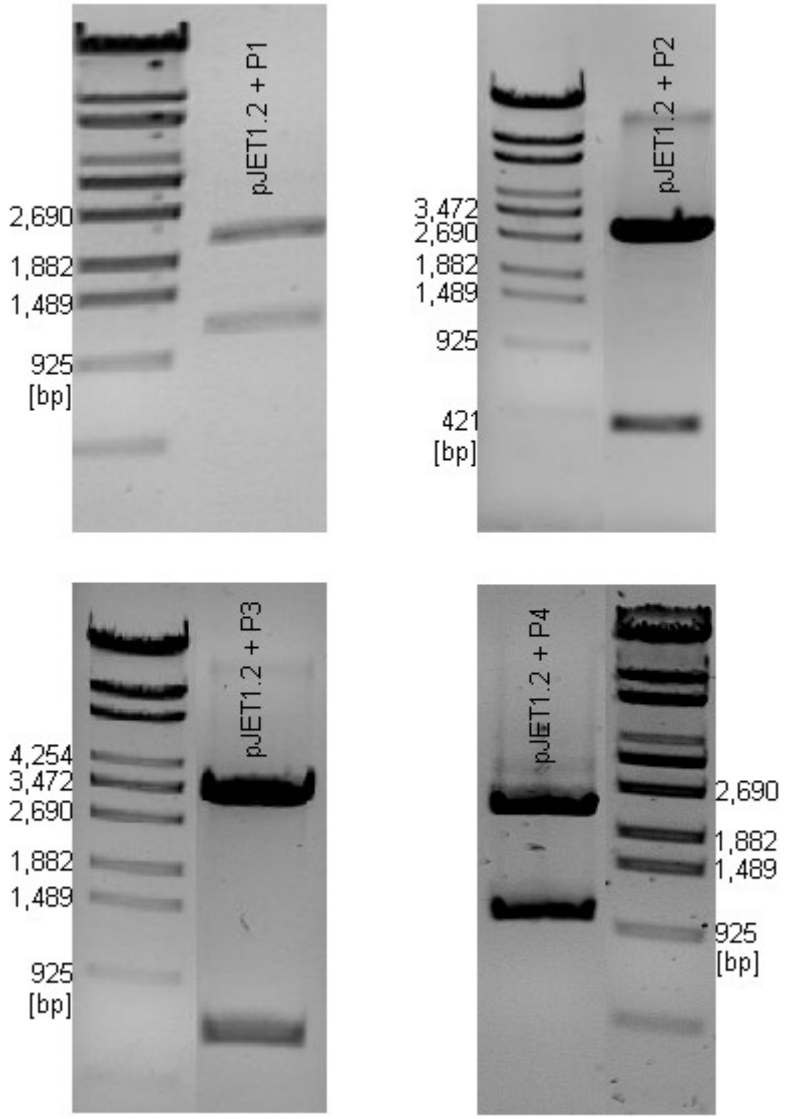

Fig. 12: Gel images of DNA fragments after digestion of the pJET1.2 entry vector incorporating peroxidase promoter fragments. Isolated plasmid DNA was digested with selected restriction enzymes: pJET1.2 + P1: *Bcu*I + *Pdm*I, pJET1.2 + P2: *Hin*dIII, pJET1.2 + P3: *Pst*I, pJET1.2 + P4: *Pst*I. Desired fragment lengths [bp] after restriction digestion were: pJET1.2 + P1: 1,158 + 2,242, pJET1.2 + P2: 511 + 3,296, pJET1.2 + P3: 603 + 3,592, pJET1.2 + P4: 1,340 + 3,055.

In addition to the truncated promoter fragments, a fifth construct excluding the putative Alfin1 BS2 regulatory element indicated by the *in silico* promoter analysis (Table 13) was generated. This was done in several steps: The fragments P_{Alf1} (upstream of Alfin1 BS2) and P_{Alf2} (downstream of Alfin1 BS2) (Fig. 13 a) were amplified separately using the peroxidase promoter P as template. The backward primer for the amplification of P_{Alf1} and the forward primer for the amplification of P_{Alf2}, both flanking the Alfin1 BS2 regulatory element, contained an additional *Bam*HI restriction site. The amplified promoter fragments were inserted into the pJET1.2 entry vector and checked first by restriction analyses (Fig. 14, left side) and second

by sequencing analysis. Sequence analysis revealed nine nucleotide exchanges at the 3'-end of P_{Alf2}. P_{Alf1} and P_{Alf2} were ligated after *Pdm*I and *Bam*HI double restriction digestion. The construct was checked by restriction analysis (Fig. 14, right side), indicating that the P_{Alf} peroxidase promoter fragment lacked the Alfin1 BS2 regulatory element (Fig. 13).

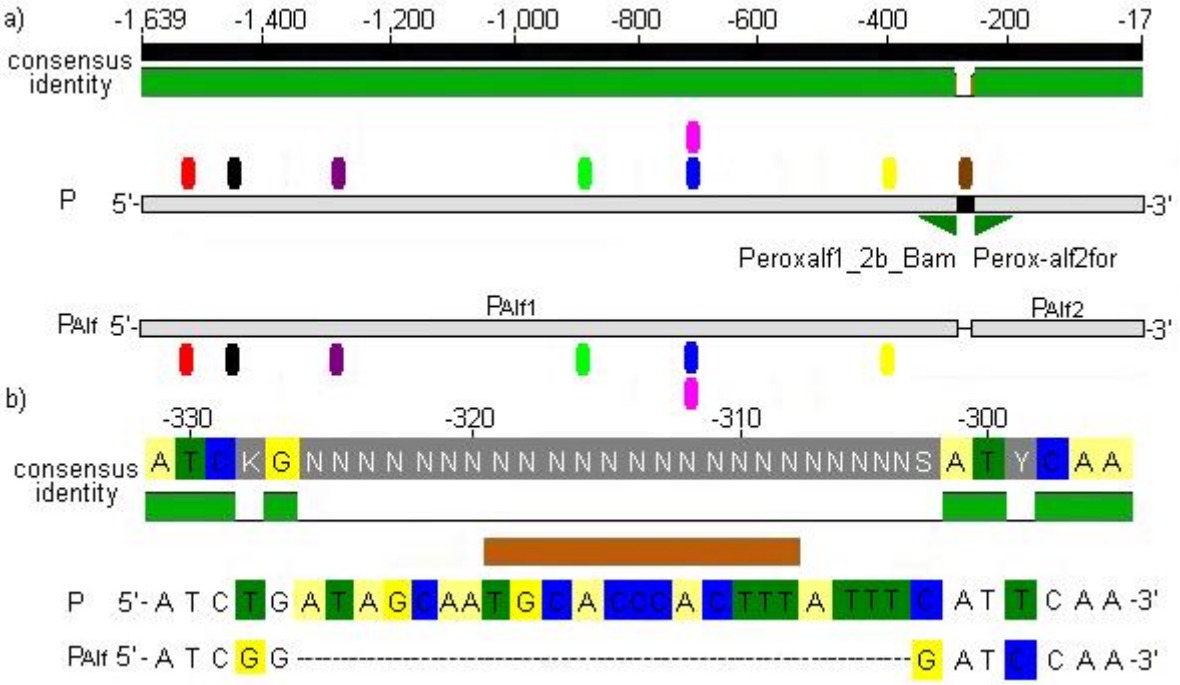

Fig. 13: Alignment of the peroxidase promoter fragment P_{Alf} with the initially obtained peroxidase promoter fragment (P) as reference. a) Alignment of P_{Alf} (1.599 bp) and P(1.622bp). Identification of P_{Alf1} and P_{Alf2}. Indication of the Alfin1 BS2 flanking primers (Peroxalf1_2b_Bam, Perox-alf2for) used for P_{Alf} amplification. b) Detail of the truncated region due to the construction process shown under a). The putative regulatory elements (Table 13), resulting from the *in silico* analysis are color indicated: red - Box II/telo-box (motif 3), black - Box II/telo-box (motif 2), purple - GA-2, green - AtEBP, blue - EM1 (CArG box 1), pink - CArG (073F), yellow - AT-rich II, brown - Alfin1 BS2.

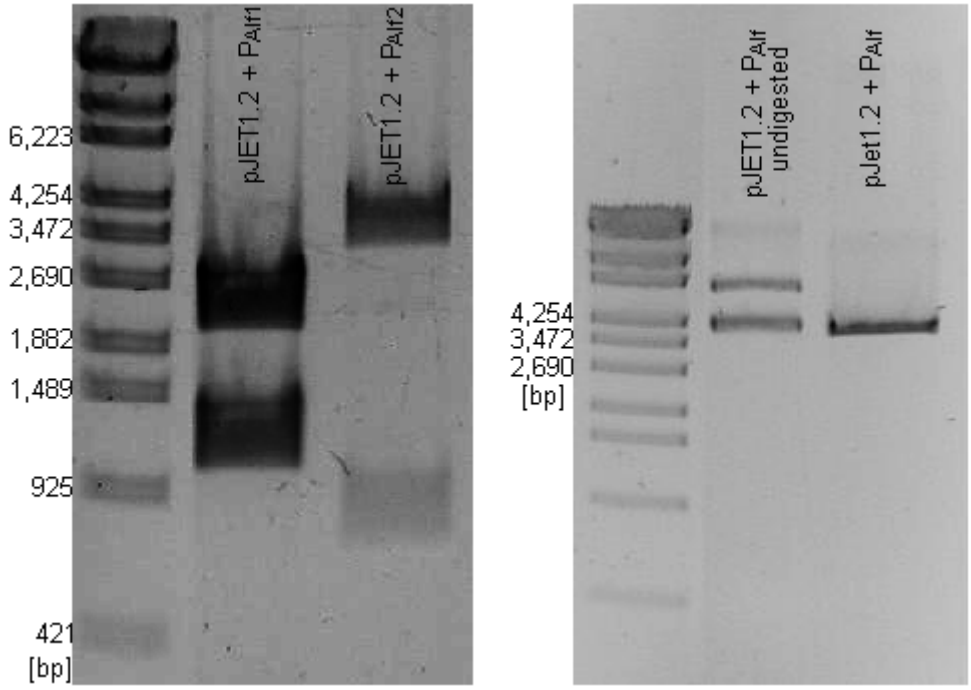

Fig. 14: Gel images of DNA fragments obtained after restriction analyses of pJET1.2 constructs containing the peroxidase promoter fragments P_{Alf1}, P_{Alf2}, and P_{Alf}. Restriction enzymes used: pJET1.2 + P_{Alf1}: *Bam*HI + *Pdm*I, pJET1.2 + P_{Alf2}: *Bam*HI + *Pdm*I, pJET1.2 + P_{Alf}: *Bam*HI. Desired fragment lengths [bp] after restriction digestion: pJET1.2 + P_{Alf1}: 1,033 + 2,231, pJET1.2 + P_{Alf2}: 755 + 3,539, pJET1.2 + P_{Alf}: 4,629.

All six peroxidase promoter fragments were cloned into the binary vector pBI121 (Jefferson et al., 1987), necessary for *Populus tremula* × P. *tremuloides* transformation as exemplary outlined in Fig. 15. Therefore, the cauliflower mosaic virus 35S promoter, driving the *GUS* gene in the pBI121 vector, was removed by *Xma*I and *Bsu*15I double digestion followed by the integration of the isolated *Kpn*2I and *Bsu*15I digested peroxidase promoter fragments yielding pBI121 + P, pBI121 + P1, pBI121 + P2, pBI121 + P3, pBI121 + P4, and pBI121 + P_{Alf}. The successful insertion of all peroxidase promoter fragments into the pBI121 vector was checked by sequencing analysis.

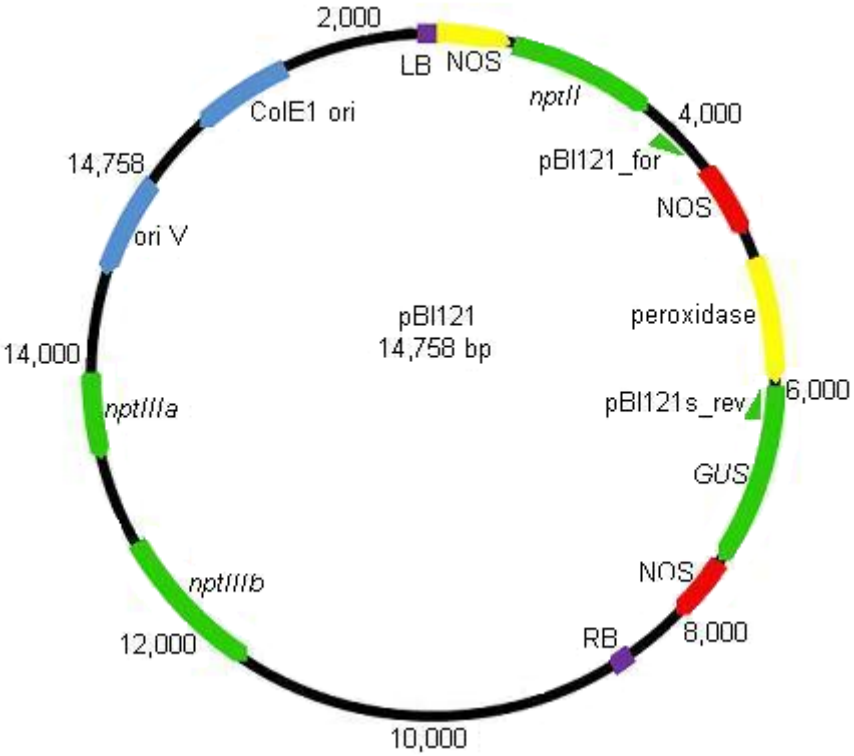

Fig. 15: Map of the plant transformation vector pBI121 (Jefferson et al., 1987) with inserted peroxiase promoter (yellow). The right and left T-DNA borders (RB, LB) are indicated in purple. In between the borders pBI121 contains a *nptII* gene expressed under control of the nopaline synthase promoter (NOS, yellow), which is terminated by the nopaline synthase polyadenylation site (NOS, red). Moreover, the β-glucuronidase encoding *GUS* gene is expressed under control of the poplar peroxidase promoter (or its respective fragments) and terminated by another NOS site (red). The backbone contains two types of the *nptIII* gene (a and b) and the replication origins ori V and ColE1 ori. The primers pBI121_for and pBI121s_rev, which were used for sequencing, are indicated.

3.2.3 Stable transformation of *Populus tremula* × *P. tremuloides* and *Populus tremula* × *P. alba* using *Agrobacterium tumefaciens*

The generated binary vectors were used for *Agrobacterium tumefaciens* (strains C58 and AGL1) transformation, which were in turn used for stable poplar plant transformation. Stable transformation can be subdivided into three different steps: the first one is the production of callus, the second step is the generation of sprouts by the callus, and the last one is the regeneration of whole plants.

To test whether callus and sprout formation was feasible and to find best conditions with *in vitro* propagated *Populus tremula* × *P. tremuloides* or *Populus tremula* × *P. alba* plants, the entire procedure was done omitting *Agrobacterium* infection and kanamycin selection. For callus formation about 7 mm long young stem and leaf stalk sections were partly sliced lengthwise at their ends with a scalpel, placed onto M1 plates and incubated between three and six days (for details see chapter 2.3).

No differences between *Populus tremula* × *P. tremuloides* and *Populus tremula* × *P. alba* plants were detected. Callus formation was not affected by an extended M1-incubation time of six days. But six day incubation resulted later in a higher number of rising sprouts in the time course. Callus formation was observed on M2 medium for stems and leaf stalks (Fig. 16 a) and sprout generation (Fig. 16 b) was observed on M3 medium proving the usability of the protocol.

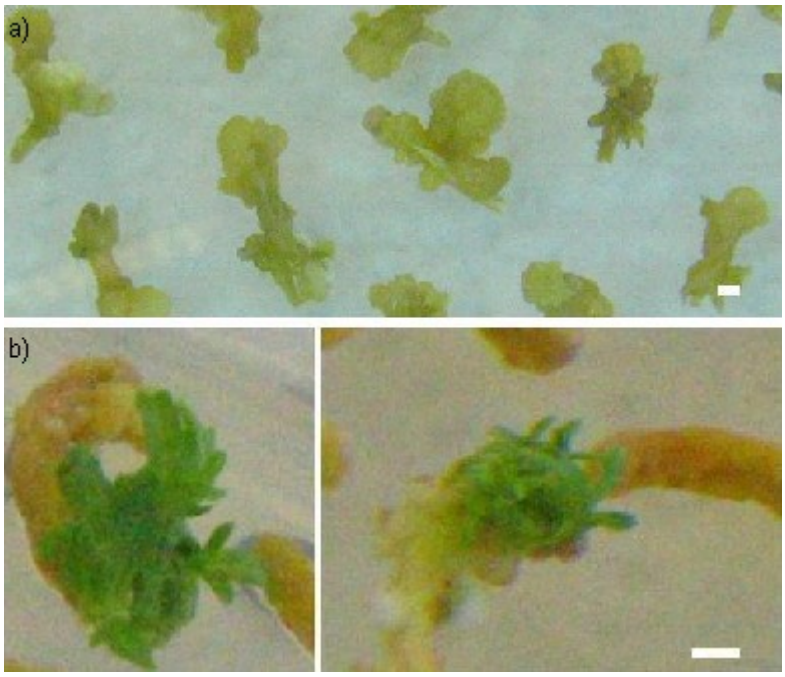

Fig. 16: Results of the entire stable transformation procedure done without *Agrobacterium* infection and kanamycin selection to test whether callus and sprout formation was feasible and to find best conditions with *in vitro* propagated *Populus* plants. a) Callus formation, observed on M2 medium; b) sprout formation, observed on M3 medium of *Populus tremula* × *P. alba* stem stalks. The first incubation on M1 plates took six days. The scale is 1 mm.

However, even when sprout formation could be induced using the protocol without *Agrobacterium*, *Agrobacterium tumefaciens* mediated stable regenerated sprouts were never observed in any transformation approach neither by using transgenic C58 nor AGL1 cells. One problem was the resistance of the *Agrobacterium* strains against tetracycline. Decontamination by using a 41.6 µM tetracycline solution for 15 min was not effective as infected shoot stalk sections were always surrounded by *Agrobacterium* cells after four to six days. Neither different tetracycline batches nor a three-fold extension of the incubation time was successful.

3.2.4 Establishment of the composite plant approach

An alternative approach, the formation of composite plants, was developed in collaboration with Dimitri Neb (University of Bremen, Germany).

Since Chilton et al. (1982) could prove that *Agrobacterium rhizogenes* is a respectably functioning tool for stable root transformation, this method was established for the generation of transgenic roots in a number of different plant species. To test the feasibility of this approach for poplar, *Populus tremula* × *P. tremuloides* cuttings were infected by *Agrobacterium rhizogenes* K599 cells transformed with the pBIN19_YFP_SNL construct (Nowak et al., 2004). The vector pBIN19_YFP_SNL leads to the expression of a yellow fluorescent protein encoding gene with a peroxisomal targeting signal at its C-terminal-end under the control of a double CaMV 35S promoter by the *Populus* hybrid. The formation of YFP expressing roots could be easily visualized under UV light using a YFP filter (Fig. 17 right side). However, not all developed roots possessed a yellow fluorescent signal (Fig. 17, lowest pair of photographs).

Populus composite plants were produced using *Agrobacterium rhizogenes* cells (strain K599) that were transformed with the pBI121, in which the CaMV 35S promoter was substituted by the peroxidase promoter or the respective fragments.

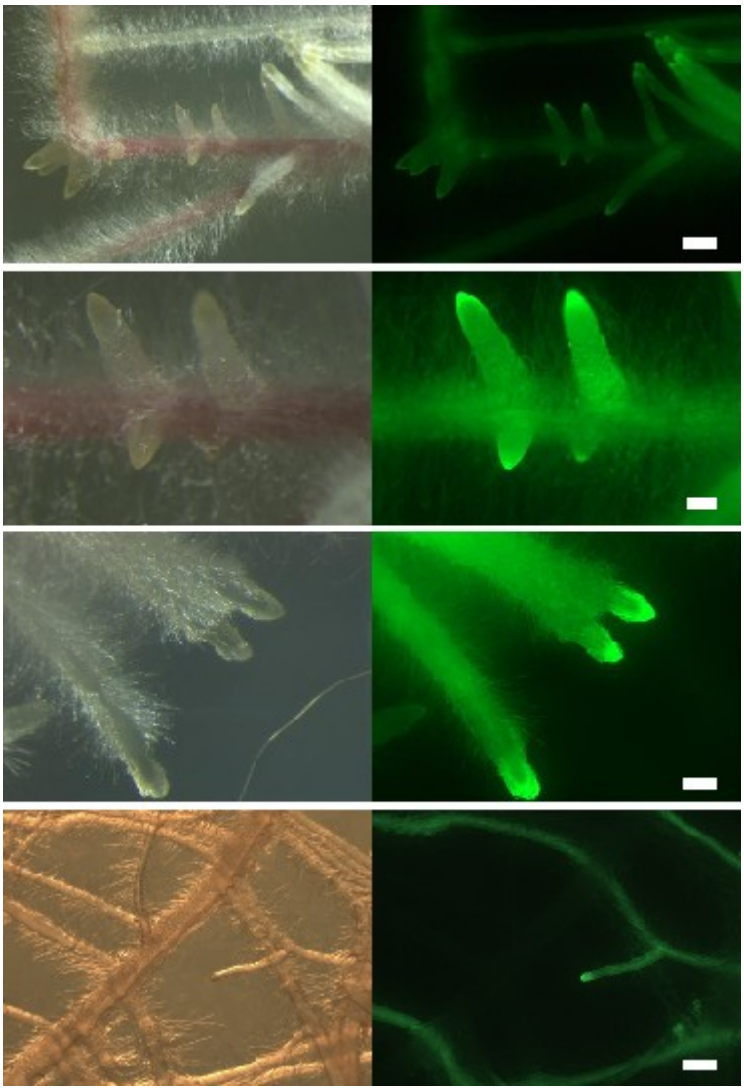

Fig. 17: Photographs of *Populus tremula* × *P. tremuloides* composite plant roots. A yellow fluorescent protein was expressed after transformation with *Agrobacterium rhizogenes* K599 cells incorporating the pBIN19_YFP_SNL vector. Left side under binocular light, right side under UV light using a YFP filter. The scale is 1 mm.

3.2.5 β-glucuronidase activity analyses using a fluorometric GUS assay

After the successful establishment of the composite plant approach all six pBI121 constructs, driving *GUS* gene expression under control of peroxidase promoter fragments, were used for generation of composite poplar plants. To investigate *GUS* gene expression by transgenic poplar roots, an enzyme activity assay was established using the longest peroxidase promoter fragment.

The β-glucuronidase assay was based on the fluorescence of 4-methylumbelliferone, released from 4-methylumbelliferyl-β-d-glucuronide by β-glucuronidase activity. To establish this technique, protein extracts derived from a) transgenic *E. coli* cells expressing a *GUS* gene and b) roots of stable transformed *Populus*

tremula × *P. tremuloides* plants (TE1A) expressing the *E. coli GUS* gene under control of the RolC promoter (Nehls et al., unpublished) were used as positive controls. Protein extract from wild-type poplar roots and water samples served as negative controls.

Unfortunately, the protein content in plant root extracts was rather low (frequently below the detection limit of the Bradford assay). When using 5 µl of the *E. coli* protein extract, 100 µl poplar protein extract of TE1A transgenic roots, and of composite plant roots (pBI121 + P), a clear increase in 4-methylumbelliferone fluorescence was observed, while fluorescence intensity remained stable when using either protein extracts from wild-type poplar roots or water (Fig. 18).

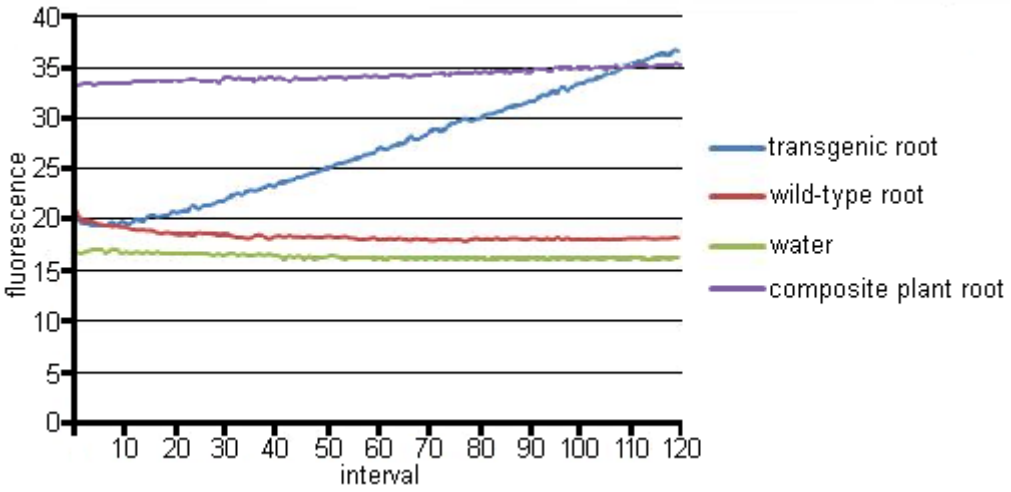

Fig. 18: GUS assay Results. 100 µl of each protein extract was used for fluorescence determination in the course of 120 intervals (each 30 s). Fluorescence generated by 5 µl of the *E. coli* protein extract is not shown due to the high values. A wild-type root protein extract and water served as control.

Compared to protein extracts obtained from transgenic *E. coli* cells, plant extracts revealed extremely low substrate turnover rates per min (Table 14). Especially the substrate turnover rate of the composite root protein extract was just at the detection limit. Together with the low protein content of composite poplar root extracts, β-glucuronidase enzyme activity turned out to be not suitable for quantification of *GUS* gene expression in composite plants.

Table 14: The protein content and maximal turnover rate of the β-glucuronidase of extracts obtained from poplar roots and transgenic *E. coli* cells. The protein amount could not be determined for all extracts due to such low contents. bLOD = below limit of detection.

Extract	Protein content in 1 µl extract [ng]	maximal turnover rate [µM substrate/min]
Wild-type root	0.43	0.046
Transgenic root	bLOD	0.064
Composite plant root	bLOD	0.048
Water	bLOD	0.045
E. coli	70	25.981

3.2.6 Identification of regulatory elements of the peroxidase promoter by *GUS* gene expression

As an alternative approach to the measurement of β-glucuronidase activity, *GUS* gene expression driven by the different peroxidase promoter fragments was analyzed. The entire root systems of two independent batches of composite plants, (at least 200 mg of fresh weight each) were used for RNA isolation. First strand cDNAs were synthesized and used for quantitative RT-PCR. A dilution series of gene-specific DNA fragments was used for determination of PCR efficiencies and for determination of template molecule numbers.

One critical aspect of composite plants is the fact that not all roots generated by poplar cuttings express the target *GUS* gene (comparison to Fig. 17, lowest pair of photographs). Therefore, a varying quantity of *GUS* gene expressing roots was taken into account. The number of template molecules in each sample was determined by quantitative RT-PCR for the *GUS* gene, *nptII* gene, which is a) part of the T-DNA and b) expressed under the control of the constitutive NOS promoter (An et al., 1990; Kim et al., 1994), and *ubiquitin*. First, the number of the *GUS* template molecules was calibrated to the number of *nptII* templates molecule in a given RNA sample. Second, this *GUS* to *nptII* ratio was calibrated to the number of *ubiquitin* template molecules, which allows a comparison between different RNA samples. The results of the *GUS* gene expression analyses are shown in Fig. 19. The *GUS* gene expression rate was similar high induced by the promoter fragments P, P4, and P2. The peroxidase promoter fragments P3 and P1 induced only very little *GUS* gene expression in *Populus* composite plant roots. No *GUS* gene expression was induced by the peroxidase promoter P_{Alf}.

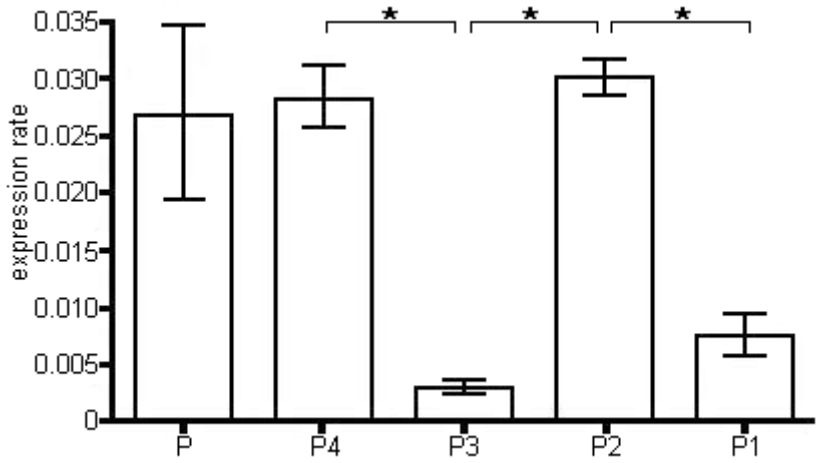

Fig. 19: Relative expression rates of the *GUS* gene driven by the peroxidase promoter or the respective fragments. P_{Alf} did not induce expression of the *GUS* gene. *GUS* gene expression rates were calculated to *nptII* gene expression rates. This ratio was calculated relatively to the *ubiquitin* expression rates. Stars indicate statistical extreme significantly differences ($P < 0.0001$). The number of technical replicates was for: P: n = 22, P1: n = 13, P2: n = 19, P3: n = 10, P4: n = 22.

The Shapiro-Wilk normality test (Sachs and Hedderich, 2009) revealed no normal distribution (*P*-value < 0.05) of the datasets for *GUS* gene expression driven by all investigated peroxidase promoter fragments (P: $P < 0.0001$; P3: $P = 0.009$, P2: $P = 0.038$, P1 $P = 0.002$) except for P4 ($P = 0.17$). Therefore, the Mann-Whitney test (Sheskin, 2004) was performed for significance analysis. The *P*-values of the non-parametric Mann-Whitney tests are listed in Table 15. A significant difference in *GUS* gene expression was accepted when $P < 0.05$. According to the Mann-Whitney test *GUS* gene expression turned out to be extreme significantly different between the peroxidase promoter fragments P4 and P3 ($P < 0.0001$), P3 and P2 ($P < 0.0001$), as well as P2 and P1 ($P < 0.0001$), while no significant difference was obtained between P and P4 ($P = 0.136$).

Table 15: Table of *P*-values of the performed Mann-Whitney tests for the *GUS* gene expression rates driven by the peroxidase promoter and the respective fragments. The number of technical replicates was P: n = 22, P1: n = 13, P2: n = 19, P3: n = 10, P4: n = 22. Green colored boxes indicate *P*-values < 0.05 whereas red ones indicate *P*-values > 0.05.

	P	P1	P2	P3	P4
P		0.040	0.139	< 0.0001	0.136
P1	0.040		< 0.0001	0.003	< 0.0001
P2	0.139	< 0.0001		< 0.0001	0.187
P3	< 0.0001	0.003	< 0.0001		< 0.0001
P4	0.136	< 0.0001	0.187	< 0.0001	

4 Discussion

4.1 Utilization of poplar hybrids

In 2006 the sequenced genome of *Populus trichocarpa* (female genotype Nisqually 1) was published. But in this work three different *Populus* hybrids were used taking advantage of crossed genetic properties.

Populus hybrids developed partially natural for example after the import of certain species to different continents but also by intended crossing of different species. For example *Populus tremula* × *P. tremuloides* is less sensitive to low temperatures because this hybrid usually grows in temperate climates whereas *Populus. tremula* × *P. alba* grows in the southern part of Europe and is less sensitive towards higher temperatures. However, inter-specific hybrids exhibit positive heterosis (evolutionary fitness, Dillen et al., 2009). So the advantage of using *Populus* hybrids for experiments is their vitality. Growth rates are clearly higher compared to sequenced *P. trichocarpa* plants.

Populus trichocarpa × *P. deltoides* was used for the amplification of SUTs because these two species are closely related. Using cDNA of this hybrid increases the variance of amplified sequences and therewith raises the possibility to obtain the DNA sequence of a functional transporter. Some hybrids are not suitable for *Agrobacterium* mediated transformation. Hence, two different hybrids (*Populus tremula* × *P. alba* and *Populus tremula* × *P. tremuloides*), which differ only slightly in their growth properties, were used for stable transformation and the composite plant approach.

4.2 Impact of sucrose transporters in ectomycorrhiza symbiosis

Which sugar is released by yet unknown mechanisms into the common apoplast of the plant/fungal interface of functional ectomycorrhizas is still an open question. The best candidate in literature is sucrose (for example Nehls et al., 2010).

Different transport mechanisms have been shown for sucrose transporters: first a proton-coupled sucrose transport mechanism for example for ZmSUT1 (Carpaneto et al., 2005) and PsSUT1 and PvSUT1 (Zhou et al., 2007), second a sucrose efflux mechanism by proton-coupled PsSUT1 and PvSUT1, under certain conditions (Zhou et al., 2007), and third a bi-directional, pH- and energy-independent, sucrose

transport mechanism was confirmed for PsSUF1, PsSUF4, and PvSUF1 (Zhou et al., 2007).

One intention of this thesis was therefore to find an answer to the question, whether sucrose transporters of the *SUT* gene family in poplar may function as sucrose efflux carriers at the plant/fungus interface in ectomycorrhizal symbiosis. The published genome of *Populus* (Tuskan et al., 2006; Wullschleger et al., 2013), a model plant that is capable to form ECM (Taylor and Alexander, 2005), allowed the identification of *SUT* homologs by BlastX search (Altschul et al., 1990) using *Arabidopsis SUT* genes as templates. A total of five *SUT* genes (*PtSUT1*, *PtSUT3*, *PtSUT4*, *PtSUT5*, and *PtSUT6*) were identified in the *Populus* genome (Nehls, unpublished) and initial expression analysis revealed reduced transcript levels of all *SUT* genes upon ectomycorrhiza formation except of *PtSUT4* (Nehls, unpublished). As the vacuolar localization of PtSUT4 (Payyavula et al., 2011) hadn't been discovered when the thesis was started, PtSUT4 was seen as a good candidate for the questioned plant root sucrose efflux carrier.

4.2.1 Correlation of *SUT* gene expression and ECM formation

Using *Populus tremula* × *P. alba* plants grown in pot culture in the green house, Payyavula et al. (2011) detected transcripts of all five *SUT* genes in roots (Fig. 3). Transcripts were also detected for all genes in non-mycorrhizal as well as mycorrhized fine roots by Nehls (unpublished results). However, only *SUT4* revealed a higher transcript level (about three-fold) upon mycorrhization (Nehls, unpublished).

Since the formation of the plant/fungus interface is a continuous process, starting in the elongation zone of a growing ectomycorrhizal fine root, the oldest part of the Hartig net, where nutrient exchange occurs, is found at the base of a mycorrhizal fine root while no Hartig net is found at the root tip. If SUTs may have a function in fungal carbohydrate support, their gene expression should to be higher at the basis of a mycorrhizal fine root compared to the root tip.

To investigate a putative PtSUT function at the plant/fungus interface, gene expression was investigated along the ectomycorrhiza. The transcript levels were only sufficient high for *PtSUT3* and *PtSUT4*. In functional mycorrhizas *PtSUT3* and *PtSUT4* revealed their highest expression in the root tip. As the root tip section is characterized mainly by the meristem, these results indicate a role of *PtSUT3* and

PtSUT4 in apical meristem carbohydrate support rather than in fungal carbohydrate nutrition in ECM symbiosis.

4.2.2 Role of *SUT*s in fungal carbohydrate support in ECM

Former gene expression analyses using mycorrhizal and non-mycorrhizal fine roots showed lower *PtSUT1* expression rates under mycorrhizal conditions (Nehls, unpublished results) implying no role of *PtSUT1* in fungal carbon support in mycorrhiza. Heterologous expression of *PtSUT1* in Susy7/ura3 mutants (Riesmeier et al., 1992) did not complement the mutant's phenotype on medium supplemented with sucrose as sole carbon source (Fig. 7). Nevertheless, a positive control was not available and hence this result might be contested. Since the yeast expression vector pDR196 was successfully tested before (Riesmeier et al., 1992) a missing expression of the target gene is not considered. However, problems often occur with regard to the localization of the protein, which should be targeted to the plasma membrane. The missing complementation of the mutant's phenotype cannot be easily attributed to the property of PtSUT1 since localization experiments are missing. Hence, further localization experiments are required to examine if PtSUT1 is situated in the plasma membrane. In addition, nothing is known about the half-life period of the expressed protein, its stability, posttranscriptional modification like glycolysation and ubiquitination, as well as folding properties, which are important characteristics in heterologous expression experiments. A possible degradation of the protein on its way to the plasma membrane cannot be excluded. Taken this information together a conclusion that PtSUT1 is not a sucrose transporter cannot be done. However, the already known reduced expression pattern of PtSUT1 under mycorrhizal conditions indicates that PtSUT1 is not involved in fungal carbon support in ectomycorrhiza.

A three-fold up-regulation of PtSUT4 under mycorrhizal conditions (Nehls, unpublished) led to the assumption that this transporter was seen as a good candidate for the questioned plant root sucrose efflux carrier in ECM. However, *PtaSUT4* revealed the highest expression rate in the non-mycorrhizal roots compared to all other *SUT*s and fusion of *PtaSUT4* to a green fluorescent protein encoding gene could show that the PtaSUT4 protein was targeted to the tonoplast (Payyavula et al., 2011). A tonoplast targeted SUT protein cannot export sucrose out of any cell due to its localization.

The answer to the question if one of the identified SUTs in *Populus* is the questioned sucrose efflux carrier in the ectomycorrhizal symbiosis cannot be answered clearly. However, some indications were given that they are presumably not the missing kind of transporter that is able to facilitate sucrose export into the common apoplast of plant root and fungus.

Since *PtSUT4* expression is up-regulated upon mycorrhiza formation PtSUT4 (Nehls, unpublished) seems to play a different role. In an acute draught stress experiment, in which the plants did not have had any time to acclimate to the water stress, stable RNAi PtaSUT4 plants revealed a higher transient tolerance compared to wild type plants resulting in a significant longer time period to initial wilt (Frost et al., 2012). *PtSUT4* expression and the associated sucrose export out of the tonoplast go along with shifts in the water status. A higher expression level of *PtSUT4* like in the mycorrhizal state represents higher sucrose concentration in the cell cytoplasm and therewith higher water availability for the plant. In contrast down-regulation of *PtSUT4* helps the plant to adjust its water balance to draught stress.

But if SUTs are not the missing sucrose efflux carrier at the functional interface, members of the recently discovered *SWEET* sugar facilitator gene family (Chen et al., 2010) come into consideration. 6 SWEETs of *Arabidopsis thaliana* (AtSWEET10 to AtSWEET15) and 2 SWEETs of *Oryza sativa* (OsSWEET11, OsSWEET14) were capable of transporting sucrose (Chen et al., 2012). The up-regulation of some *SWEET* genes upon ectomycorrhiza formation in poplar fine roots (Nehls, unpublished) led to the hypothesis that this kind of transporter might be involved in fungal carbohydrate support in ECM. In *Populus trichocarpa* 26 *SWEET* genes were identified and some of them were highly up-regulated upon ECM formation (Nehls et al., unpublished). Therefore, four constructs, expressing the entire open reading frames of *SWEET* genes under the control of a constitutive yeast promoter were analyzed for the capability of the respective proteins to complement the sucrose transport deficiency of the yeast mutant Susy7/ura3. As none of these constructs was capable in conferring yeast growth on sucrose (Fig. 8 b), sucrose transport capability of the investigated proteins is rather unlikely. However, as a positive control and protein localization experiments (see above) were missing this result is contestable. Furthermore, none of the investigated *SWEET* ORFs was able to cause yeast growth on maltose (Fig. 8 c). However, glucose was taken into consideration as alternative

fungal carbon source delivered by infected poplar fine roots as supposed by Nehls and Bodendiek (2012) and Nintemann (2012).

Taken the given information together *PtSUT* expression analyses pointed only to PtSUT4 to be the questioned sucrose exporter that is responsible for fungal carbon support in mycorrhiza. However, localization experiments of Payyavula et al. (2011) debilitated this idea. Today, some SWEETs are known to be highly up-regulated upon mycorrhiza formation (Nehls et al., unpublished). Since some of them were shown to transport glucose (Nintemann, 2012), SWEETs and their substrate glucose come into the center of research. Glucose might be exported by a/some certain SWEET/s at the symbiotic interface out of the plant's fine root into the common apoplast of plant and fungus and might be taken up directly by the fungal hyphea.

4.3 Peroxidase promoter analysis

A tool that allows the investigation of root-specific processes even under mycorrhizal conditions without affecting the other plant organs is of great importance for research. For the investigations of root-specific processes promoters are required that cause only root-specific gene expression, which is not affected by mycorrhiza formation. These promoters can for example be used to suppress gene expression of target genes using the RNAi approach.

As described by Krützmann (2010), the promoter of the poplar *Potri.003G214500* gene, coding for a putative peroxidase, provokes a strong root-specific and mycorrhiza-independent expression pattern together with a high transcript level. Provoking a mycorrhiza-independent expression enables the investigation of genes under mycorrhizal and non-mycorrhizal conditions. To gain first information how root-specific gene expression and gene regulation are mediated and to check if the peroxidase promoter is an appropriate tool for root-specific investigations it was analyzed.

In silico analyses of the promoter region -1,639 to -17 bp upstream of the TSS of the corresponding *peroxidase* gene of *Populus trichocarpa* × *P. deltoides* indicated a TATA-box from base -491 to -477. The attachment of the TATA-box binding protein initiates the generation of the transcription initiation complex, an essential step for the transcription. TATA-boxes are usually situated about 25 bp upstream of the TSS. Hence, the TSS would be situated around -452 resulting in a calculated 452 bp long

5’-untranslated region. This is rather unlikely because 5’-untranslated regions in plants are about 80 to 150 bp long. It must therefore be concluded, that the prediction of this TATA-box is not reliable. CAAT-boxes are usually situated around 80 bp upstream of the TSS. The predictions of the two CAAT-boxes by the MatInspector tool around -1,376 and -1,224 are hence also not reliable. Furthermore, eight putative regulatory elements were predicted by the *in silico* analyses (Table 13, Fig. 10). To identify arrays where transcription factors operate, four different peroxidase promoter fragments (P4, P3, P2, and P1) with progressively truncated 5’-ends compared to the initial peroxidase promoter P were used for investigation. Moreover, the peroxidase promoter fragment P_{Alf} was generated lacking the putative Alfin1 BS2 regulatory element.

By the use of a plant expression vector the strength of the peroxidase promoter and the respective fragments were examined using the expression of the *GUS* marker gene. The constructs were used for the generation of *Populus tremula* × *P. tremuloides* composite plants. However, transformation of *Populus* plants mediated by *Agrobacterium rhizogenes* K599 cells was considered to not result in a fully *GUS* gene expressing root system (compare Fig. 17, lowest pair of photographs), what means that in some cells the *GUS* gene containing T-DNA was not integrated. T-DNA integration of the Ri-plasmid and the used constructs (pBIN19_YFP_SNL and pBI121 incorporating the different peroxidase promoter fragments) happen independent of each other and can only occur in areas of wounded plant stem cells.

The peroxidase promoter driven *GUS* gene expression, using the longest fragment P, in roots of *Populus* composite plants caused only low β-glucuronidase activity (Table 14). However, the gene expression rate does not necessarily reflect actual protein content. Posttranscriptional modifications might lead to protein degradation or problems might occur regarding folding of foreign proteins leading to protein instability. There is no information given with regard to bacterial *GUS* expression rate in a foreign organism and amount of functional β-glucuronidase protein correlation. Expression data of interconnected pathways (glycolysation and ubiquitination, protein folding) in combination with analysis of β-glucuronidase content and function, as well as metabolite contents are necessary to give reliable information. Nevertheless, the *GUS* gene expression was directly determined (Fig. 19) although,

One drawback of composite plants is the fact that not all roots generated by the poplar cuttings were expressing the *GUS* gene. As transgenic roots couldn't be easily visualized with the constructs used in this work different proportions of transgenic roots from single composite plants had to be taken into account. The *nptII* gene, which is transferred in parallel to the *GUS* gene into the plant genome by *Agrobacterium*, served as marker for the proportion of *GUS* gene expressing roots. *NptII* gene expression was driven by the nopaline synthase promoter that enables constitutive expression in roots (An et al., 1988; An et al., 1990; Kim et al., 1994). To calibrate *GUS* gene expression to the root proportion, where the T-DNA of the used vector was integrated, in a given sample the number of *GUS* template molecules was divided by the number of *nptII* template molecules.

Only the promoter fragment, in which the Alfin1 BS2 regulatory element had been deleted, revealed no *GUS* gene expression, while P3 and P1 showed very low, and P, P4, and surprisingly P2, showed similar strong *GUS* gene expression rates (Fig. 19).

Within the 230 bp long sequence, ΔP-P4, two regulatory elements were predicted by *in silico* analysis. However, no significant differences in *GUS* gene expression were obtained between both peroxidase promoter fragments making it likely that no important regulatory element is located upstream of P4.

Deletion of the following 207 bp, ΔP4-P3, resulted in an extremely significant reduction of the *GUS* gene expression by the peroxidase promoter fragment P3. The regulatory element predicted by the *in silico* analysis within ΔP4-P3 is called GA-2 and the transcription factor BASIC PENTACYSTEINE 1 (BPC1) is known to bind to this element (Monfared et al., 2011). BPC1 belongs to a subgroup of a plant-specific transcription factor encoding gene family (Monfared et al., 2011). Members of this family are known to regulate a wide range of genes in multiple plant organs but they have not been described to be involved in root-specific gene expression (Meister et al., 2004). However, binding of BPC proteins to a *cis* element in *Arabidopsis thaliana* was described to induce gene transcription responsible for ovule development (Meister et al., 2004).

Interestingly, a truncation of the following 388 bp, ΔP3-P2, resulted again in a similar strong *GUS* gene expression rate as induced by the peroxidase promoter P and the fragment P4. Hence, the presence of a regulatory element in the 388 bp promoter

sequence, ΔP3-P2, is assumed. The transcription factor that binds to this element had a silencing effect, which was abolished by a putative enhancer that bound to the transcription factor binding site within the 207 bp long sequence ΔP4-P3. This was indicated by the almost equal *GUS* gene expression rate obtained by the peroxidase promoter fragments P2 and P4. The *in silico* predicted regulatory element of the peroxidase promoter region in the truncated ΔP3-P2 sequence is called AtEBP. The *Arabidopsis thaliana* ethylene-responsive element binding protein, AtEBP, belongs to a large gene family (Yang et al., 2009) and contains an AP2/EREBP binding domain and binds to GCC boxes of promoters (Büttner and Singh, 1997). A suppressive effect of the AtEBP protein regarding the expression of an in cell death involved gene was provided (Pan et al., 2001). A suppressing effect is also assumed with regard to the transcription factor that binds to the regulatory element in ΔP3-P2. However, AtEBP involvement in root-specific gene expression has not been described.

Further 396 bp truncation of the promoter sequence, ΔP2-P1, resulted again in a statistical extremely significant reduction of the *GUS* gene expression rate. The regulatory elements predicted by *in silico* analysis of the peroxidase promoter in the ΔP2-P1 region are EM1 (CArG box 1), CArG (073F), and AT-rich II. The transcription factors that bind to these regulatory elements are MADS-box proteins, RIN, and an unknown nuclear factor. MADS-box proteins form a large transcription factor gene family and play a key role in a lot of plant developmental processes (Parenicova et al., 2003). Several MADS-box proteins are expressed in the root (Parenicova et al., 2003) and are hence capable to drive root-specific gene expression. The ripening inhibitor, RIN, is a member of the MADS-box transcription factor family and is essential for fruit ripening as described for *Lycopersicon esculentum* (Martel et al., 2011; Shima et al., 2013).

Lacking the Alfin1 BS2 regulatory element of P_{Alf} completely omitted *GUS* gene expression, indicating this regulatory element as a major switch for the expression of the *Potri.003G214500* gene in *Populus*. However, presence of the Alfin1 BS2 regulatory element alone was not capable in inducing *GUS* gene expression since P1, which still contained the Alfin1 box, induced only very little *GUS* gene expression. *Alfin1* encodes a protein with a putative zinc-binding domain and hence a role in transcriptional regulation was assumed (Bastola et al., 1998). In transgenic *Medicago sativa L*. plants overexpression of Alfin1 increased the expression of the root-specific

MsPRP2 gene, which contains the sequence for the *Medicago sativa* transcription factor Alfin1 (Winicov, 2000). Overexpression of both Alfin1 and MsPRP2 in *Medicago sativa* resulted in enhanced overall root growth in transgenic plants (Winicov, 2000). Hence, Alfin1 can be assumed to have a similar function in the regulation of the *peroxidase* gene expression in poplar.

The different *GUS* gene expression rates induced by the different peroxidase promoter fragments indicate that several regulatory elements and hence transcription factors are involved in the transcriptional regulation of the *Potri.003G214500* gene expression in *Populus*. The *in silico* analysis of the 1,639 to -17 bp peroxidase promoter region of *Populus trichocarpa* × *P. deltoides* indicated eight putative regulatory elements. However, by using six different promoter sequences four regulatory regions of the peroxidase promoter were identified using *GUS* marker gene expression in this work. Literature data provided evidence that transcription factors that bind to the *in silico* predicted regulatory elements present in the regulatory regions investigated experimentally provoke changes in gene expression patterns.

Since all transcription factors that bind to the predicted cis-elements are encoded in *Populus'* genome they all (but even other ones) have to be taken into consideration to be responsible for the root-specific expression of *Potri.003G214500.* However, a direct link between the *in silico* predictions and the available *GUS* gene expression results cannot be given. To determine if the *in silico* predicted regulatory elements are the ones identified in the promoter regions by *GUS* gene expression and if these elements and the binding transcription factors are involved in root-specific gene expression further investigations are necessary.

5 Outlook

The results of this work do not give any evidence that the fungal carbon support in mycorrhizal roots is mediated by sucrose transporters. Hence, further investigations regarding sucrose transporters are not advisable. Since some of the recently discovered *SWEET* genes are up-regulated upon mycorrhiza formation (Nehls, unpublished data) and two poplar members have already been shown to be capable of transporting glucose (Nintemann, 2012), SWEET transporters have to be considered for the fungal carbon support in mycorrhiza (Nehls and Bodendiek, 2012).

Dependent on the question to be addressed, suppression of gene expression, e. g. by RNAi, needs to be root-specific. For the production of transgenic plants a root-specific promoter e. g. the peroxidase promoter investigated in this work is required. However, a missing experiment in this work is the proof, that the investigated promoter fragments really allow root specific gene expression. As composite plants have been used for analysis, it cannot be excluded, that elements further upstream of the fragments used in this work are needed for root specificity. Therefore, poplar plants have to be transformed at least with the longest construct used in this work by an *Agrobacterium tumefaciens* based strategy.

6 Summary

Forests play an outstanding role in ecology. The ability of certain fungi to explore the soil for nutrients and to mobilize them, as well as the ability of plant roots to exudate carbohydrates are the basis for a symbiosis that enables forest ecosystems to cover about 30 % of the Earth's terrestrial surface. In this ectomycorrhizal symbiosis the fungus gets easily degradable carbohydrates in exchange for nutrients. *Populus* is a representative organism for woody perennials, participating in mycorrhiza formation.

It was commonly accepted that carbohydrates are exuded in form of sucrose into the common apoplast. Sucrose transporters, also known as SUTs, were long time favored to be the questioned transporters mediating sucrose efflux at the functional interface. In this work selected *SUT*s of the model plant *Populus trichocarpa* × *P. deltoides* were analyzed. *PtSUT3* and *PtSUT4* did not reveal their highest expression in the area of the functional interface in mycorrhizal poplar roots. Growth tests using the heterologous yeast expression system could not prove the ability of PtSUT1 to transport sucrose. Therefore, the obtained data in this work do not indicate a role of sucrose transporters and sucrose as carbon source for the fungal partner.

For investigation of root-specific processes, promoters are required that cause only root-specific gene expression. The question how root-specific gene expression is mediated was addressed in this work. The promoter of a previously identified, root-specific expressed *Populus trichocarpa* peroxidase gene has been investigated for indication of regulatory regions in this thesis. An *in silico* analysis of the peroxidase promoter region indicated eight putative regulatory elements within a region of 1622 bp. To investigate whether these regulatory elements play a role in *peroxidase* gene expression, six truncated peroxidase promoter fragments driving *GUS* gene expression were examined. Two regulatory regions, one responsible for induction, one for repression of gene expression could be identified in the peroxidase promoter region in this work. Furthermore, a putative Alfin1 BS2 regulatory element identified within the promoter region turned out to be essential for peroxidase gene expression.

7 Zusammenfassung

Wälder spielen eine außerordentliche Rolle in der Ökologie. Ihre Böden sind reich an komplexen Kohlenhydraten deren Abbau jedoch ein langsamer Prozess ist. Die Fähigkeit der Pilze, den Erdboden nach Nährstoffen zu durchforschen und sie zu mobilisieren, und die Fähigkeit der Pflanzen, große Mengen an Kohlenhydraten photosynthetisch herzustellen und sie anteilig über die Wurzel in den Erdboden abzugeben, sind die Basis der Symbiose, die die Wälder etwa 30 % der terrestrischen Erdoberfläche einnehmen lässt. Diese Symbiose, in der die Pflanze Nährstoffe durch den Pilz bekommt und dieser dafür einfach verwertbare Kohlenhydrate von der Pflanze erhält, wird Mykorrhiza genannt. Ein repräsentativer Organismus für mehrjährige Gehölze, der an der Mycorrhizabildung beteiligt ist, ist die Pappel.

Allgemein akzeptiert ist, dass in der Mykorrhiza Symbiose Kohlenhydrate in Form von Saccharose aus der Pflanzenwurzel in den gemeinsamen Apoplasten der Pflanze und des Pilzes abgesondert werden. Saccharose Transporter, die auch SUTs genannt werden, waren lange Zeit die favorisierten Transporter, die Saccharose am funktionellen Interface exportieren. In dieser Arbeit wurden SUTs des Modelorganismus *Populus trichocarpa* × *P. deltoides* untersucht. *PtSUT3* und *PtSUT4* sind nicht im Bereich des funktionellen Interface am höchsten exprimiert. Wachstumstests, die mittels heterologer Expression in Hefezellen durchgeführt wurden, konnten kein Hefewachstum durch Saccharose Transporter PtSUT1 zeigen. Daher deuten die in dieser Arbeit erhaltenen Daten nicht auf eine Rolle von Saccharose Transportern und Saccharose als Kohlenstoffquelle der pilzlichen Kohlenhydratversorgung in der Mykorrhiza hin.

Um Prozesse wurzelspezifisch zu manipulieren, werden Promotoren benötigt, die lediglich eine wurzelspezifische Expression erlauben. Dabei stellt sich die Frage, wie eine solche wurzelspezifische Genexpression erreicht wird. In der vorliegenden Arbeit wurde zur Analyse dieser Fragestellung ein Pappelpromotor analysiert, der eine wurzelspezifische Genexpression erlaubt. Eine *in silico* Analyse ergab acht mögliche Bindestellen für Transkriptionsfaktoren. Um die Rolle dieser Bindestellen in Bezug auf die Regulation des *Peroxidase* Gens in *Populus* zu untersuchen, wurde ein *GUS* Marker Gen hinter sechs verschieden lange Peroxidase-Promotor Fragmente kloniert. Hierdurch konnten in der vorliegenden Arbeit zwei Bindebereiche

für mögliche Transkriptionsfaktoren identifiziert werden, von denen einer eine induzierende und einer eine reprimierende Wirkung auf die Expression des Peroxidasegens hat. Weiterhin konnte gezeigt werden, dass eine von *Medicago sativa L* bekannte Transkriptionsfaktorbindestelle (Alfin1 BS2) für die wurzelspezifische Expression des untersuchten Pappelperoxidasegens essentiell ist.

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9 Appendix

List of abbreviations

A	Alanine
A.	*Agrobacterium*
a.a.	Amino acid
ADH	Alcohol dehydrogenase terminator
AtEBP	*Arabidopsis thaliana* ethylene-responsive element binding protein
BPC1	BASIC PENTACYSTEINE 1
CaMV 35S	Cauliflower mosaic virus 35S promoter
cDNA	Complementary DNA
C-terminal-end	Carboxy-terminus of a protein
ddNTPs	Di-deoxyribonucleoside triphosphate
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxyribunucleoside triphosphate
E	Glutamic acid
ECM	ectomycorrhiza
E. coli	*Escherichia coli*
Fig.	Figure
g	Gravity acceleration
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
LB	Lysogenic broth

M	Methionine
MMN	Modified Melin-Norkrans
MS	Murashige & Skoog
n.m.	Not measurable
NOS	Nopaline synthase promoter
n.t.	Nucleotide
OD	Optical density
ORF	Open reading frame
P	Proline
P.	*Populus*
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pma1	Plasma membrane ATPase promoter
PtSUT	Sucrose transporter of *Populus trichocarpa*
Q	Glutamine
R	Arginine
Ri	Root inducing
RIN	Ripening inhibitor
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
S	Serine
SUT	Sucrose transporter
T	Threonine
Ti	Tumor inducing
TL	T-DNA left border
TR	T-DNA right border
TSS	Transformation and storage solution

UV	Ultra violette
V	Valine
Y	Tyrosine
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose

Primer properties

Table 16: Names, sequences and annealing temperatures of selected primers for the amplification of genes and the peroxidase promoter fragments done in this work. Sequences are given in 5`→3` direction.

Amplificated fragment	Forward primer name	Forward primer sequence 5'→3'	Reverse primer name	Reverse primer sequence 5'→3'	Annealing temperature [°C]
PtSUT1	PtSUT1.1hin	GAGTGATAGCCCACATGG	PtSUT1.1rck	CAGATCAATGGAATGCAGC	53.5
PtSUT3	PtSUT1.2hin	CCATGCAAACCCGCTTCC	PtSUT1.2rck	GCAAGTAATGGAATGCAGC	50.2
PtSUT4	PtSUT4.1hin	CCATGTCAGTCGCTAACC	PtSUT4.1rck	CTCATGAGAAGACCATGG	52.6
PtSUT5	PtSUT2.1hin	GAAGAAGATGGAGTCGGC	PtSUT2.1rck	CACGGTTTGGTGGCTAGCC	55.4
PtSUT6	PtSUT2.2hin	AGAAGAAGATGGAGTCGG	PtSUT2.2rck	CATGGTGTGGTGGTTAGCC	50.7
PtSUT4	SUT-pJet_ Xma_FOR	TGGCCCGGGTTTTTCAGCAAG	SUT-pJet_ Xho_REV	ATCTCTCGAGAAGATCTCCTACAATATTCTC	57
PtSUT5					
PtSUT6					
P1	pJetMCXmaI	CAGATCTCCCGGGTGG	PtPeroxP_P1	TCAGTACCATCGATTTTAA	50.5
P2	pJetMCXmaI	CAGATCTCCCGGGTGG	PtPeroxP_P2#2	GGAATATGATCGATTACCTCGTT	55.0
P3	pJetMCXmaI	CAGATCTCCCGGGTGG	PtPeroxP_P3#2	ACGTTAATAATCGATTTTCAG	49.0
P4	pJetMCXmaI	CAGATCTCCCGGGTGG	PtPeroxP_P4	CTGACAATCGATGGATG	50.4
PAlf1	pJet1.2_TYFP_for	GGCGTAATACGACTCACTATAGG	Peroxalf1_2b_Bam	ATTGGATCCAAATTGTTTGTCGG	60.8
PAlf2	Perox-alf2for	TTGGGATCCGATTCAATGTAATTTAAGC	pJET1.2_rev	AAGAACATCGATTTTCCATGGCAG	56.2

Table 17: Names, sequences and annealing temperatures of selected primers for gene expression analyses done in this work. Sequences are given in 5`→3` direction.

Amplificated fragment	Forward primer name	Forward primer sequence 5‘→3‘	Reverse primer name	Reverse primer sequence 5‘→3‘	Annealing temperature [°C]
PtSUT1	PoptrSUT1.1-2a	GAGGGCTGCCACCAGTAGTAG	PoptrSUT1.1-3b	GGAAAGAAGTTTTAAAAAAGG	50
PtSUT3	PoptrSUT1.2-2a	TGTTGCAGCCGCAGTTAG	PoptrSUT1.2-2b	TTATTACATTTTTGCAAG	50
PtSUT4	PtSUT4.1-1a	CGAACATCTTAATGGCTC	PtSUT4.1-1b	AATTACCACAATGCTATCTG	50
PtSUT5	PoptrSUT2.1-2a	TCACAATGCTGACATCAAT	PoptrSUT2.1-2b	ATCTCTGTGAATGCAAG	50
PtSUT6	PoptrSUT2.2-2a	TCAACATGTTGACATCGAAG	PoptrSUT2.2-2b	CCATCTCTCTGACTGTAGAC	50
GUS	gusA_end_FW	ATCTCTTTGATGTGCTGTGC	gusA_end_REV	ATCCCTTTCTTGTTACCGC	54
ubiquitin	UBQ-for	GTTGATTTTTGCTGGGAAGC	UBQ-rev	GATCTTGGCCTTCACGTTGT	54
nptII	NTP2_FOR	GGACATAGCGTTGGCTACC	NTP2_REV	TCAGAAGAACTCGTCAAGAAGGCG	54

List of chemicals

Table 18a: List of chemicals used in this work and names of their providers. Due to their purity all chemicals are appropriate for molecular biological techniques. The list is completed on the following page.

Name	Provider
Acetosyringone	Fluka
Agar	Serva
SeaKem® LE Agarose	FMC BioProducts
TopVision™LE GQ Agarose	Thermo Scientific
Albumin from bovine serum	Carl Roth
Ampicillin sodium salt	Sigma Aldrich
Biotin	Duchefa
Calcium chloride dihydrate	Merck
Calcium pantothenate	Duchefa
Carbenicillin disodium	Duchefa
Casein Hydrolysate	Duchefa
Cefotaxime sodium salt	Duchefa
L-Cysteine hydrochloride monohydrate	Fluka
Deoxyribonuclease I from bovine pancreas	Sigma Aldrich
Cesium chloride	Carl Roth
2′-Deoxyadenosine 5′-triphosphate disodium salt	Thermo Scientific
2′-Deoxycytidine 5′-triphosphate disodium salt	Thermo Scientific
2′-Deoxyguanosine 5′-triphosphate trisodium salt	Thermo Scientific
3′-Deoxythymidine 5′-triphosphate sodium salt	Thermo Scientific
Diethyl pyrocarbonate	Carl Roth
3′,5′-Dimethoxy-4′-hydroxyacetophenone	Fluka
Dimethyl sulfoxide	Sigma Aldrich
Disodium hydrogen phosphate dihydrate	Fluka
DL-Dithiothreitol	Carl Roth
DNA from fish sperm	Serva
Ethanol	Sigma Aldrich
Ethidium bromide	Carl Roth
Ethylenediaminetetraacetic acid	Serva
Gentamicin sulfate	Carl Roth
Glucose D(+)	Duchefa
L-Glutamine	Sigma Aldrich
Glycerol	Merck
Guanidinium isothiocyanate	Serva
L-Histidine	Carl Roth
Hydrochloric acid	Carl Roth
Indol-3-Acetic-Acid	Sigma Aldrich
Kanamycin sulfate	Serva
N-Lauroylsarcosine sodium salt	Sigma Aldrich
L-Leucine	Carl Roth

Table 18b: Second part of the list of chemicals used in this work and names of their providers. Due to their purity all chemicals are appropriate for molecular biological techniques.

Name	Provider
Lithium acetate	Carl Roth
Magnesium chloride hexahydrate	Fluka
Magnesium sulfate heptahydrate	Carl Roth
D-(+)-Maltose monohydrate	Merck
2-Mercaptoethanol	Carl Roth
4-Methylumbelliferone	Carl Roth
4-Methylumbelliferyl-β-d-glucuronid dihydrate	Carl Roth
4-Morpholineethanesulfonic acid	Carl Roth
Murashige & Skoog medium including vitamins	Duchefa
1-Naphthaleneacetic acid	Duchefa
Peptone, soy derived	Carl Roth
Poly(ethylene glycol) 3,350	Sigma Aldrich
Poly(ethylene glycol) 4,000	Serva
Polyvinylpyrrolidone 10	Sigma Aldrich
Potassium acetate	Riedel
Potassium chloride	Riedel
Potassium hydroxide	Riedel
2-Propanol	VWR
Ribonuclease A from bovine pancreas	Sigma Aldrich
Sodium acetate	Riedel
Sodium chloride	Fluka
Sodium dihydrogen phosphate monohydrate	Merck
Sodium dodecyl sulfate	Carl Roth
Sodium hydroxide	Merck
Sucrose	Sigma Aldrich
Tetracycline	Sigma Aldrich
Thidiazuron	Duchefa
Tris(hydroxymethyl)aminomethane	Sigma Aldrich
Triton X-100	Serva
Uracil	Serva
Yeast Extract	Difco
Yeast Nitrogen Base with amino acids	BD Difco™
Zeatine, trans isomer	Duchefa

Amino acid sequences of the amplified *PtSUT*s (N- to C-terminal end)

>*PtSUT1*

MESGVRKENNPPSSSFSLQQQPPATNPSPLRKIIMVASIAAGVQFGWALQLSLLTPYVQLLGIPHTWA
AFIWLCGPISGMLVQPTVGYYSDRCTSRFGRRSPFIAAGAGFVAISVFLIGYAADIGHLSGDSLTKTAK
PRAIAVFVVGFWILDVANNMLQGPCRAFLADLSGTDHKKTRTANAFYSFFMAVGNVLGFASGSYTHL
YRIFPFSRTKACDVYCANLKSCFFISIALLLTLTILALSYVREKPWSPEGSSGDGGNEEEKEVEGGEAK
ESTPAPFFGEIVAALKNLQRPMRILLLVTCLNWVAWFPFLLFDTDWMGREVYGGDSSRNADQLKMYD
RGVRAGALGLLLNSVVLGFTSLGVEVLARGVGGVKRLWGIVNFILAICLAMTILITKVAQSNRRYTTVN
GGTHLLPPPSGVKAGALALFAVMGIPQAITYSIPFALASIFSNTSGAGQGLSLGVLNLSIVIPQMVVSVA
AGPWDALFGGGNLPAFVVGAVAAAASGILAFTMLPSPPPDIPSNKRAATSSTAAFH

>*PtSUT4*

MSVANPEPHRPSRSRQTNRPPSTRHHQQQSSRVPLRQLLRVTSIAGGIQFGWALQLSLLTPYVQELG
IPHKWASIIWLCGPLSGLFVQPLVGVISDRCTSRFGRRRPFIVAGSLLIAISVLIIGHSADIGWWLGDRG
GVRPRAIGAFVFGFWILDVANNMTQGPCRALLADLTGKDHRRTRVANAYFSLFMAIGNILGFATGSYN
GWYKVFPFTITSACNIDCANLKSAFYLDVVFMAITACISISAAQESPLDLPARSMLADEEMPGQSNSEQ
EAFLWELFGTFRCFPSTVWIILLVTALNWIGWFPFLLFDTDWMGREIYGGKPNEGQNYNTGVRMGAF
GLMFNSVILGVTSVLMEKLCSKWGAGFLWGLSNILMALCFLSMLVLYYVASHIGYMGHNLPPDSIVVIA
LVIFAVLGMPLAITYSVPYAMVSSRIESLGLGQGLSMGVLNLAIVLPQVVVSLGSGPWDQIFGGGNSPA
IAIGALAAFAAGIIAILGIPRSGVQKPMVFS

>*PtSUT5*

MESAPIRVPYRNLKKEIEVEMVGLEMESPPSPPRIQSPHTHNSDADLRSQSTRHHISLITLVLSCTVAA
GVQFGWALQLSLLTPYIQTLGIGHAFSSFIWLCGPITGLVVQPCVGIWSDKCSSKFGRRRPFILAGSLM
ISVAVIIIGFSADIGYVLGDTEEHCSKFKGTRTWAAFVFVIGFWMLDLANNTVQGPARALLADLSGPDQ
HNLSNAVFCSWMAVGNILGFSAGASGSWNRWFPFLMNRACCEACGNLKAAFLVAVVFLTFCTLVTLY
FADEVPLNVNQPRHLSDSAPLLNGSQQNGHELSTSESHLPGLDNLSGNGNNHDHELRMNSKHANSV
GDQNENFSDGPGAVLVNLLTSLRHLPPGMHSVLVVMALTWLSWFPFFLFDTDWMGREVYHGDPKG
NSNEVELYDQGVREGAFGLLLNSVVLGISSFLIEPMCRRLGSRFVWAMSNLIVFVCMAGTAVISLISVG
EYSEGIEHVIGGNAPIRIAALIVFALLGFPLAITYSVPFSVTAELTADSGGGQGLAIGVLNLAIVIPQMIISIG
AGPWDALFGGGNIPAFVLASVSALAAGVIATLKLPNLSSRSFQSGFHFG

>*PtSUT6*

MESAPIRVPYRKLKKEIEVEMVSLEVESSPSPSPPPPRIQSPLNPNSNGDFRSQTTKHQISFTTLVLSC
TVAAGVQFGWALQLSLLTPYIQTLGIGHAFSSFIWLCGPITGLVVQPCVGIWSDKCSSKFGRRRPFILA
GALMISIAVIIIGFSADIGYVLGDTEEHCSKFKGTRMRAAFVFVIGFWMLDLANNTVQGPARALLADLSG
PDQHNISNAVFCSWMAVGNILGFSAGASGSWSRWFPFLMNRACCEACGNLKAAFLVAVVFLLFCTLV
TLYFADEVPLNVNQPRHLSDSAPLLNDPQQNSHELSKSEFHTPGLDNLSGNSTDHDYEPSMNSKHAN
SVGGQNENFSDGPGAVMVNLLTSLRHLPPGMHSVLLVMALTWLSWFPFFLFDTDWMGREVYHGDP
KGTSNEVKLYDQGVREGAFGLLLNSVVLGISSFLIEPMCKKLGSRLVWAMSNFIVFVCMAGTAIISLISV
GEYSEGIEHVIGGNAPIRIASLIVFALLGFPLAITYSVPFSVTAELTADTGGGQGLAIGVLNLAIVIPQMIVS
IGAGPWDALFGGGNIPAFVLASVCALAAGVYAALKLPNLSSSSFSGFHFG

Nucleotide sequences of the amplified peroxidase promoter and its fragments (5'→3')

>P

CTTATTCAGTTACGTGTCGTCTGTTTGAAGATCCTGATTTTTCATAGAACACTTCAAATTAATCCCC
CTGTTAGGGTTTTTCTCAATGGAGTTTCCAAACTACTGATATTCTCAATCGTGTTCCACCATCCTTC
ATTCTTTCCCATTTGGATCGCTATGATATTCTGATATAAGGGTGGAAAAAGGGGCCAGTCAATTAG
GTCCTAGTTACCTCGATCAGATAACATTAACTGACAGTCGATGGATGTTGATGAAGGGACCCAAT
TAGGAACTGATAAAATATGAGGGACCTAATAAATAAAATCAATAAATAGTTTGAAGAAAGAGACTA
ACAGCTTCTCTGTTGAAGCTGTAATATCTATCAGATAGCTGCTGATGTTTAGAATGGACATGCAGA
CATGGAAATATAAGCATTGGTGCTATCCTTAAGGACAGGCACGTTAATATTCGAGTTTCAGTTGTT
GGTTTCTAAGGATTTAATTCTATAATTTTGATCTTTGTGTTGCATTGATAATCTGAGATGTTCTTCAT
ATTTTCCTGCAAAGATTTGTATTGATCTTCATCTTATGGTTGTGCATTTAAAGCATGCAAGTGTTGG
TCTGGCTTTGAGATGCACTGCTTGGTGCTAGACAGCTGCTTACTTTAGCTTGTTCTTTTTATGGTT
TGTCCTGTTGACTTGCTGCAGGTCTGGCCTTCTGGTTTTGCTTTCGAAGAAGTAAAATAGAAGAG
CTCCAACCATTCACTTCAGAACACATTGAAATGTGGCTGGCTAGCATTATCATGCCAAGATGTTTC
TGATGACGCAAAACATCTCTTGCATCATAAGGAATATGCTCGAGTACCTCGTTATGTGGCTAGCTA
GCACTAGCTAGCTGATCCAAGACCAAAAAAAGGTATTGCAGAAAGGATATAATTTATGGTTTCTAG
TGGCTGGATAATGTTAGAGGTTGTAAGGTGGCTCTGTCATCAGTCCACTTCCATATTATTCTTCAT
AATTAATAGCCGTAGTTGAAGTGAAGCTGCCATACACTTAATGTTCATGGCAATTGCCAGTTATTT
AATCAAGCAGAAGTCAGCTCCAGCTGCTACTCTCTAATTAGATATCAGAATTGAGAAATTATCCTT
ATCATGTTTAGTTGCTGCCATTAAATTATAAAAACATGTCCGTATTCGGGTGGCTAAGCCTGTCAG
GTACAGACAAAATTAGAATTCTTCTTAATCTCAGTACCTTCGATTTTAATATGACACTAGTGCTCTT
AAGGGTGCTTTGATTCATGAACTTGCAAATAAATGCTTAAATTACATTGAATCTGATAGCAATGCA
CCCACTTTATTTCATTCAAATTGTTTGTCGGTAATTTTAAATATTCAAGTCTGTAAAATCTATGCAAA
TGGTAAAGCTTAGTACAGCCTTTTGAGCACAGAAACCAGCCGCAGCCGCCATACATCACCAGCAC
TAATACTAAATAGAACCATGTGCTATATTAGTGTACACAAGTTGAGATTAACTTTGTCCATGATGTT
GGTTATAAATAGAACTTCAAGTTTTTTTCAATGCTTAAGTATCTGATCCTTAATCTTGTTAGAACAA
GTTTTCTTTCAACACGTCTTGTGAGAGAACT

>P1

TCAGTACCTTCGATTTTAATATGACACTAGTGCTCTTAAGGGTGCTTTGATTCATGAACTTGCAAAT
AAATGCTTAAATTACATTGAATCTGATAGCAATGCACCCACTTTATTTCATTCAAATTGTTTGTCGG
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T

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AGTGAGCC

Exemplary calculation of relative *GUS* gene expression rate driven by the peroxidase promoter P

Raw data from quantitative RT-PCR (Cp-values):

GUS: 25.37

nptII: 28.36

ubiquitin: 22.63

According to a dilution series of all three gene-specific DNA fragments linear equations for determination of the number of template molecules are available:

y represents the Cp-value

x represents the logarithm of number of template molecules

GUS: y = -3.5897x + 44.538

nptII: y = -3.361x + 42.825

ubiquitin: y = -3.3003x + 31.78 (Nehls, unpublished)

The numbers of template molecules due to the above mentioned equations are:

GUS: 218,636.16

nptII: 20,126.98

ubiquitin: 592.21

The *GUS*/*nptII* ratio of template molecules was calibrated to exclude diminishing effects on *GUS* gene expression of non-transgenic roots. This ratio was calibrated to the number of *ubiquitin* template molecules allowing a comparison between different RNA samples:

$$\text{relative } GUS \text{ expression} = \frac{(\text{number of } GUS \text{ templates}/\text{number of } nptII \text{ templates})}{\text{number of } ubiquitin \text{ templates}}$$

The relative *GUS* gene expression rate driven by the peroxidase promoter P in this exemplary calculation is 0.018.

Exemplary calculation of the ß-glucuronidase activity in the enzyme assay using the longest promoter fragment P

Raw data: fluorescence of 4-methylumbelliferone, released from 4-methylumbelliferyl-β-d-glucuronide by β-glucoronidase activity applying 5 µl of *E. coli* protein extract

Tab. 19: Raw data of the fluorescence of 4-methylumbelliferone applying 5 µl of *E. coli* protein extract.

interval	raw data
1	31.22
2	49.89
3	81.06
4	134.07
5	212.43
6	314.70
7	455.03
8	602.83
9	800.17
10	1019.00

Calculation of the slope using Excel across the 10 intervals: 107.98

Calculation of the slope across 10 intervals was done for all 120 intervals:

Tab. 20: Slope calculation across 10 intervals using the raw data of applying 5 µl of *E. coli* protein extract. aLOD = above detection limit.

interval	slope
1 – 10	107.98
11 – 20	251.45
21 – 30	172.98
31 – 40	80.82
41 – 50	aLOD-
51 – 60	aLOD
61 – 70	aLOD
71 – 80	aLOD
81 – 90	aLOD
91 – 100	aLOD
101 – 110	aLOD
111 - 120	aLOD

Maximal slope across 10 intervals: 251.45

According to a dilution series of 4-methylumbelliferone a linear equation for determination of produced 4-methylumbelliferone is available:

x represents amount of produced 4-methylumbelliferone
y represents the maximal slope across 10 intervals
$y = 1.9389x – 0.4329$

The amount of produced 4-methylumbelliferone is 129,91 µM across 10 intervals. The maximal turnover rate of the substrate 4-methylum-belliferyl-β-d-glucuronide by β-glucoronidase resulting in 4-methylumbelliferone per minute (2 intervals) is 25.981 µmol/min.