CLE/RLK regulated vascular signalling pathways in plants

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Abstract

Entire postembryonic production of plant tissues are maintained by meristems. These specialized structures provide a pool of undifferentiated stem cells and a limited population of proliferating cells which are destined for differentiation in order to generate a variety of tissues in the plant body. For the forest trees, a large part of the biomass is produced by a secondary meristem called vascular cambium. Vascular cambium forms a continuous cylinder of meristematic cells in the stem, producing both secondary phloem and secondary xylem or wood. Maintenance and differentiation of meristems are much conserved and strictly regulated for the production of correct tissues and organs.

Receptor-like kinases (RLKs) are characterized by the presence of a signal sequence, a putative amino-terminal extracellular domain connected to a carboxyl-terminal intracellular kinase domain with a trans-membrane domain. They control a wide-range of physiological processes, including development, disease resistance, hormone perception, and self-incompatibility. Leucine-rich repeat receptor-like kinases (LRR-RLKs) represent the largest group of RLKs in the Arabidopsis thaliana genome, with more than 200 members.

Several LRR-RLKs and their putative ligands CLAVATA3 (CLV3)/ Endosperm Surrounding Region (ESR)-related (CLE) peptides have been found to be involved in the regulation of vascular development. In the current study, the main aim was to study the tissue-specific expression patterns of LRR-RLK genes in A. thaliana by generating promoter::GUS transcriptional fusions. The results confirmed that these genes are expressed in the vasculature of the plants. Moreover, Populus orthologs of the CLE genes were detected by bioinformatic tools as putative ligands of LRR-RLKs and an extensive quantitative Real-Time Reverse Transcriptase PCR (qRT-PCR) analysis was performed to test for significant changes in transcript levels across different tissue types. As a result, a collection of potential candidate genes for vascular development were identified.
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I. Introduction

Wood is a crucial component for the conduction of water and solutes in the plant body as well as for the mechanical support in trees and increase in the plant biomass. It is also harnessed frequently as a crude material in many aspects of human life such as construction, manufacturing, energy generation and pulp industry.

In forest trees wood formation (xylogenesis) is initiated in a lateral meristem called vascular cambium. The vascular cambium is a derivative of the procambium and interfascicular cambium and arranged in radial files of proliferating meristematic cells (Ubeda-Tomas et al. 2007). Each file in the radial zone supposedly harbors one initial cell analogous to the stem cell in animals which when divided generates two daughter cells; one (the initial) keeping the meristematic ability and staying in the cambium, and another one (the derivative of the initial) becoming a pre-destined mother cell. This implies that cells that are programmed to become phloem are called phloem mother cells, whereas cells which are programmed to become xylem are called xylem mother cells (Mellerowicz et al. 2001). The initial cells of vascular cambium exist in two morphologically distinct forms: fusiform initials and ray initials, which generate the axial and radial system, respectively (Ubeda-Tomas et al. 2007). Due to the difficulties in discriminating the initial and the mother cell types ultra-structurally from each other, these initials and mother cells are overall referred as the cambial zone. Periclinal (tangential) divisions of the cells in the cambial zone eventually generate the secondary phloem tissue on the outside and secondary xylem or wood on the inside (Schrader et al. 2004).

Although many anatomical studies have been performed on the cambial zone and its derivatives, very little is currently known about the molecular and genetic mechanisms regulating the wood development. Despite the unquestionable importance of mutant isolation and analysis in meristem biology; a classical genetic approach and mutant screening are particularly hard to execute on trees due to several reasons including their long generation times, physical size, slow growth rate, and relatively large genome sizes. Therefore, other potential possibilities for studying the mechanisms behind the cambial growth and wood formation were considered. Eventually the utilization of herbaceous species instead of trees has become the method of interest (Schrader et al. 2004; Ubeda-Tomas et al. 2007). Recent studies on the model annual plant *A. thaliana* have revealed that, *Arabidopsis* hypocotyls can
undergo secondary growth under appropriate conditions. Moreover the development of both vascular and cork cambium were also observed. When the anatomy of Arabidopsis hypocotyls with secondary growth is compared to the stem of an angiosperm tree such as poplar, the resemblance between the cellular organizations is remarkable (Chaffey et al. 2002). Unfortunately during Arabidopsis secondary growth, parenchyma rays are not generated. As a result, Arabidopsis can be considered as a model system to address some essential aspects of wood development in an accelerated pace (Nieminan et al. 2004).

Apart from the anatomical similarities in the secondary growth, the sequencing and constant assembly of poplar genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) and the generation of large expressed sequence tag (EST) resources for transcript profiling of poplar genes (http://www.populus.db.umu.se/) have persuaded the scientists to utilize poplar as a model tree system (Tuskan et al. 2006). Furthermore, it also let them to compare the poplar genome comprehensively with other plant genomes such as Arabidopsis. Therefore, identification and characterization of the orthologs of poplar genes in Arabidopsis can be used as initial approaches to identify the prospective functions of poplar genes during wood formation (Schrader et al. 2004; Ubeda-Tomas et al. 2007).

The following report describes my project for the characterization of certain LRR-RLKs and their putative ligands CLE proteins involved in vascular development of Arabidopsis and Populus. During the project, the tissue-specific expression patterns of several LRR-RLK genes in A. thaliana were investigated by generating promoter::GUS transcriptional fusions and the basic trans-genetic modification techniques were practiced. The candidate genes for this study were previously chosen by my supervisor Bo Zheng, and post-doctoral researcher Linbin Zhang using the data from detailed transcript profiling of the poplar wood-forming tissues (http://www.populus.db.umu.se/).

In addition, Populus orthologs of the Arabidopsis CLE genes were systematically identified by using bioinformatic tools and differential expression of CLE plant peptide hormones in Populus were determined by quantitative qRT-PCR using a collection of 43 gene-specific primers. As a result, a collection of potential candidate genes for vascular development were identified.
II. Background

1. Shoot Apical Meristem (SAM)

In plant species positional cues rather than cell lineages establish the identity of the stem cells and the developmental destiny of the stem cell derivatives; which implies that, environmental factors, besides the existing tissues and organs affect the determination and differentiation of new tissues and organs (Schrader et al. 2004). Therefore most of the research focuses on these positional cues in order to understand the molecular mechanisms lying beneath.

Without any doubt, one of the evidences regarding the existence of the positional cues comes from the studies on the shoot apical meristem (SAM). SAM supplies the cell pools for the formation of all the aerial organs in the plant body following germination. It is initially generated at embryogenesis; during the maturation of the embryo it takes a dome shaped structure (Nakajima et al. 2002).

The SAM of the mature embryo has been separated into 3 domains according to the histological differences: the central zone, the peripheral zone and the rib zone. The central zone is rather a small region which resides in the center near the top of the SAM and harbors a stem cell population which has a slow rate of cell proliferation. Following the cell division, the peripheral progenies of these stem cells are recruited progressively towards the peripheral and rib zones. Cells residing in the peripheral and rib zones have the ability to divide fast. In the later phases of development they contribute to the production of lateral organs and stem, respectively (Nakajima et al. 2002).

Based on the clonal relationships and cell fate, the SAM of the mature plants is divided into 3 groups: the epidermal, subepidermal and underlying cells (L1, L2 and L3 layers). Cells residing in the L1 and L2 layers show anticlinal division (perpendicular to the surface plane) pattern, and give rise to the epidermal and subepidermal layers of the lateral organs such as leaves and flowers. On the other hand, cells in the L3 layer reveal a more complicated proliferation pattern and give rise to the inner tissues of the lateral organs (Nakajima et al. 2002; Miwa et al. 2009).
In order for repetitive initiation of shoot structures during plant development, the balance between the number of stem cells and the programmed differentiation of their descendants needs to be sustained consistently. This self perpetuation of the stem cell population in the central zone of SAM is assured by a feedback loop between WUSCHEL (WUS) homeodomain transcription factor and CLAVATA (CLV1, CLV2 and CLV3) genes (Brand et al. 2000; Schoof et al. 2000).

2. WUS Function in SAM

Loss-of-function wus mutants reveal growth malfunction throughout the development of the plant (Bäurle et al. 2005). In wus mutants the embryonic shoot apical meristem is initiated normally, whereas the development terminates prematurely after the growth of a few lateral organs. Lateral organs are usually established ectopically from the center of the SAM. In the further phases of development, wus mutants initiate new SAMs at the base of the cotyledons and leaves, but all of them fail to develop. This stop-and-start development prototype gives a bushy appearance to the plant, which also persists during the floral stages (Sharma et al. 2003). These observations showed that in the absence of WUS, the ability to control the cell proliferation is lost in SAM, and stem cells are recruited for the formation of lateral organs prior to replenishing themselves. Moreover, the expression studies for WUS mRNA showed that the expression is confined to a few cells lying beneath the stem cells and these cells act as an organizing centre (OC); which proposes that WUS promotes stem cell proliferation in a non-cell-autonomous manner in SAM (Mayer et al. 1998).

3. CLV Function in SAM

clavata (clv) loss-of-function mutants show an opposite phenotype compared to the wus loss-of-function mutants. They produce excess amounts of stem cells in the SAM and therefore generate enlarged meristems. These observations indicated that CLV (CLV 1, CLV 2 and CLV3) genes restrict meristematic cell accumulation and suggested that they trigger the recruitment of stem cells into differentiation (Clark et al. 1993; Clark et al.1995; Kayes et al. 1998). All three CLV genes have been studied and their products discovered to encode components of a single signal transduction pathway in SAM (Jun et al. 2008). These findings correlate with the fact that mutants from all three genes show similar phenotypes.
The *CLAVATA 1 (CLV1)* encodes a LRR-RLK protein. RLKs are characterized by the presence of an amino-terminal extracellular domain connected to a carboxyl-terminal intracellular kinase domain with a trans-membrane domain. They are involved in a broad variety of mechanisms such as development, disease resistance, hormone perception, and self-incompatibility. They are the largest group of RLKs in the *A. thaliana* genome, including more than 200 members (Shiu et al. 2001; Jun et al. 2008).

*CLAVATA2 (CLV2)* encodes a receptor-like protein with an extracellular LRR, connected to a short cytoplasmic tail with a trans-membrane domain. It shows a similar domain organization to CLV1, however lacks the intracellular kinase domain (Jun et al. 2008).

The interactions between the CLV1 and CLV2 proteins come from the experiments with protein gel blot analysis. The experiments revealed that the CLV1 protein exists in two protein complexes with different molecular weights, ~450 kDa and ~185 kDa. The ~185 kDa complex is composed of a CLV1-CLV2 heterodimer linked by di-sulfide bonds; whereas the ~450 kDa complex includes two other subunits besides the ~185 kDa complex: a kinase-associated protein phosphatase (KAPP) and a Rho GTPase–related protein (Rop). KAPP is thought to be a negative regulator of CLV1 signaling and Rop is considered to participate downstream of this signal transduction pathway (Trotochaud et al. 1999).

*CLAVAT3 (CLV3)* encodes a 96-amino acid long polypeptide which is a member of the CLE protein family. During regulation of stem cell identity in SAM, CLV1 and CLV2 form a receptor complex to which CLV3 binds as a ligand, initiates the assembly of receptor complex and promotes differentiation (Shiu et al. 2001; Jun et al. 2008).

The expression studies on different *CLV* mRNAs revealed that *CLV3* mRNA is confined in the L1 and L2 layers of central zone in SAM, and *CLV1* mRNA exists in the cells just beneath, in the L3 layer. Therefore CLV3 is secreted into the extracellular space in order to interact with the CLV1-CLV2 complex in a non cell-autonomous manner (Trotochaud et al. 1999; Clark et al.1995; Shiu et al. 2001; Jun et al. 2008).

CLE proteins are presently the best understood family of small polypeptides in plants. Members of this family exist in many different plant species and share several common properties. They encode small polypeptides (≤15 kDa). They include a short hydrophobic
amino acid signal sequence at their amino-termini for directing the peptide to the secretory pathways. Their nucleotide and amino acid sequences are largely dissimilar but they all possess a conserved 12 amino acid domain known as the CLE domain at or near their carboxyl termini. Although this conserved region has long been proposed to be the active mature form of CLV3, there was no direct evidence to prove this (Jun et al. 2008). Recently, a mature form of CLV3 which consists of 12 amino acids corresponding to the conserved region has been identified by in situ matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Kondo et al. 2006). Moreover, the application of chemically synthesized 12 amino acids mature form of CLV3 to the plants gave a phenotype similar to clv3 over-expression phenotype, which is now consistent with the idea that the mature form is the secreted active form during the regulation of the stem cell identity in SAM (Miwa et al. 2009).

CLE peptides can be classified into two groups according to their functional diversification and conserved domain organization: A-type CLE peptides (CLV3 like) and B-type CLE peptides (CLE 41-44 like). A-type CLE peptides promote the cell differentiation in root and shoot apical meristems, in contrast B-type CLE peptides do not. CLE41 and 44 have the same conserved CLE region with tracheary element differentiation factor (TDIF) which suppresses the differentiation of the stem cells into tracheary elements (TE) in xylogenic cell cultures of Zinnia elegans. Moreover, it has been recently showed that TDIF is synthesized and secreted from phloem and its neighboring cells, binds to a LRR-RLK called PXY (phloem intercalated with xylem) on procambial vascular cells in A. thaliana. This interaction eventually promotes the proliferation of procambial cells whereas it delays differentiation into xylem cell lineages (Whitford et al. 2008; Hirakawa et. al 2008).

4. Regulatory Loop between WUS and CLV

The self maintenance of the stem cell population in the central zone of SAM is assured by a negative feedback loop between WUS and CLAVATA genes (Brand et al. 2000; Schoof et al. 2000). The CLV3 peptide is synthesized in the L1 and L2 layer of cells in the central zone of the SAM, cleaved into the 12 amino acid mature form, and secreted to the apoplastic space, where it is perceived by the CLV1-CLV2 receptor complex residing in the L3 layer. This receptor complex thenconveys the signal to inhibit the WUS transcription. Hence, the proliferation of the stem cells in the OC ceases and cell differentiation in the peripheral zones.
is initiated. Conversely, *WUS* also acts non-cell autonomously to promote the *CLV3* expression (Figure 1) (Miwa et al. 2009).

Recently, the detailed transcript profiling of the poplar wood-forming tissues (http://www.populus.db.umu.se/) has been accomplished and differential regulation of *LRR-RLKs* and *CLV3*-related genes across the cambial zone of hybrid aspen has been detected, which supports the possibility that a similar mechanism to SAM maintenance might be active during vascular development as well (Schrader et al. 2004).

**Figure 1: Signal Transduction in SAM.** *CLV3* peptide is synthesized, maturated, and secreted to the apoplastic space, where it is perceived by the *CLV1-CLV2* receptor complex. This receptor complex then conveys the signal to inhibit the *WUS* transcription.
III. Materials and Methods

1. Expression Analysis of LRR-RLK Genes

A. Plant Materials and Growth Conditions

*A. thaliana*, ecotype Columbia (Col) seeds were sown in soil: vermiculate (3: 1) mixture and vernalized for 4 days at 4º C in the dark for the simultaneous germination of all seeds. Following germination, the plants were cultivated in a growth chamber with long day photoperiod (23° C and 16 h light, 18º C and 8 h dark, under 70% humidity). Transformed plants were also grown under same conditions till seed formation.

To determine the GUS activity of the seedlings, sterile transformant seeds (T₁ generation) were germinated and selected on Murashige-Skoog (Duchefa Biochemie) medium supplemented with 10 g/L Sucrose, 8 g/L Plant Agar (Duchefa Biochemie) and 50 µg/mL kanamycin in an *in vitro* culture room with long day photoperiod (23° C and 16 h light, 18º C and 8 h dark, under 70% humidity) after 4 days of vernalization in the dark at 4º C. As a control, wild-type seeds of *A. thaliana*, ecotype Columbia (Col) were used.

B. Preparation of Plant Genomic DNA

The purification of plant genomic DNA using Plant DNAzol Reagent (Invitrogen) was performed as recommended by the manufacturer. Fresh *A. thaliana* leaves pulverized in liquid nitrogen were mixed with Plant DNAzol (Plant DNAzol: plant tissue = 0.3 ml: 0.1 g) and incubated at 25º C with shaking for 5 min. Chloroform (Chloroform: plant tissue = 0.3 ml: 0.1 g) was added to the resulting mixture, and it was further incubated at 25º C with shaking for another 5 min. Following this, the solution was centrifuged at 12000 x g for 10 min, and the resulting supernatant (the aqueous phase) was transferred to a fresh tube. For the precipitation of DNA, the aqueous phase was mixed with 0.225 ml of 100% ethanol (100% ethanol: the aqueous phase = 2: 1). The precipitated DNA was sedimented at 5000 x g for 4 min, and the resulting supernatant was removed. The DNA pellet was washed with 0.3 ml of Plant DNAzol-ethanol solution (Plant DNAzol: ethanol = 1: 0.75) and 0.3 ml of 75% ethanol, and then centrifuged at 5000 x g for 4 min. After removal of ethanol by decanting, the DNA pellet was air-dried and dissolved in 100 µl of Elution Buffer (Qiagen). The concentration and
quality of the DNA solution was determined by Thermo Scientific NanoDrop™ Spectrophotometer.

C. Ethanol Precipitation of DNA

1/10 volume of 3M NaOAc and 3 volumes of 99% ethanol was added to 1 volume of DNA solution and left at -20°C overnight. The solution was centrifuged for 30 min at 17000 x g to pellet the DNA. The resulting supernatant was discarded. The DNA pellet was washed in 70% ethanol for 1 h with shaking and centrifuged for 30 min at 17000 x g. After removal of ethanol by decanting, the DNA pellet was air-dried and dissolved in 100 µl of Elution Buffer (Qiagen). The concentration and the quality of the DNA solution were determined by Thermo Scientific NanoDrop™ Spectrophotometer.

D. BP Recombination Reaction and Generation of Entry Clones

Polymerase Chain Reaction

The promoter regions of LRR-RLK genes were amplified and flanked by attB sites by the following PCR program; 94°C for 3 min; 94°C for 45 sec, 56°C for 30 sec, 72°C for 2 min 30 sec, 4 cycles; 94°C for 45 sec, 62°C for 30 sec, 72°C for 2 min 30 sec, 32 cycles; 72°C for 10 min; 10°C for 30 min; with gene specific forward and reverse primers containing attB sites (Supplemental Data Table 1). The PCR mixtures contained 0.4 µM of each PCR primer and 25 µL of Phusion® Flash High-Fidelity PCR Master Mixture (2X, Finnzymes) in 50 µL of total reaction volume. During the PCR, genomic DNA extracted earlier was used as a template. Approximately 2-3 µL of the PCR products mixed with loading dye were loaded onto a 1% agarose 1X TAE (w/v) gel containing 0.0001 µg/µL of ethidium bromide and separated by gel electrophoresis. The gel was examined under shortwave UV light.

PCR Purification

After testing the accuracy of the PCR by gel electrophoresis, the desired DNA fragments, which did not show unspecific amplification, were purified by using QIAquick PCR Purification Kit (Qiagen). 5 volumes of Buffer PBI to 1 volume of PCR sample was added into each tube and mixed. QIAquick spin columns were placed into the provided 2 mL
collection tubes. To bind the DNA to the column, the samples were applied to the QIAquick columns, and centrifuged for 1 min at 17000 x g. Flow-through was discarded. To wash the DNA, 0.75 mL Buffer PE was added into the QIAquick columns, and centrifuged for 1 min at 17000 x g. Flow-through was discarded. The columns were centrifuged for an additional 1 min at 17000 x g to remove residual wash buffer. The QIAquick columns were placed into clean 1.5 mL micro-centrifuge tubes. To elute the DNA, 50 µL of Elution Buffer was added to the center of the column membranes. The columns were let to stand for 5 min at room temperature and then centrifuged for 1 min at 17000 x g. The concentration and the quality of the DNA solutions were determined by Thermo Scientific NanoDrop™ Spectrophotometer; meanwhile the presence of the desired fragments was confirmed by agarose gel electrophoresis. Approximately 2-3 µL of the purified products mixed with loading dye were loaded onto a 1% agarose 1X TAE (wt/v) gel containing 0.0001 µg/µL of ethidium bromide and separated by gel electrophoresis. The gel was examined using shortwave UV light.

**Gel Extraction**

After testing the accuracy of the PCR by gel electrophoresis, the desired DNA fragments, which showed unspecific amplification, were extracted from the agarose gel by using QIAquick Gel Extraction Kit (Qiagen). Approximately 45-47 µL of the PCR products mixed with loading dye were loaded onto 1% agarose 1X TAE (wt/v) gel and separated by gel electrophoresis. The DNA fragments were excised from the agarose gel with a clean, sharp scalpel. The gel slices were weighed in colorless tubes and for each tube 3 volumes of Buffer QG were added to 1 volume of gel (Buffer QG: gel slice = 300 µL: 100 mg). Tubes were incubated at 50º C for 10 min until the gel slices had completely dissolved. To help dissolve the gel slices, the tubes were vortexed every 2-3 min. QIAquick spin columns were placed into the provided 2 mL collection tubes. To bind the DNA to the column, the samples were applied to the QIAquick columns, and centrifuged for 1 min at 17000 x g. Flow-through was discarded. To wash the DNA, 0.75 mL Buffer PE was added into the QIAquick columns, and centrifuged for 1 min at 17000 x g. Flow-through was discarded. The columns were centrifuged for an additional 1 min at 17000 x g to remove residual wash buffer. The QIAquick columns were placed into clean 1.5 mL micro-centrifuge tubes. To elute the DNA, 50 µL of Elution Buffer was added to the center of the column membranes. The columns were let to stand for 5 min at room temperature and then centrifuged for 1 min at 17000 x g. The concentration and the quality of the DNA solutions were determined by Thermo Scientific
NanoDrop\textsuperscript{TM} Spectrophotometer; meanwhile the presence of the desired fragments was confirmed by agarose gel electrophoresis. Approximately 2-3 µL of the purified products mixed with loading dye were loaded onto a 1% agarose 1X TAE (wt/v) gel containing 0.0001 µg/µL of ethidium bromide and separated by gel electrophoresis. The gel was examined using shortwave UV light.

**BP Recombination Reaction**

In order to create entry clones, BP reaction was performed for all PCR and/or gel purified DNA fragments according to Gateway\textsuperscript{®} BP Clonase\textsuperscript{TM} II Enzyme Mix Catalog no. 11789-100 and Karimi et al., 2002. The BP Clonase\textsuperscript{TM} II Enzyme Mix (Invitrogen) was thawed on ice for about 2 min and vortexed briefly twice. 1 µL of BP Clonase\textsuperscript{TM} II Enzyme Mix (Invitrogen), 150 ng of DNA fragments, and 1 µL of pDONR\textsuperscript{TM}201 (Invitrogen) vector were added to clean PCR tubes, mixed well and the reactions were incubated at room temperature overnight. Plasmids carrying the insertions of the promoter regions of \textit{LRR-RLK} genes were then transformed into \textit{E. coli} DH5\(\alpha\) competent cells. 50 µL of competent cells and 5 µL of BP reaction solutions were mixed and kept on ice for 30 min. The cells were heat-shocked for 90 sec in 42\(^\circ\)C water-bath for the entry of the DNA into the competent cells. 250 µL of SOC medium was immediately added to each tube after heat-shock. Transformed cells were incubated at 37\(^\circ\) C for 1 h with vigorous shaking for recovery. 100 and 200 µL of culture for each transformation was spread aseptically on selective LA (Kanamycin 25 µg/mL) plates and incubated at 37\(^\circ\) C overnight.

**Colony Polymerase Chain Reaction**

The colonies were screened for the transformation of the plasmids carrying the insertions of the promoter regions of \textit{LRR-RLK} genes by a PCR. Colonies were used as templates for PCR reaction. The following PCR program; 94\(^\circ\) C for 3 min; 94\(^\circ\) C for 50 sec, 56\(^\circ\) C for 30 sec, 72\(^\circ\)C for 2 min 30 sec, 36 cycles; 72\(^\circ\) C for 10 min; 12\(^\circ\) C for 30 min was performed with gene specific forward and reverse primers containing \textit{attB} sites (Supplemental Data Table 1). The PCR mixtures contained 0.4 µM of each PCR primer and 12.5 µL of GoTaq\textsuperscript{®} Green Master Mix (Promega) in 25 µL of total reaction volume. 5 µL of the PCR products were loaded onto a 1% agarose 1X TAE (wt/v) gel containing 0.0001 µg/µL of ethidium bromide and separated by gel electrophoresis. The gel was examined using shortwave UV light.
**Plasmid Isolation**

Plasmids carrying the insertions of the promoter regions of *LRR-RLK* genes were then isolated from the selected positive colonies by using QIAprep® Spin Miniprep Kit (Qiagen). For each positive colony, 3 mL of selective liquid LB medium (Kanamycin 25 µg/L) was inoculated with its corresponding colony solution, and incubated at 37°C with vigorous shaking for 12-16 h. The bacterial cells were centrifuged for 45 sec at 17000 x g and then re-suspended in 250 µL of Buffer P1. To lyse the cells, 250 µL of Buffer P2 was added into each tube and mixed thoroughly by inverting the tube 4-6 times. For neutralization, 350 µL of Buffer N3 was added into each tube and mixed immediately and thoroughly by inverting the tube 4-6 times. The samples were centrifuged for 1 min at 17000 x g. QIAprep spin columns were placed into the provided 2 mL collection tubes. The supernatants were applied to the QIAprep spin columns, and centrifuged for 1 min at 17000 x g. Flow-through was discarded. To wash, 0.75 mL of Buffer PE was added into the columns, and centrifuged for 1 min at 17000 x g. Flow-through was discarded. The columns were centrifuged for an additional 1 min at 17000 x g to remove residual wash buffer. The QIAprep columns were placed into clean 1.5 mL micro-centrifuge tubes. To elute the plasmid DNA, 50 µL of Elution Buffer was added to the center of the column membranes. The columns were let to stand for 5 min at room temperature and then centrifuged for 1 min at 17000 x g. The concentration and the quality of the DNA solutions were determined by Thermo Scientific NanoDrop™ Spectrophotometer. Isolated plasmids were sent to sequencing prior to further experiments. Sequencing service was provided by Eurofins MWG Operon Company (Germany).

**E. LR Recombination Reaction and Generation of Expression Clones**

**LR Recombination Reaction**

In order to create expression clones, LR reaction was conducted for all the purified and sequenced plasmids carrying the insertions of the promoter regions of *LRR-RLK* genes according to Gateway® LR Clonase™ II Enzyme Mix Catalog no. 111791-100 and Karimi et al., 2002. The LR Clonase™ II Enzyme Mix (Invitrogen) was thawed on ice for about 2 min and vortexed briefly twice. 1 µL of LR Clonase™ II Enzyme Mix (Invitrogen), 100 ng of plasmid constructs, 1 µL of pKGWFS7™ vector (Invitrogen) having GUS::GFP fusion, and 2 µL of ddH$_2$O were added to clean PCR tubes, mixed well and the reactions were incubated at
room temperature overnight. Plasmids carrying GFP::GUS fusions under the control of the promoter regions of LRR-RLK genes were then transformed into E. coli DH5α competent cells as described previously, and 100 and 200 µL of culture for each transformation was spread aseptically on selective LA (Spectinomycin 50 µg/mL) plates and incubated at 37° C overnight. The colonies were then screened for the transformation of the plasmids by a PCR, and plasmids were isolated from the selected colonies as described previously.

**F. Agrobacterium Transformation**

Plasmids carrying the GUS::GFP fusion under the control of the promoter regions of LRR-RLK genes were transformed into Agrobacterium PMP 90RK strain as a binary vector. 20 µL of competent Agrobacterium cells and 0.1-1 µg of plasmid constructs in Elution Buffer (Qiagen) were added in the pre-cooled 2mm cuvette and mixed well by tabbing the cuvette. The mixtures were electroporated at 1.5 kV 400 olm 25 µF. 250 µL of SOC medium was immediately added to each cuvette after electroporation. Transformed cells were transferred to a clean micro-centrifuge tube, and incubated at 28° C for 4h with vigorous shaking for recovery. 10 µL of culture for each transformation was spread aseptically on selective YEB (Kanamycin 25 µg/mL, Spectinomycin 50 µg/mL) plates and incubated at 28° C for 2 days.

**G. Feeder Culture Preparation and Polymerase Chain Reaction**

Feeder culture for each Agrobacterium transformation was prepared in order to inoculate the large scale plant transformation culture. A single Agrobacterium colony was inoculated aseptically into 3 mL of selective liquid LB medium (Kanamycin 25 µg/mL, Spectinomycin 50 µg/mL) for selection. The cultures were incubated at 28° C overnight with vigorous shaking. Before the inoculation of the large scale transformation cultures, feeder cultures were screened by preparing a PCR. The following PCR program; 95° C for 3 min; 95° C for 30 sec, 59° C for 30 sec, 72° C for 1 min, 40 cycles; 72° C for 10 min; 12° C for 30 min was performed with gene specific attB forward and reverse primers. The PCR mixtures contained 0.4 µM of each PCR primer and 12.5 µL of GoTaq® Green Master Mix (Promega) in 25 µL of total reaction volume. 5 µL of the PCR products were loaded onto a 1% agarose 1X TAE (wt/v) gel containing 0.0001 µg/µL of ethidium bromide and separated by gel electrophoresis. The gel was examined using shortwave UV light.
H. Large Scale Transformation Culture Preparation and Floral Dipping

Transformation of plants was conducted on *A. thaliana*, ecotype Colombia (Col) plants after they began to bolt and floral inflorescences, according to Zhang et al., 2006. The day before floral dipping, each feeder culture was used to inoculate 200 mL of liquid LB medium supported by appropriate antibiotics (Kanamycin 25 μg/mL, Spectinomycin 50 μg/mL). The cultures were incubated at 28°C overnight with vigorous shaking until their OD<sub>600</sub> values were between 0.8-1.0. *Agrobacterium* cells were collected by centrifugation at 4000 x g for 10 min at room temperature. The cells were gently re-suspended in 200 mL of freshly made 5% (wt/vol) Sucrose, 0.03% Silwet L-77 (vol/vol) solution. Plants were inverted and aerial parts of the plants were dipped in the *Agrobacterium* cell suspension (10 sec) with gentle agitation. All bolts even the shorter auxiliary floral buds were submerged into the *Agrobacterium* cell suspension. Transformed plants were covered with plastic bags to maintain the high humidity for 48 h and grown in a growth chamber with long day photoperiod till the seed formation (23°C for 16 h light, 18°C for 8 h dark, under 70% humidity) (Figure 2).

**Figure 2: Stages during floral dip transformation method.** (A) Plants were grown until they contain approximately 20-30 inflorescences and some maturing siliques. (B) The aerial parts of the plants were dipped in to the *Agrobacterium* cell suspension for 10 s. (C and D) Plants were covered with plastic bags for 48h and grown in the growth chamber till seed formation.
1. Histochemical Analysis of GUS Expression

6-days old seedlings were vacuum-infiltrated for 2 min in GUS solution including 1 mM X-gluc, 50 mM Sodium phosphate pH 7.0, 0.1% Triton X-100, 1 mM Potassium ferricyanide and 1 mM Potassium ferrocyanide. The samples were then incubated at 37°C over night. De-staining of the seedlings was performed by incubation at 55°C in 0.24 M HCl and 20% MeOH solution for 15 min, and at room temperature in 7% NaOH and 60% EtOH solution for 15 min. The samples were dehydrated through ethanol series (40%, 20%, and 10%) and mounted in glycerol for further microscopic studies. Mounted samples were analyzed by bright field transmitted microscopy using an Axioplan 2 microscope (Carl Zeiss Inc.) and images were then captured by AxioCam HRc and Axiovision software (AxionVs40 V4.5.0.0).

2. Populus Genomics

A. Identification of CLE genes

The genome mining for Populus trichocarpa CLE gene family was performed by blastp searches against the Joint Genome Institute Populus Genome Sequence Database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) (Tuskan et al. 2006). A set of known 32 CLE sequences from A. thaliana and 34 sequences from P. trichocarpa were used as queries (Jun et al. 2008; Oelkars et al. 2008). The blastp searches were repeated until no additional candidate was identified.

B. Sequence Alignment and Phylogenetic Tree Construction

In Arabidopsis, 12 amino acid residues at the C-terminus of CLE peptides are assumed to be the functional site and peptide sequences were highly conserved within and between species (Jun et al. 2008). Hence this was set as the criteria to designate the Populus CLE gene family. Sequences from the blastp searches, as well as Arabidopsis CLE gene sequences were aligned using Clustal X (version 2.0) (Larkin et al. 2007). First iteration of alignment was executed on the whole predicted protein sequences. CLE domains of the peptide sequences were determined manually and false positives which did not contain CLE domains were eliminated from the further analysis. Second iteration of alignment was executed on the highly conserved CLE domain sequences. Phylogenetic trees were constructed using Clustal X software
Neighbour-joining (NJ) was the main method to deduce phylogenetic relationships between the CLE genes. Final phylogenetic trees were analyzed using MEGA (version 4.0) (Kumar et al. 2008).

3. Transcript profiling of CLE genes

A. Plant Material and Growth Conditions

Experiments were executed on 6 month-old wild type hybrid aspen (Populus tremula L. x Populus tremuloides Michx; clone T89). The plants were cultivated in the growth chamber under natural light. Tissue samples including shoot apex; young stem 1, just below the shoot apex; young stem 2, 5 cm below the shoot apex; 5th leaf as the younger leaf representative; 10th leaf as the older leaf representative; phloem; xylem; primary roots; secondary roots and root tips were collected between 10.00 and 12.00 on the dates 09.02.14; 09.03.29; and 09.05.09. For the sampling of phloem and xylem, bark was peeled and tissues were scraped from both exposed surfaces with a scalpel.

B. Total RNA Extraction and cDNA Synthesis

The total RNA from greenhouse grown trees was extracted individually from pooled samples of 5 independently grown trees using an Aurum Total RNA Mini kit (Bio-Rad). Tissue samples were grinded with a mortar and a pestle containing liquid nitrogen. For each sample, up to 60 mg of grinded plant tissue was transferred into an RNase-free 2.0 mL capped microcentrifuge tube. 700 µL of lysis solution (Aurum lysis solution supplemented with 2% polyvinylpyrrolidone-40) was added into each tube and the samples were disrupted by pipetting up and down. The lysate were centrifuged for 3 min at 12000 x g, and the supernatants were transferred into a new 2.0 mL capped microcentrifuge tubes. 700 µL of 70% ethanol was added into each tube and mixed thoroughly until no bilayer was visible. The homogenized lysate were decanted into RNA binding columns and centrifuged for 3 min at 12000 x g. Flow-through was discarded. 700 µL of low stringency wash solution was added to each RNA binding column and centrifuged for 3 min at 12000 x g. Flow-through was discarded. For each column processed, 5 µL of reconstituted DNase I was mixed with 75 µL of DNase I dilution solution and added to the membrane stack at the bottom of the column. The digestion was allowed to incubate at room temperature for 15 min. The columns were
centrifuged for 3 min at 12000 x g. Flow-through was discarded. 700 µL of high stringency wash solution was added to each RNA binding column and centrifuged for 3 min at 12000 x g. Flow-through was discarded. 700 µL of low stringency wash solution was added to each RNA binding column and centrifuged for 3 min at 12000 x g. Flow-through was discarded. The columns were centrifuged for an additional 1 min at 12000 x g to remove residual wash buffer. The RNA binding columns were transferred to 1.5 mL capped microcentrifuge tubes and 80 µL of pre-warmed elution solution was pipetted on the membrane stacks at the bottom of the RNA binding columns. The solution was allowed for 3-5 min to saturate the membranes. The columns were centrifuged for 3 min at 12000 x g to elute the RNA. The concentration and the quality of the RNA solutions were determined by Thermo Scientific NanoDrop™ Spectrophotometer and then kept at -80°C for further studies. cDNA synthesis was performed according to qScript™ cDNA synthesis kit (Quanta Biosciences) Catalog no. 95047-100 using 300 ng total RNA per reaction.

C. Initial Primer Testing

For the transcript profiling, gene specific primers were designed for every member of poplar CLE gene family according to annotated P. trichocarpa gene models with freely available web-based software Primer3 (http://primer3.sourceforge.net/). For an efficient RT-qPCR, amplicon range was adjusted between 75 bp-200 bp. Although the homology between P. trichocarpa and hybrid aspen is relatively high and the coding sequences of genes are identical up to 98%, the primer pairs for each gene was tested with PCR by using genomic DNA isolated from both species as template prior to RT-qPCR experiments.

For the extraction of genomic DNA, young plant leaves and apical buds were pulverized in liquid nitrogen and mixed with freshly prepared Rapid Extraction Buffer including 50 mM Tris-HCl pH 8.0, 25 mM EDTA, pH 8.0, 250 mM NaCl and 0.5% (w/v) SDS. The solutions were further incubated at 65°C with shaking for 30 min. One volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the tubes, vortexed and centrifuged for 10 min at 13000 x g. The upper phase was taken and the step was repeated until no opaque disc of proteins was visible. 1/10 volume of 3M NaOAc and 3 volumes of 99% ethanol was added to 1 volume of DNA solution and left at -20°C overnight. The solutions were centrifuged for 15 min at 13000 x g to pellet the DNA. Resulting supernatant was discarded. The DNA pellet was washed in 70% ethanol for 30 min with shaking and
centrifuged for 15 min at 17000 x g. After removal of ethanol by decanting, the DNA pellet was air-dried and dissolved in 80 µl of Elution Buffer (Qiagen). The concentration and the quality of the DNA solutions were determined by Thermo Scientific NanoDrop™ Spectrophotometer.

The following PCR program; 94°C for 3 min; 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, 36 cycles; 72°C for 10 min; 12°C for 30 min was performed for testing the gene specific forward and reverse primers. The PCR mixtures contained 0.4 µM of each PCR primer and 6.25 µL of GoTaq® Green Master Mix (Promega) in a 12.5 µL of total reaction volume. 5 µL of the PCR products were loaded onto 2% agarose 1X TAE (wt/v) gel containing 0.0001 µg/µL of ethidium bromide and separated by gel electrophoresis. The gel was examined using shortwave UV light separated by gel electrophoresis and examined using shortwave UV light for determination of correct amplicon size and specificity of the amplification.

**D. Quantitative RT-qPCR Assays**

RT-qPCR assays were performed on LightCycler ® 480 Real Time Systems platforms (Roche Applied Sciences), and primer efficiencies and relative gene expressions of populus CLE genes were determined.

To evaluate the primer set efficiency, 4-fold serial dilutions of T89 genomic DNA was used as template, giving a range of concentration from 20 ng/µL to 0.3125 ng/µL. Each PCR mixture contained 5 µL of DNA, 10 µL of LightCycler ® 480 SYBR Green I Master (Roche Diagnostics), 0.5 µM of gene specific forward and reverse primers and 3µL of ddH2O in a 20 µL of total reaction volume. PCR assays included 5 minutes pre-incubation at 95°C for the activation of enzyme and 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 15 seconds. After amplification, melting curve analysis was performed. The reactions were performed as duplicates, and standard curves were generated using LightCycler ® 480 Software (Roche Applied Sciences).

For the determination of relative gene expression levels, RT-qPCR was performed using 5 µL of cDNA (corresponding to 1.25 ng of total RNA), 7 µL of LightCycler ® 480 SYBR Green I Master (Roche Diagnostics), 0.5 µM of forward and reverse primers and 0.6 µL of ddH2O in a
14 µL of total reaction volume. PCR assays included 5 minutes pre-incubation at 95°C for the activation of enzyme and 50 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 15 seconds. After amplification, melting curve analysis was performed. The reactions were performed for 3 biological and 2 technical replicates; also 3 internal standards were used for each 96 plate, to acquire the normalization between different plates. Poplar 18S and UBQ were used as reference genes for the calculation of relative gene expression levels. The average Cₚ value of each CLE gene was subtracted from the corresponding average Cₚ value of 18S and UBQ separately to acquire normalized Cₚ values. The relative expression levels compared to two different reference genes were calculated by the 2⁻Cₚ method.

**IV. Results**

1. **Expression analysis of LRR-RLK Genes**

In this study a reverse genetics approach was utilized. The candidate genes were previously chosen by my supervisor Bo Zheng, and post-doctoral researcher Linbin Zhang using the data from the detailed transcript profiling of the poplar wood-forming tissues (http://www.populus.db.umu.se/). The main approach was the determination of differently expressed poplar genes in vascular tissues, further systematical identification of orthologs in *Arabidopsis* and gene characterization.

The experiments were executed on 4 different LRR-RLK genes; At4g39400; At1g55610; At3g13380; and At5g01890. In order to determine the tissue specific expression of these LRR-RLKs, promoter::GUS reporter fusions were generated by Gateway® cloning (Invitrogen). Whole list of gene members with AGI accession numbers, gene descriptions and primer sequences used during experiments are given in Supplemental Data Table 1.

The application of Gateway® technology throughout cloning resulted in high efficiency and precision. During the experiments, after each step the accuracy of promoter regions were controlled by PCR. All agarose gel results are not presented in this report. As a representative of the cloning part, the final step, PCR results following the Agrobacterium transformation was included. Maps of the constructs generated by LR reaction for promoter regions of LRR-
RLK genes and amplification of expected sized fragments are illustrated in Figure 3. It can be easily recognized that desired fragments were successfully inserted into the expression vector, and transformed into the *Agrobacterium* strain.

During the GUS analysis, the main concern was to find a consistent expression pattern within the same lines. After microscopic analysis it was found that, all 4 lines showed the GUS activity along the vascular strands in either some or all organs examined, such as cotyledons, hypocotyls, leaf primordia, roots and SAM (Figure 4). This observation is consistent with the
idea that these LRR-RLKs were putative orthologs of poplar genes expressed in vascular tissues. Moreover, the expression patterns of different genes were different from each other.

Figure 4: Vascular tissue-specific expression of At4g39400, At1g55610, At3g13380, and At5g01890. (A, E, I, and M) pAt4g39400::GUS; (B, F, J, and N) pAt1g55610::GUS; (C, G, K, and O) pAt3g13380::GUS; (D, H, L, and P) pAt5g01890::GUS. (A-D) Cotyledons; (E-H) Shoot apical meristems and leaf primordia; (I-L) Hypocotyls; (M-P) Roots. (Scale bars 50 µM).
Figure 4 above demonstrates that all lines showed GUS activity in cotyledons, and this activity was restricted to the vascular strands. However, in lines pAt4g39400::GUS, and pAt3g13380::GUS, the expression was discontinuous and weaker than the other two lines. It was also observed that pAt4g39400::GUS, and pAt1g55610::GUS lines revealed a strong GUS expression in SAM and leaf primordia. When the expression patterns along the hypocotyls were compared between 4 lines, pAt4g39400::GUS, pAt1g55610::GUS, and pAt5g01890::GUS lines showed GUS expression, however line pAt3g13380::GUS did not reveal any GUS activity. In the root tissues, the GUS expression was observed for all the lines. Interestingly, in the line pAt4g39400::GUS, the expression in the root not only spread through the elongation zone but also examined in the root cap. Overall, these results correlate with the data obtained from the expression atlas of Arabidopsis development (http://jsp.weigelworld.org/expviz/expviz.jsp).

2. Populus Genomics

In the *P. trichocarpa* genome, 50 putative CLE gene models were identified from the version 1.0 and 1.1 of genome assembly (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) (Tuskan et al. 2006). Whole list of CLE genes is given in Supplemental Data Table 2. The number of CLE genes identified in poplar is much higher compared to the previously published data (Oelkers et al. 2008). 16 putative gene models were additionally obtained, and 7 previously published gene models were re-annotated according to version 1.1 genome assembly. However, 4 gene models previously proposed as poplar CLEs by Oelkers et al. (2008) could not be found during blastp searches. These sequences were not ignored in this study. In order to analyze the sequences in a systematic way and distinguish these 4 old gene models (version 1.0) from newly annotated ones, the protein IDs were assembled together with letters Pt and the versions that they have been determined such as Pt1_1_protein ID or Pt1_0_protein ID.

First iteration of alignment was executed on the whole predicted protein sequences (Supplemental Data Figure 1). The results showed that CLE proteins consist of short and largely unrelated sequences. It has been observed that several hydrophobic amino acid residues at the amino termini are highly similar in almost all sequences, including more abundantly Isoleucine (I) and Valine (V), less abundantly Methionine (M). On the other hand, the alignment also showed a strong conservation of sequences at or near the carboxyl termini,
except Pt1_0_85301, Pt1_1_777016, Pt1_1_292182, Pt1_1_762220, Pt1_1_779771, Pt1_1_549191, Pt1_1_554332 gene models. This conservation is consistent with the existence of a CLE domain (Jun et al. 2008). The phylogenetic analysis using whole predicted amino acid sequences did not give a clear separation of putative orthologs (Supplemental Data Figure 2). Therefore, CLE domains of the peptide sequences were determined manually and 6 false positives which did not contain CLE domains were eliminated from further analysis. Interestingly, one of the predicted gene models, Pt1_0_85301, which did not show any conservation during the alignment, included a CLE domain pattern near the carboxyl end.

Figure 5: Phylogenetic tree on the conserved CLE domain of putative CLE genes in P. trichocarpa. B-type CLE peptides are indicated within the box. Phylogenetic tree was constructed using Clustal X software (version 2.0) (Larkin et al. 2007).
Second iteration of alignment was executed on the highly conserved 44 CLE domain sequences (Supplemental Data Figure 3). It was observed that Glycine (G)-6 and Proline (P)-9 amino acid residues are absolutely conserved in almost all *Populus* and *Arabidopsis* CLE peptides. Moreover, Valine (V)-3, Asparagine (N)-8, and N-12 are slightly less but still conserved residues. It was also found that Histidine (H)-1 is exclusive among the B-type CLE peptides (CLE41–CLE44 like). The phylogenetic analysis using just conserved CLE domain sequences gave a much better resolution between orthologs and showed the separation between the A-type CLE peptides which promote cell differentiation in root and shoot apical meristems, and the B-type CLE peptides (CLE41–CLE44 like) that promote cell proliferation in vascular tissues (Whitford et al. 2008; Hirakawa et al. 2008) (Figure 5).

3. Transcript profiling of CLE genes

*Populus* EST sequencing provides valuable information in describing the type of genes expressed in wood forming cells; however in CLE case it does not supply enough information. Therefore transcript profiling of *Populus* tissues was performed in order to comprehend the existence and differential expression patterns of CLE genes.

To identify candidate CLE proteins involved in vascular signaling, 43 primer pairs (not given) for the whole CLE gene family of *Populus* were designed and 43 independent quantitative RT-qPCR analysis were performed on shoot apex, 2 types of young stem, younger and older leaves, phloem, xylem, primary and secondary roots and root tips of hybrid aspen (described in Materials and Methods). The total RNA from greenhouse grown trees was extracted individually from pooled samples of 5 independently grown trees. The quantitative RT-qPCR reactions were performed for 3 biological and 2 technical replicates; also 3 internal standards were used for each plate, to acquire the normalization between different plates. During the analysis the main concern was to find an almost identical expression pattern between the biological and technical replicates. In addition all of the same primer pair should have only one melting curve. If a primer did not produce a nice triplicate for a certain type of cDNA that primer pair was assigned to run for a second time. Poplar 18S and UBQ were used as reference genes for the calculation of relative gene expression levels. At the end of the analysis it has been observed that UBQ normalization gave results with minor standard deviation.
Quantitative RT-qPCR assays were conducted on several different tissue types. However, we were particularly interested in cambial tissues including phloem and xylem. Overall, the expression strength of CLEs varied largely among different tissue types. The data obtained was not described entirely here but some major observations are described below.

Firstly, *Pt1_1_821595* transcript levels were considerably higher in the shoot apex comparing to the other tissues. Other than primary and secondary roots, almost no expression of *Pt1_1_821595* was observed (Graph 1). *Pt1_1_821595* is one of the predicted orthologs of *CLV3* according to the results of bioinformatic analysis described previously in *Populus* genomics section (Figure 5).

![Graph 1: Relative expression of *Pt1_1_821595* normalized against *UBQ*. X-axis shows different tissue types tested, Y-axis shows relative expression levels, standard deviations are calculated by Microsoft Excel.](image)

It has been also determined that putative ortholog of *CLE40, Pt1_1_750766* was expressed remarkably abundant in root tissue types (Graph 2).
All the quantitative RT-qPCR data so far were analyzed and expression of 5 gene models belong to the B-type CLE peptides were observed in vascular tissues. It has been determined that out of these 5 transcripts; expressions of Pt1_1_569594, Pt1_1_639958, Pt1_1_547959, and Pt1_1_751056 were up-regulated to some extent in the phloem (Graphs 3 to 6).

Graph 2: Relative expression of Pt1_1_750766 normalized against UBQ. X-axis shows different tissue types tested, Y-axis shows relative expression levels, standard deviations are calculated by Microsoft Excel.

Graph 3: Relative expression of Pt1_1_569594 normalized against UBQ. X-axis shows different tissue types tested, Y-axis shows relative expression levels, standard deviations are calculated by Microsoft Excel.
**Graph 4**: Relative expression of *Pt1_1_639958* normalized against *UBQ*. X-axis shows different tissue types tested, Y-axis shows relative expression levels, standard deviations are calculated by Microsoft Excel.

**Graph 5**: Relative expression of *Pt1_1_547959* normalized against *UBQ*. X-axis shows different tissue types tested, Y-axis shows relative expression levels, standard deviations are calculated by Microsoft Excel.
However, the most significant expression overall was determined for \textit{Pt\_1\_558094} gene model. Its expression levels were significantly up-regulated both in the phloem and xylem (Graph 7).

Graph 6: Relative expression of \textit{Pt\_1\_751056} normalized against \textit{UBQ}. X-axis shows different tissue types tested, Y-axis shows relative expression levels, standard deviations are calculated by Microsoft Excel.

Graph 7: Relative expression of \textit{Pt\_1\_558094} normalized against \textit{UBQ}. X-axis shows different tissue types tested, Y-axis shows relative expression levels, standard deviations are calculated by Microsoft Excel.
V. Discussion

1. Expression analysis of LRR-RLK Genes

In this part of the project, the tissue-specific expression patterns of certain LRR-RLK genes in *A. thaliana* were studied by generating promoter::GUS transcriptional fusions. Transcriptional fusions consist of promoterless reporter genes fused to the promoter of the gene of interest. Therefore, the expression of the reporter gene corresponds to the expression of the gene of interest. It is really a convenient and frequently used method to assist studies of gene expression and promoter activity.

Throughout the experiments the constructs were generated by Gateway® cloning. It was determined that, the application of Gateway® technology resulted in high efficiency and precision. These high-throughput results mostly depend on the attachment sites which flank the region desired to be cloned into the expression vector. Therefore, initial primer design which makes BP reaction possible is essential.

As the final step, positive plants which were germinated successfully on the selective media were tested for GUS activity. During GUS analysis, the main concern was to find a consistent expression pattern within the same lines. However it should be kept in mind that in T1 generation of plants, GUS expression can be unstable due to heterozygosity. Moreover, the candidate genes in this case were proposed to be active during vascular development. Therefore, further examinations of the tissue-specific expressions should be done using different tissue types such as leaf, different stem parts and flowers both from T1, T2 and T3 generations for more reliable results.

2. Populus Genomics and Transcript Profiling of CLE Genes

In this part of the project, I systematically identified *Populus* orthologs of the *Arabidopsis* CLE genes by using bioinformatic tools. Recent studies among CLE peptides revealed that in *Arabidopsis*, 12 amino acid residues at the C-terminus assumed to be the functional site and CLE peptide sequences were highly conserved within and between species. Moreover these peptides show a functional redundancy (Jun et al. 2007). Hence this was set as the criteria to designate the poplar CLE gene family.
The blast searches and initial alignment results confirmed the well-known fact that CLE proteins consist of short and largely unrelated sequences (Miwa et al. 2009). Moreover, it was observed that several hydrophobic amino acid residues at the amino termini are highly similar in almost all sequences, including more abundantly I and V, less abundantly M. These hydrophobic amino acid residues were previously proposed to direct the peptides to the secretory pathways (Jun et al 2008). It was also found that H-1 is exclusive among the B-type CLE peptides (CLE41–CLE44 like) and may give a distinct biological activity.

Through a genome-wide search, 50 putative orthologs of CLE peptides in *P. trichocarpa* were initially identified. In 44 of these sequences a high similarity between either A-type CLE or B-type CLE domains was observed suggesting the evolutionary importance of this domain.

*Populus* EST sequencing provides valuable information in describing the type of genes expressed in wood-forming cells; however in the CLE case it does not supply enough information. Therefore transcript profiling of *Populus* tissues was performed in order to comprehend the existence and differential expression patterns of CLE genes.

During the maintenance of stem cells in SAM, CLV3 peptide is synthesized in the L1 and L2 layer of cells, secreted to the apoplastic space, where it is perceived by CLV1-CLV2 receptor complex. This receptor complex then conveys the signal to inhibit the *WUS* transcription. Hence, the proliferation of the stem cells in the organizing center stops and cell differentiation of the cells in the peripheral zone is initiated (Miwa et al. 2009). The expression of poplar genes were examined and it has been determined that a putative ortholog of *CLV3*; *Pt1_1_821595* was expressed at high levels in SAM, which might suggest a similar role for this candidate in the SAM of hybrid aspen in the future.

Studies in root apical meristem showed that similar mechanisms for the regulation of stem cell identity are both active in SAM and root apical meristem (RAM) (Miwa et al. 2009). Quiscent center (QC) in RAM corresponds to the organizing center in SAM. The cells lie in QC expresses a *WUS* homolog, *WOX5*, which triggers the proliferation of stem cells that surrounds QC. On the other hand differentiating progenies of the stem cells in RAM, expresses *CLE40* (a homolog of *CLV3*) which regulates *WOX5* expression in a non-cell autonomous manner and promotes cell differentiation. CLE40 is also perceived by a receptor kinase ACR40 (Stahl et al. 2009). At the end of this study it has been observed that a putative
ortholog of CLE40; Pt1_1_750766 was expressed at highly elevated levels only in root tissues. The identification of a CLE40 ortholog in root tissues might propose such a similarity in hybrid aspen as well; however the potential function of Pt1_1_750766 needs to be examined carefully.

Recent identification of tracheary element differentiation inhibitory factor (TDIF, a B-type CLE peptide) in Zinnia elegans, promotion of procambial cell proliferation by in vitro application of TDIF to Arabidopsis seedlings, and identification of PXY locus in Arabidopsis genome which encodes a LRR-RLK and binds specifically to TDIF raised the speculation that a CLE-based stem cell maintenance might be active during vascular development (Whitford et al. 2008; Hirakawa et. al 2008). Throughout the study I also determined that several B-type CLE genes were significantly up-regulated in the vascular tissues phloem and xylem. Therefore the cambial expression of these poplar genes provides valuable information and candidates for further studies.

However, function of a gene cannot be predicted based on sequence or gene expression pattern alone. Moreover; it is yet not known that whether transcripts for the above-mentioned genes are also translated. Therefore; potential functions of these genes should be studied more carefully in the future. A good initial approach for this purpose might be the application of synthetic peptides to Populus seedlings and observation of development, which might eventually lead to more precise determination of cambial markers. Finally, these data shed light on potential candidates for the regulation of vascular signaling and provide us with a portfolio of hybrid aspen CLE genes that may be major players in the control of wood formation.
VI. Acknowledgements

I would especially like to thank to my supervisor Bo Zheng for his amazing supervision, never-ending patience and belief in me throughout this project. I am also very grateful to Linbin Zhang for teaching and helping me with everything in the laboratory. Moreover, I would like to thank to the members of my research group as well, especially Ove Nilsson, for improving my discussion skills and making this time period in UPSC so pleasant. Thanks to my wonderful parents and friends for their permanent support and belief in me during my educational life. I know that they will be always there for me with their love. Last but not least thanks to all the people in UPSC and Molecular Biology Department of Umeå University, you were always so nice and helpful!

VII. References


VIII. Supplemental Data

Supplemental Data Table 1: *LRR-RLK* genes used for promoter analysis. Table shows the AGI/gene accession numbers, description of the genes and primer sequences without 5′*attB* sites.

<table>
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<th>AGI/Gene Accession No</th>
<th>Description</th>
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<td>At4g39400</td>
<td>Encodes a plasma membrane localized leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction.</td>
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<td>F: AATAAGTCAGAGAGGCAATAG R: GTTATAGCCCAAAAGTGTTCC</td>
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</table>
Supplemental Data Table 2: List of CLE genes in *P. trichocarpa*

The genome mining for *P. trichocarpa* CLE gene family was performed by blastp search against the Joint Genome Institute Populus Genome Sequence Database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) (Tuskan et al. 2006). Table shows gene model, chromosomal location, JGI protein ID, and best hit results against *A. thaliana* genome.

<table>
<thead>
<tr>
<th>Gene Model</th>
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Supplemental Data Figure 1: First iteration of alignment on the whole predicted protein sequences of putative CLE genes in *P. trichocarpa*. Light grey color indicates similar amino acid residues; black color indicates conserved amino acid residues. Multiple sequence alignment was conducted using Clustal X software (version 2.0) (Larkin et al. 2007). The shading was performed by BOXSHADE (version 3.21).
Supplemental Data Figure 2: Phylogenetic tree on the whole predicted protein sequences of putative CLE genes in *P. trichocarpa*. Phylogenetic tree was constructed using Clustal X software (version 2.0) (Larkin et al. 2007). Neighbour-joining (NJ, 1000 bootstrap replicates) was the main method to deduce phylogenetic relationships between the CLE genes. Phylogenetic tree was analyzed using MEGA (version 4.0) (Kumar et al. 2008).
Supplemental Data Figure 3: Second iteration of alignment on the highly conserved CLE domain sequences in *P. trichocarpa*. Light grey color indicates similar amino acid residues; black color indicates conserved amino acid residues. Multiple sequence alignment was conducted using Clustal X software (version 2.0) (Larkin et al. 2007). The shading was performed by BOXSHADE (version 3.21).