Sveriges lantbruksuniversitet
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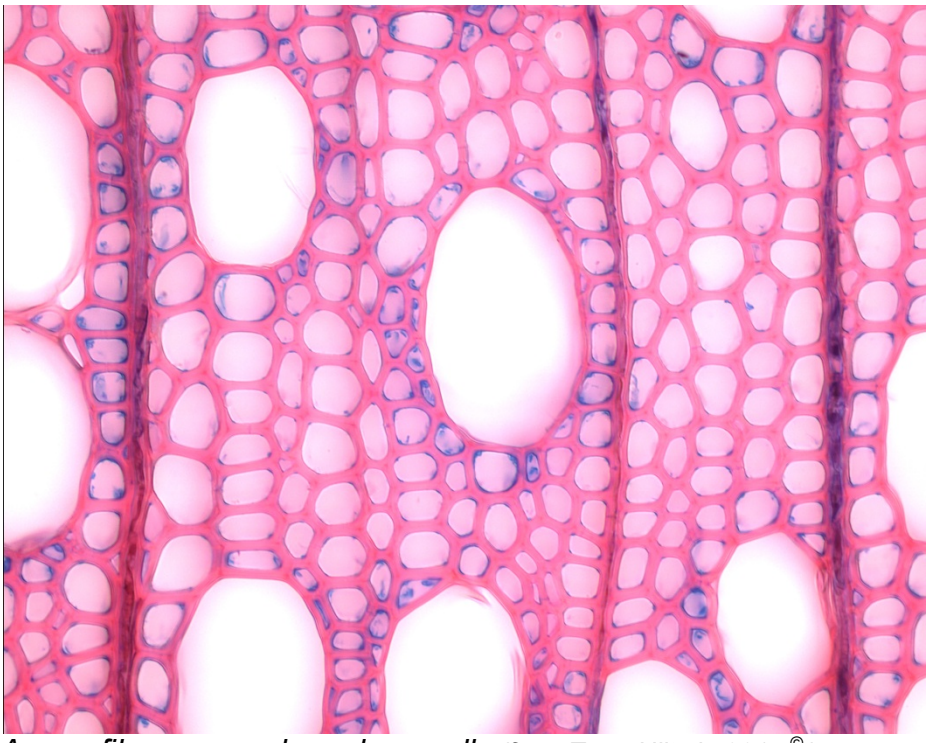
Faculty of Forest Sciences

Subcellular Localization of a Neutral Invertase from hybrid aspen (*Populus tremula x tremuloides)*

Subcellulär lokalisering av ett neutralinvertas från hybridasp (*Populus tremula x tremuloides*)

Marcus Andersson



Aspen fibers, vessels and ray cells (from Totte Niittylä 2014 ©)

Department of Forest Genetics and Plant Physiology
Master´s thesis • Examensarbete • 30 hp

Umeå 2014

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Credits: 30 hp
Level: Second cycle, A2E
Course title: Master's degree thesis in Biology
Course code: EX0767
Programme/education: Jägmästarprogrammet

Place of publication: Umeå
Year of publication: 2014
Number of part of
Online publication: http://stud.epsilon.slu.se

Keywords: Developing wood, Hybrid aspen, Localization, Neutral invertase, Populus

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Acknowledgements

I would like to thank Dr. Totte Niittylä for the enduring support I had from the start of the thesis work. A very much appreciated support in discussing and improving skills and theory, as well as providing appreciated tips.

I would also like to thank Umut Rende who helped in the start up the thesis work and reviewing the thesis. Umut also thankfully provided the construct which made the results seen in the confocal microscope possible.

I would also like to thank Valentina Floran, the people working in the Cell Wall lab, and all the nice people in the fika-room. Big thanks go out Dr. Concetta Valerio who has been very helpful with laboratory tips and supportive during my thesis work.

Special thanks to my family and friends and who have been very supportive and good friends during this thesis period!

Abstract

Neutral Invertases are sucrose hydrolyzing enzymes whose role in plants remains to be understood. This thesis focuses on the subcellular localization of a neutral invertase from hybrid aspen (*Populus tremula* × *Populus tremuloides),* which shows increased transcript levels during secondary cell wall formation. The study is made with a yellow fluorescent protein (YFP) construct fused to either the C- or N-terminal end of the neutral invertase protein. The constructs were transiently expressed in tobacco (*Nicotiana tabacum)* leaf epidermis cells. The YFP-signal was mainly seen along the border of the cells, corresponding to the cytoplasm of the epidermal cells. The pattern from the YFP-signal appears aggregated, indicating that the neutral invertase is associated with a compartment located in the cytoplasm. From the interpretation of the pattern and the subcellular prediction of the location based on the peptide sequence, the neutral invertase is plausibly associated with the Golgi-apparatus and the synthesis of hemicellulose and pectin. Experiments that would be able to confirm the results would be: co-localization of the neutral invertase-YFP signal with Golgi compartment markers, ideally in hybrid aspen cells, and Western blots to confirm the correct size of the fusion protein. Hybrid aspen neutral invertase *RNAi* lines should be characterized for their cell wall properties to investigate the role of neutral invertase during wood biosynthesis.

Introduction

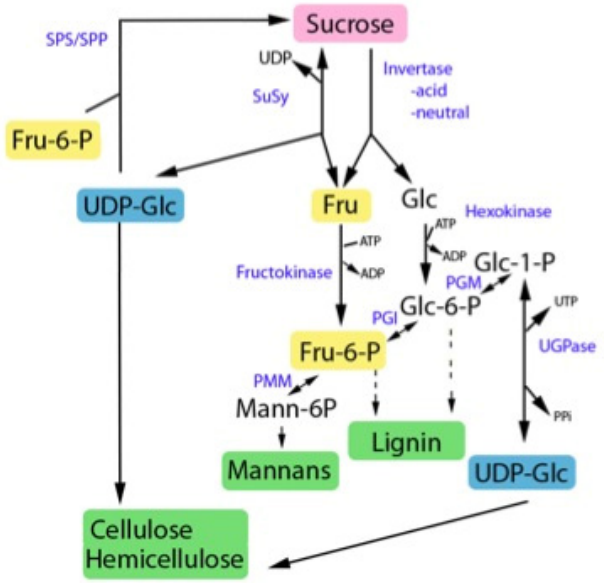
Sucrose metabolism in developing wood

Plants use sugars as both building blocks and energy source. Sugars are synthesized in source tissues by photosynthesis from the CO_2 in the air, and are then transported through the phloem to sink cells where the sugars are stored and later metabolized. The primary way to transport carbon in the phloem is in the form of sucrose. Sucrose is a non-reducing disaccharide formed by a linkage via an ether bond between C1 on a glucose subunit, and C2 on the fructose subunit. In addition to being an important metabolite, sucrose also acts as a signaling molecule that can alter the gene expression, growth development and physiology in plants (Koch 2004).

The cell wall in developing wood consists of three main components, cellulose, hemicellulose and lignin (Albersheim et al. 2010). Cellulose is a long polysaccharide chain and forms the major part of the cell wall structure (Pettersen 1984). The hemicelluloses are matrix polysaccharides that together with pectin form a ground to bind the cellulose in the cell wall structure (Albersheim et al. 2010). Studies have concluded that the dry weight of wood generally consists of between 40-50% of cellulose, 25-35% of hemicelluloses, and 25-30% of lignin (Pettersen 1984). The biosynthesis of polysaccharides such as cellulose and hemicellulose mainly use activated sugars as building blocks (Albersheim et al. 2010). Since sucrose is the main carbon source in the sink cell, a large portion of the cell wall structure is derived from a sucrose source (Albersheim et al. 2010; Koch 2004).

Overview of the sucrose metabolism pathway

When the sucrose enters a sink cell, sucrose is cleaved by enzymes to be used in the cell metabolism. According to current understanding, there are two different forms of sucrose cleaving enzymes; sucrose synthase and invertase (Joshi & Mansfield 2007; Koch 2004). Sucrose synthase performs a reversible reaction $(sucrose + UDP) \leftrightarrow fructose + UDP - glucose$. Invertase cleaves sucrose in an irreversible reaction $(sucrose + H_2O) \rightarrow fructose + glucose$.



Enzyme abbreviations:
PMM: Phosphomannomutase
PGM: Phosphoglucomutase
PGI: Phosphoglucose isomerase
UGPase: UGP-glucose-pyrophosphorylase
SuSy: Sucrose synthase

Figure 1. Sucrose metabolism pathway in sink cells leading to carbon allocation to the cell wall.

There are several possible pathways leading towards carbon allocation for the cell wall biosynthesis, one of them begins with sucrose cleavage by invertase. After invertase cleaves sucrose into fructose and glucose, hexokinase turns the glucose into glucose-6-phosphate. Phosphoglucomutase (PGM) then turns the glucose-6-phosphate into glucose-1-phosphate. UGP-glucose-pyrophosphorylase then catalyzes the reactions where glucose-1-phosphate is turned into UDP-glucose (Kleczkowski et al. 2010). The fructose cleaved by invertase is turned into fructose-6-phosphate by fructokinase, fructose-6-phosphate, may be used to synthesize lignin, and cell wall carbohydrates (figure 1) (Roach et al. 2012).

There is evidence that reduced fructokinase levels also affect the carbon partitioning and the cell wall. An RNAi mediated reduction of fructokinase in developing wood in aspen showed decreased levels of hexosephosphates and UDP-glucose. Wood fibers also had a thinner cell walls, and there was a reduction in cellulose (Roach et al. 2012).

The UDP-glucose is used by cellulose synthase complex as a substrate to synthesize cellulose (Joshi and Mansfield 2007). Sucrose synthase can also cleave sucrose directly into UDP-glucose and possibly provide UDP-glucose directly to the cell synthase complex (Joshi and Mansfield 2007) The origin of the UDP-glucose for the cellulose biosynthesis is disputed. Possible enzymes responsible for supplying UDP-glucose for cell wall biosynthesis are sucrose synthase and UGP-glucose-pyrophosphorylase (Somerville 2006; Kleczkowski et al. 2010). Overexpression of the UDP-glucose-pyrophosphorylase in hybrid aspen showed an increase in cellulose content. Plants also showed reduced height growth and reduced stem diameter (Coleman et al. 2007). Also overexpression of the UDP-glucose-pyrophosphorylase in Jute (*Corchorus capsularis*), showed increased cellulose content in stem tissues while the tree height growth was increased (Zhang et al. 2013). Since invertase can supply substrate for UDP-glucose-pyrophosphorylase, there might be a connection and interplay between the two enzymes regarding the sucrose metabolism in developing wood.

Among the enzymes active in the cell wall biosynthesis are the glycosyltransferases. The glycosyltransferases are active in the biosynthesis and modification of the cell wall polysaccharides and glycoproteins (Rosén et al. 2004). The glycosyltransferases can be divided into 95 families (http://www.cazy.org/GlycosylTransferases.html), and among those are the cellulose active subunits (CESA) and Golgi-localized type II integral membrane proteins included (Somerville, 2006 ; Geshi et al., 2004). Golgi-localized type II integral membrane proteins are able to synthesize the pectin backbone (Bacic 2006), adding side chains to polysaccharides (Persson et al. 2007) and synthesizing xylan (Faik et al. 2000; Peña et al. 2007; Cooper 2000). Hence glycosyltransferases and the Golgi-localized type II integral membrane proteins play an important role in the biosynthesis of parts of the cell wall.

Sucrose synthase

Sucrose synthase (SUSY) can reversibly cleave sucrose in sink cells (Koch 2004). Some evidence points to that sucrose synthase could be directly involved in the cellulose biosynthesis. SUSY has been found to be associated with the plasma membrane in developing cotton fibers (Amor et al. 1995). SUSY associated with the plasma membrane has also been shown to be oriented in similar pattern as the cellulose microfibrils, seen in a immunolocalization study in cotton fibers (Haigler et al. 2001). The study concludes that SUSY associated to the plasma membrane possibly could supply carbon directly to the cellulose synthesis (Haigler et al. 2001).

An immunoprecipitation study made in poplar suggests that SUSY co-immunoprecipitated with the cellulose synthesis complex (Song, Shen, and Li 2010)**.** The author (Song, Shen, and Li 2010) also states that it is difficult to harvest pure cellulose synthase (CESA) complex, and that some nearby proteins could have been contaminating the samples, which could influence the results. A similar study in Azuki bean (*Vigna angularis*) found that immunolabeled SUSY with attached gold particles, was associated with a plasma membrane structure similar to Cellulose active subunits (Fujii, Hayashi, and Mizuno 2010). However it was not clearly established if it was a CESA structure or not due to an uncertainty of the identification of the SUSY protein (Fujii, Hayashi, and Mizuno 2010). Proteins may have many variants that look similar but is slightly different in structure and function, these variants are called isoforms. A study concluded that a SUSY isoform SusC, that was identified in the original Amor et al. (1995) study, is mostly located in near the cell wall (Brill et al. 2011). Considering the finding of the SUSY isoform near the cell wall, it could indicate that SUSY possibly is associated to the cell wall biosynthesis metabolism (Brill et al. 2011).

Interestingly there are findings that strongly indicate that SUSY is redundant in sucrose metabolism in sink cells and cell wall biosynthesis, and supports invertase's role in sucrose metabolism instead. T-DNA mutant lines in *Arabidopsis* with a quadruple null-mutant construct of four SUSY isoforms SUS 1-4, showed that the null mutant SUSY did not have any major effects on the growth and development (Barratt and Derbyshire

2009). T-DNA mutant lines in *Arabidopsis* roots with cytosolic invertase, cinv1/cinv2 null-mutant constructs were also made, and invertase mutant lines showed shorter root length (Barratt and Derbyshire 2009). These results show that SUSY is redundant and invertase possibly is essential in development and growth for sink cells in *Arabidopsis*. However a repeated study in Arabidopsis made in similar conditions, claim that there are SUSY activity in the null mutants, and that SUSY is therefore not redundant for cellulose synthesis (Baroja-Fernández et al. 2012). A later study have found that SUSY is not essential for cellulose biosynthesis in poplar, but that SUSY affect the cell wall density and carbon allocation to wood (Gerber et al. 2014). These findings raise the question which of the two sucrose cleaving enzymes, sucrose synthase or invertase, that provides carbon for cellulose synthesis in developing wood.

Invertases

To be able to function in different compartments invertases have different pH-optima, depending of their subcellular localization and function. The invertases can be divided into alkaline/neutral invertases and acidic invertases (Vargas and Salerno 2010). Invertases can also be organized after their subcellular organization and function (Koch 2004). Vacuolar invertases have been linked to sink initiation and generating vacuolar hexoses leading to expansion, respiration and hexose-based sugar sensing (Koch 2004). Cell wall invertases have been linked to functions such as; continued sink initiation and cell expansion (Koch 2004). Further studies in *Arabidopsis* have shown vacuolar invertase to possibly regulate the osmotic potential (Sergeeva et al. 2006; Roitsch and González 2004). Another study on the other hand speculate that invertase does not regulate the osmotic potential, because sucrose accounts for only 1-2% of total sap osmolarity in *Arabidopsis* roots (Ruan et al. 2010). The assumption is that when invertase cleaves sucrose into two molecular compounds, invertase doubles the amount of molecules and thus affects the osmotic pressure.

Alkaline/neutral invertases

Neutral invertase has been shown to be important for plant growth and development. A mutation of an alkaline/neutral invertase in rice homologue to *Arabidopsis* AtCyt-inv1, showed shorter root growth, delayed flowering and partial sterility (Jia et al. 2008). In the mutant lines there was also an accumulation of sucrose and a reduction of hexose. Supplying glucose exogenously could rescue the mutant (Jia et al. 2008). In *Lotus japonicas* a mutant of a neutral/alkaline isoform LjINV1 showed severed growth in the roots, shoots and impaired flowering (Welham et al. 2009). The shoot tips in LjINV mutants showed a 9-fold increase in sucrose levels (Welham et al. 2009).

Apart from being located in the cytosol, neutral invertase has also been found to have peptide signals that target proteins in mitochondria and chloroplasts (Ji et al. 2005). Gene analysis and localization studies in rice have shown invertase to be localized in mitochondria and plastids (Murayama and Handa 2007). It has also been shown in *Arabidopsis* and spinach that invertase is active inside chloroplasts (Vargas, Pontis, and Salerno 2008). Another study in *Arabidopsis* examines two different alkaline/neutral invertases, one mitochondrial and one cytosolic. The absence of either the invertases is associated with an oxidative stress defense gene expression. While an overexpression of the invertase genes down-regulated the oxidative stress-responsive *ascorbate* peroxidase 2 (APX2) promoter (Xiang et al. 2011). This could imply that sucrose has a regulatory role in plastids or that the sucrose pathway in plastids is more extensive than previously thought (Murayama and Handa 2007).

Invertases in poplar

In poplar 24 different types of invertase have been identified (Bocock et al. 2008). Of them 16 isoforms were neutral invertases predicted to be localized with a cytosolic localization. The rest of the invertase isoforms in poplar were acidic invertases, three of them predicted to be localized to the vacuole and five cell wall invertases (Bocock et al. 2008). In *Arabidopsis* for comparison there has only been found 9 neutral invertase isoforms (Nonis, Ruperti, and Pierasco 2008). The fact that poplar have almost the double number of neutral invertase isoforms compared to *Arabidopsis* (Bocock et al. 2008), could indicate that neutral invertase play a more important role in trees and possibly wood formation.

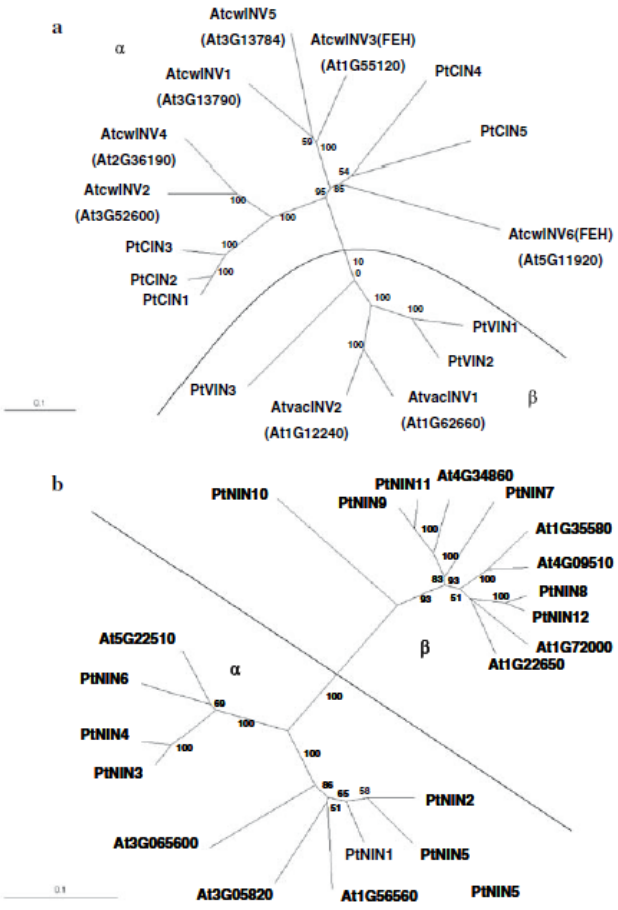


Figure 2. Analysis of the similarities between invertases in *Populus trichocarpa* and *Arabidopsis*, based on full-length sequences. The invertases are divided in α- and β clades based on different physical properties. (a) The protein similarity tree above is from of eight poplar and eight *Arabidopsis* acid invertases. (b) The protein similarity tree to the below is illustrating the difference between 12 poplar and 9 *Arabidopsis* alkaline/neutral invertases. Poplar has more neutral invertase isoforms, while *Arabidopsis* has more cell wall invertase isoforms (Bocock et al. 2008).

Neutral invertase expression in developing wood

In poplar a neutral invertase (NIN) protein has been found to be interesting in a screening of different genes during different stages of wood development (Unpublished UPSC, RNA sequencing program). The NIN-gene which we are interested in is clearly up-regulated during the secondary cell wall synthesis during the wood formation (see figure 3). This suggests that the NIN-gene potentially could be involved in formation of developing wood, and be part of the cell-wall synthesis. The aim of this study is to investigate and try to localize this NIN-protein.

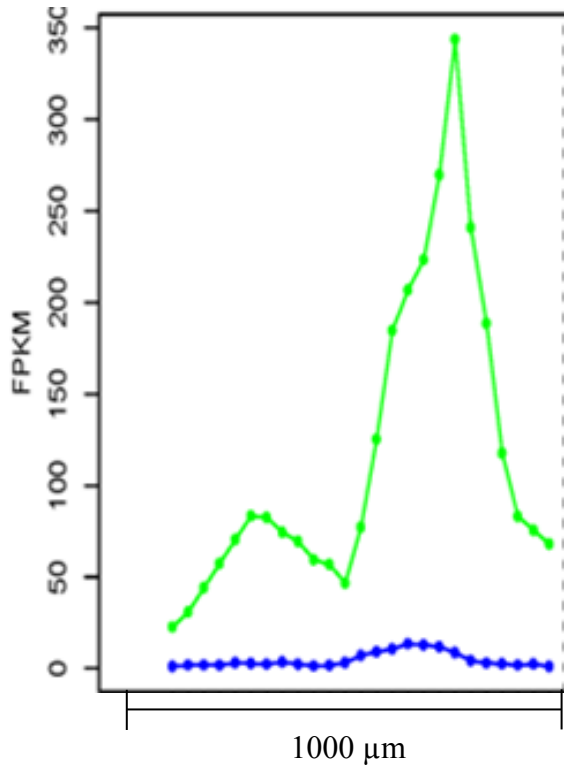


Figure 3a. Expression profile of the neutral invertase enzyme in developing wood in from a representative sample (Unpublished, UPSC RNA sequencing program). The samples are vertical slices taken from different parts of the developing wood. The Y-axis represents the strength of signal of the expression FPKM (Fragments Per Kilobase of transcript per Million mapped reads). The X-axis is in micrometers where in the developing wood profile the samples are extracted, from the left to the right, starting from the cambium to the maturation of the wood fibers (see figure 3b). The NIN peak expression profiles correspond to the secondary cell wall formation stage of wood development.

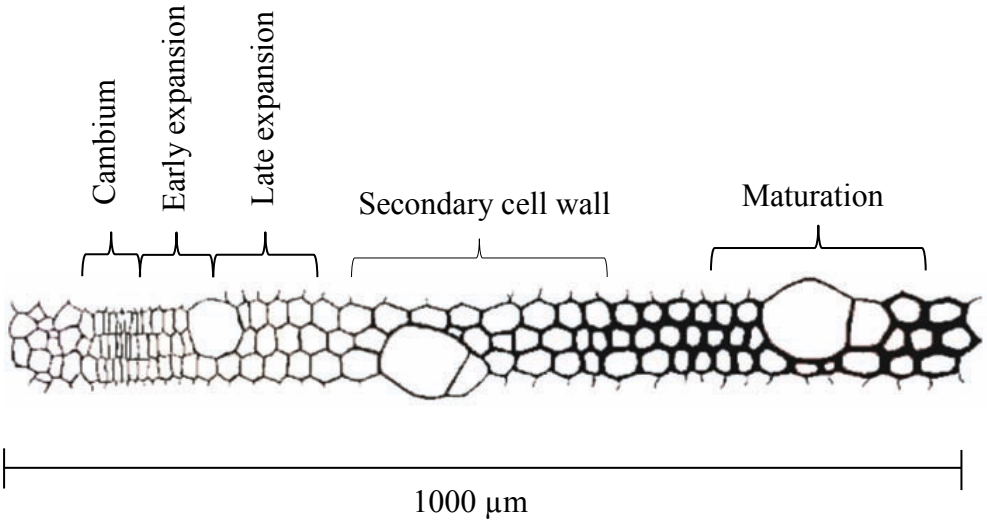


Figure 3b. Horizontal profile of the developing wood, adapted from (Mahboubi et al. 2013). The figure starts from the left with the cambium, early expansion, late expansion, secondary cell wall, and maturation. The figure can be read so that the developmental zones and scale bar corresponds with the scale bar in figure 3a.

Methods and materials

To understand more about the neutral invertase (NIN) and its role in biosynthesis of the cell wall, a subcellular localization study was proposed. A construct was created with the NIN-gene and an attached YFP-construct at the N- and C-terminal side of the protein. Both N- and C-terminal constructs were used because the targeting sequence for subcellular localization can be found on either side of the protein, usually at the N-terminal side. The constructs was made by using the Gateway technology™ and cloning process. With the constructs made, it was possible to transform them into *Agrobacterium* and infiltrate tobacco leafs, and then visualize YFP-signal in a confocal-microscope**.** The confocal-microscope works by exciting the YFP by a laser and causing it to emit light a certain wavelength, the emitted light then reflects back to the microscope. This makes it possible to see fluorescence from the specific part of the cell where the YFP is located.

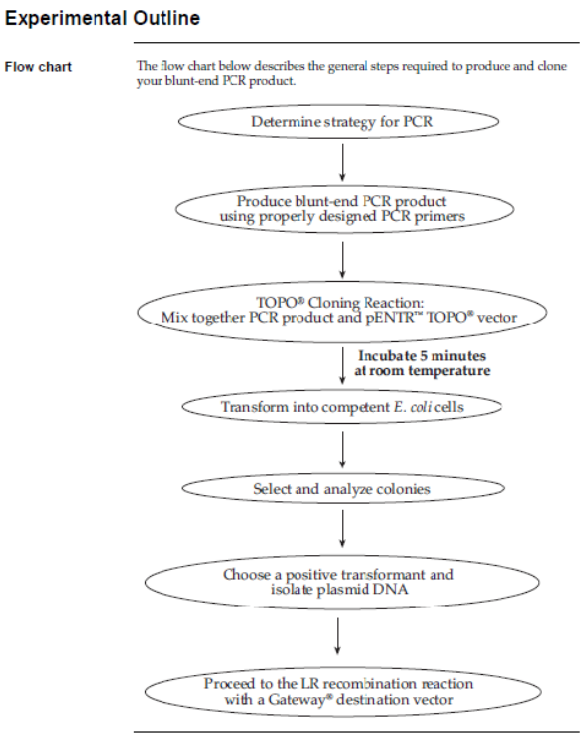


Figure 4. Flow chart for Gateway cloning, process and steps from the Gateway pENTR D'TOPO manual. The end result is the binary plant transformation vector used in the transformation of *Agrobacterium,* and later the infiltration of tobacco plants.

Amplification

The NIN-gene was amplified from a cDNA library prepared from developing wood. The amplification was made by two primers, the forward NIN primer was CACCATGGATGGGACTAAAGAGATGGG, and the reverse primer was GCAAGTCCAAGAAGATGATCTCCTGAG. The amplification was done with Invitrogen® Phusion taq enzyme. The PCR reaction was done by denaturation at 98 °C for 30 sec. Then the amplification was done in 35 two-step cycles, with an initial denaturation step at 98°C for 7 sec and extension step at 72°C for 1 min. Then after the 35 cycles the PCR reaction was ended with an extension step in 72°C for 10 min and after put on hold at 8°C.

The PCR products were then put onto a 100 ml 1,5% agarose gel with 5 µL GelRed®. Then 50 µL of PCR products were put on the gel and run at 120 V for 2 hours. Then a band was cut out from the gel corresponding to the NIN gene size at 1674 bp. The gel fragment was then purified using a DNA gel extraction kit from Thermo dynamics®. After the purification the concentration of DNA was estimated by using Nanodrop®.

D'TOPO reaction

The NIN gene was inserted into a pENTR™ D'TOPO® cloning kit from Invitrogen™. Estimations from the Nanodrop® assay were used to match the ratio between the D'TOPO® vector and the PCR product to 1:1. After insertion the construct was transformed into chemically competent One Shot® TOP10 *E.Coli* cells. The One shot® cells were incubated on ice for 30 min. The construct was added into two TOP10 cell aliquots. One aliquot with 2 µL of D'TOPO® reagent was prepared, and in the other aliquot 6 µL D'TOPO® reagent was added, this is ensure that enough colonies will spawn. The cells were then heat shocked for 45 sec at 42°C. After SOC medium addition it was incubated on ice for 2 minutes and then incubated at 37 °C for one hour. The cells were put onto plates with 25 µg/mL kanamycin. There were two plates for each aliquot one with 50 µL of transformants and one with 150 µL with transformants. After plating the plates were incubated at 37°C overnight.

D'TOPO reaction information

The Gateway D'TOPO® reactions use an enzyme Topoisomerase, which can bind to duplex DNA at CCCTC sites. Topoisomerase originates from *Vaccinia* virus (Shuman 1991). When the Topoisomerase binds to the site on the D'TOPO® construct, it cleaves the phosphodiester backbone in one strand. When the cleavage is done the energy is conserved by a covalent bond formation between the 3´ phosphate of the cleaved strand, and a tyrosyl residue (Tyr-274) of topoisomerase (Shuman 1994). After the cleavage the 5´ hydroxyl of the original strand can attach the phosphor-tyrosyl bond between the DNA and topoisomerase. The reaction can then be reversed and the topoisomerase is released from the DNA strand (Shuman 1991). This process is used in the D'TOPO reaction to insert a construct into an entry vector. The construct can then be transferred from entry vector to a destination vector in a Gateway LR /BP ® reaction (Gateway cloning manual).

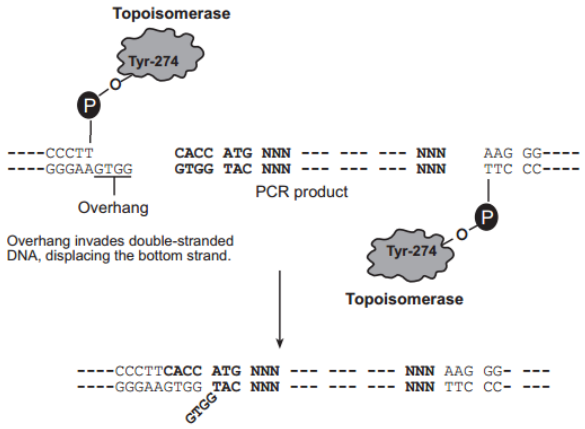


Figure 5. Illustrating how the DNA strand looks like before and after the the D´TOPO reaction.

Preparing LB culture

After overnight incubation, six colonies were inoculated in 5 ml LB culture containing 50µL/ml Spectinomycin. The cultures were then incubated overnight with shaking at 225 rpm at 37°C. The Miniprep Qiagen kit® was used for extracting the plasmids, and the plasmid concentrations were measured with Nanodrop®.

LR reaction

The LR-reaction was done using the Gateway® LR Clonase™ II Enzyme Mix kit. The incubation time for the final LR reaction was prolonged to overnight. The LR reaction products were amplified in *E.coli* and the selection of the complete construct was done using 50µL/ml Spectinomycin. See figure 6 and 7 on the following pages for how the plasmid maps of the destination vectors looks like.

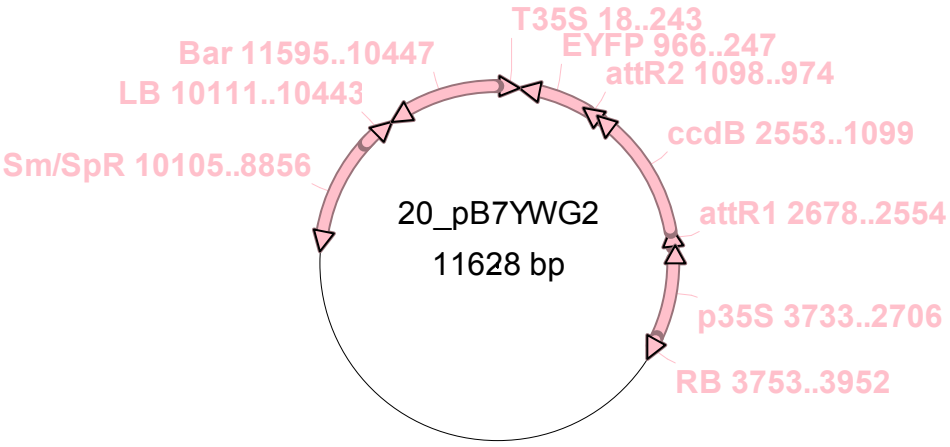


Figure 7. Showing the plasmid map for the empty destination vector from A Plasmid Editor®. The insert would later be placed between the attR1 and attR2 sites between the EYFP and T35 sites. The map is without any inserts.

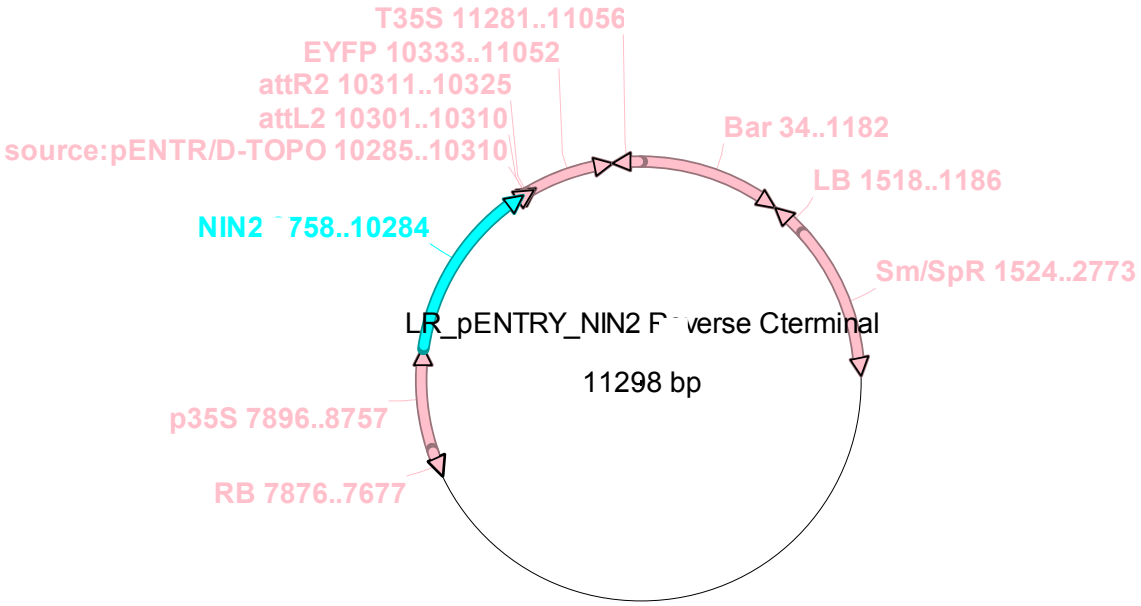


Figure 8. Showing the plasmid map for the destination vector with the inserted NIN gene, from A plasmid editor®. The insert with the NIN-gene is showed in blue color. This is the expected result of how the destination vector with an insert would look like after the LR reaction.

LR reaction information

The LR reaction is a transfer of a construct between the entry clone and the destination vector done by LR Clonase™ II Enzyme. The LR reaction and Gateway® system relies on mutated variants of the lambda *att* sites. The variants can be made so attB1 recombines with attP1, but not with attP2, or attP3. This means that there are little cross talk and no recombination between non-specific sites (Katzen 2007). In the LR reaction the construct of interest first is situated in an entry clone between two *att* sites, and then transfers to the destination vector. The destination vector have two *att* sites that are specific to the *att* sites in the entry clone (Hartley, Temple, and Brasch 2000).

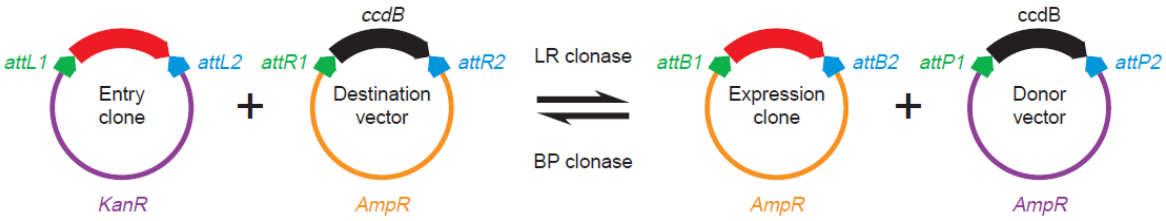


Figure 6. The red strand represent the construct of interest (Katzen 2007).

To ensure that the destination vector with the construct is selected and not the entry clone, the destination vector and entry clones have different antibiotic resistance genes. The destination vector also transfers *ccdB* gene to the entry clone in the LR-reaction. The *ccdB* gene is a counter selectable marker with gyrase-mediated double-stranded DNA breakage, which selects cells of the entry clone that does not have the *ccdB* gene (Katzen 2007)

Restriction Digest

The plasmids were tested with restriction digest using the FastDigest® enzymes from Fermentas® and FastDigest® Green Buffer from Fermentas®. The enzymes were Nco1, Nto1 for the D'TOPO construct, and Sal2 for the destination vector. Then the digested plasmid products 50 µL, were put onto gel 100 ml 1,5% agarose with 5 µL GelRed®, and was run at 120 V for 2 hours.

Sequencing

Plasmids were submitted in a premixed sample, 50 µg/µL concentrations of plasmids and 10 µMol/ml of primer. The two primers used for sequencing were, YFP reverse primer sequence: ACACGCTGAACTTGTGGCCGTTTA, and the sequence for the P35S primer: CCACTATCCTTCGCAAGACCCTTC. The two samples were submitted to Eurofins-MWG Operon in Munich.

Transformation of tobacco plants

Plant material

Tobacco plants (*Nicotiana tabacum)* were grown in 16 hours daytime and 8 hours nighttime for three weeks. The temperature was 20°C and relative humidity was 70%.

Agrobacterium transformation

The *Agrobacterium* infiltration started with transforming the expression vector into *Agrobacterium tumefaciens* strain GV3103 (pMP90). The *Agrobacterium* cells were thawed on ice in a 100 µL suspension. Then 1uL with 2-3 ng of plasmid DNA were added by the 100 µL suspension by mixing. The *Agrobacterium* suspension was transferred to an electroporation cuvette, the cuvette was prechilled on ice.

The *Agrobacterium* suspension was then electroporated at a voltage adjusted to the cuvette size. Then 1 ml of LB media was added to the cuvette and transferred to a 2 ml eppendorf tube, and was grown at 28°C with shaking for 14 hours. The solution was then plated on LB media with $Gent^{25}$, Rif^{50} and Spectinomycin resistance. The plates were prepared and grown at 28°C for two days. Then the culture was scaled up to 250 ml and was grown until the OD_{600} reached to 1.0.

Tobacco infiltration

The *Agrobacterium* colonies from the expression vector transformation was put in 5 ml falcon tubes with Rif^{50} and Spectinomycin resistance, and were grown for 28°C with shaking overnight. Then 25 ml of the LB media is grown at 28°C with shaking for 12-16 hours with 20µM Acetosyringone added. The bacteria was precipitated at 10.000 rpm for 15 min and adjusted to a final OD_{600} of 0.4. Then the culture was incubated in room temperature for 3 hours. The infiltration was done with a syringe with the nozzle pressed against the lower (abaxial) epidermis of the leaf. The injection was done while exerting a counter-pressure by pressing a finger on the other side of the leaf. The borders of the infiltrated area were marked by a permanent pen and the plants were incubated in normal growing conditions for 2 days.

Confocal microscopy

A small square was cut from the infiltrated area was cut from the tobacco leafs, and placed on a slide with water added. The slide were covered with a cover-slide and put under the confocal-microscope. The confocal microscope used was a Leica TCS SP2 AOBS spectral system with laser excitation lines at 51/364, 405, 458, 476, 488, 496, 514, 561 and 633 nm mounted on an inverted microscope.

Subcellular localization prediction

The peptide sequence was tested in programs predicting the subcellular localization. Three programs were used for general subcellular localization (1-3). When the results from the fluorescence from the confocal-microscope pointed to a possible Golgi localization, a Golgi specific localization program were also used (4). Prediction programs 1-4:

1. Extensive feature detection of N-terminal protein sorting signals iPSORT http://ipsort.hgc.jp/index.html (Bannai et al. 2002)
2. Subnuclear Compartments Prediction System http://array.bioengr.uic.edu/subnuclear.htm (Lei and Dai 2005)
3. CELLO- subCELlular LOcalization predictor. http://cello.life.nctu.edu.tw/cgi/main.cgi) (Su et al. 2007)
4. Prediction of Golgi Localized Transmembrane Proteins. http://ccb.imb.uq.edu.au/golgi/golgi_predictor.shtml) (Yuan and Teasdale 2002).

Results

The results were achieved using a construct of the neutral invertase gene attached with YFP-tags fused at the N- and C-terminal side of the protein. The constructs were transformed into *Agrobacterium* and infiltrated in tobacco leafs, and the visualized in a confocal microscope to see the YFP-spectrum (see figure 15-19). Prediction of the subcellular prediction was made using the peptide sequence of the neutral invertase. The result section is ordered by first showing the laboratory results, followed by the microscopy results and the prediction of the subcellular localization results.

Construct laboratory results

There were several steps in the cloning procedure; from the PCR amplification to the Gateway cloning reactions. The cloning was not initially successful during the experimentation period, a colleague in the group thankfully could help after with providing a cloning of the C-and N-terminal constructs, so that I could start the tobacco infiltration with a working construct as well as visualizing the YFP-signal with the confocal microscope. The laboratory results below are the initial attempt of cloning of the C-terminal construct. In the initial attempt of cloning the C-Terminal construct, the sequencing of the products from the LR reaction did not contain any evidence that the NIN-gene was inside the vector at the correct location. PCR testing with NIN gene primer suggested that a construct with the size of the NIN gene could be inside the D'TOPO vector. The sequencing results were on the other hand convincing and it is likely that the insert was not inside the destination vector.

The reason for the laboratory results is probably an error somewhere between the first PCR and the D'TOPO cloning. One example of a laboratory error could be a use of too small amounts of D'TOPO vector compared to the recommended amount, due to a limited D'TOPO vector supply at the time. There are numerous of other possibilities such as handling of bacteria wrong or giving the wrong amount of an antibiotic.

Concluding which of the possibilities that led to the initial result is difficult, but because of the D'TOPO vector seemed faulty and the initial PCR that seemed correct, it is probable that the laboratory error happened before or during the first D'TOPO cloning.

Results PCR

The picture from the electrophoresis seems to confirm that the initial amplification and PCR was successful. The PCR result showed a band approximately at 1700 bp (see figure 9), this is close to the equivalent of the NIN gene size of 1674 bp.

Digestion of the products from the D'TOPO reaction

The gel pictures from the D'TOPO reaction showed ambiguous results. The digestion and the pictures from the gel showed that there was not a correlation between what the gels showed, and the prediction made from the plasmid maps (see figure 10). The restriction digestion was made with enzymes Nco1 and Nto1. The gel showed one band around 5000 bp instead of the expected three bands (see figure 11). This could mean that the NIN-gene insert was not there, the plasmid map was not correct or perhaps the plasmid was cut only once and the plasmid were linear.

Restriction Digest

Restriction digestion of the plasmid product from the Gateway LR reaction with enzyme Sal2, produced two bands (see figure 12). The bands are lower in size than the expected (see figure 13). This means that the digestion of the product from the LR reaction is not in correlation with the expected sizes.

PCR – LR-reaction

A PCR was also made for the different vectors. Both the bacterial E.*coli* colonies from the D'TOPO-reaction and from the colonies from the LR-reaction were tested in a PCR-reaction. Overall there seems to be bands in the correct region of around 1700 bp for both LR destination vector products and D'TOPO reaction products (See figure 14). One colony from the LR reaction didn't produce any bands at all. In some lanes there is a

band in the 1700 region, but it's of bit unclear size and structure. There are also artifacts in some lanes, which mean that the results from the PCR might be questioned.

Results sequencing

Results from the sequencing showed that there was not any inserts in the destination vector. The sequencing was made by using primers from the promoter 35:S and the YFP-gene. The YFP and promoter 35:S is next to the NIN gene according to the plasmid map (see figure 8, Methods and materials). The sequencing results showed both correlations with the opposite genes. In the sequencing results from the forward p35:S primer the YFP gene sequence could be seen, and in the sequencing results from the reverse YFP primer the p35:S gene sequence could be seen. This result indicates that the correct destination vector is sequenced, but it is empty without an inserted NIN gene. Two different colonies from the LR-reaction were tested and both showed similar results.

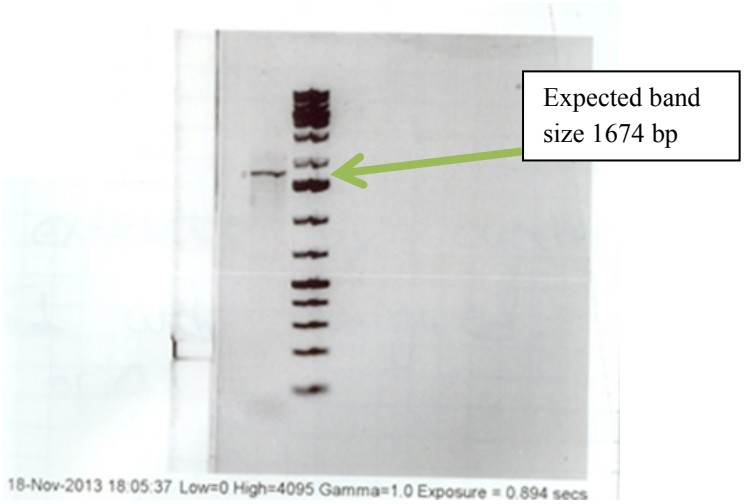


Figure 9. Picture of the agarose gel after the PCR reaction. The green arrow indicates where the expected band from the PCR is. The ladder used are 1kb plus ladder from Thermo Scientific. The band in the left lane is situated between the ladder bands in the right lane of 1500 bp and 2000 bp.

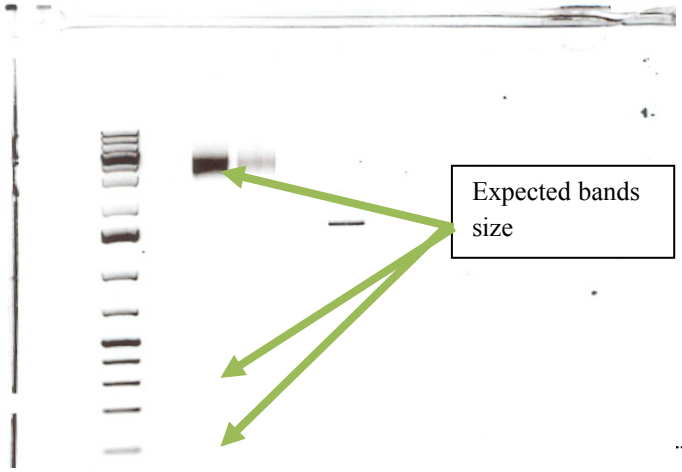


Figure10. The gel from a restriction digest of the D'TOPO vector after the D'TOPO reaction with restriction enzymes Nco1 and Nto1. The green arrows represent the expected band sizes after a restriction digest according to the plasmid map prediction (see figure 11). As seen the bands showing in the picture does not match with the expected band sizes shown by the green arrows.

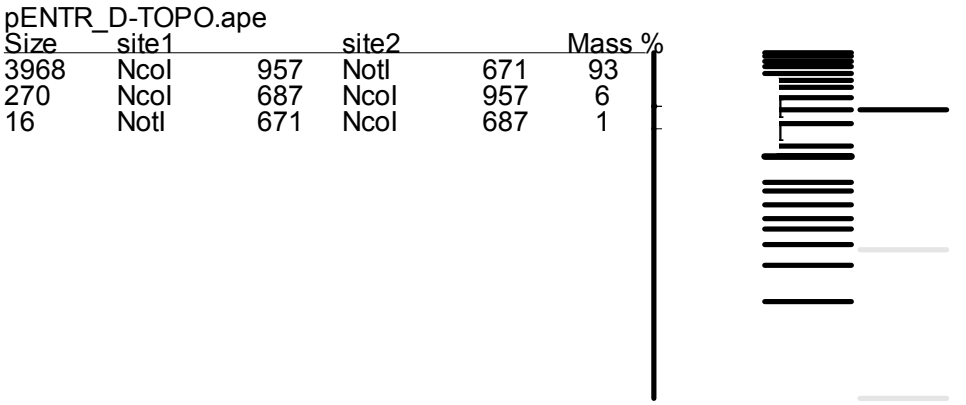


Figure 11. Expected band distribution after the digestion of the DTOPO vector with restriction enzymes Nco1 and Not1, with a prediction made from "A plasmid editor®".

Figure 12. The gel from restriction digest of the LR reaction products. The green arrows represent the expected band sizes after restriction digest, with a prediction made from the plasmid map (figure 8, materials and methods) (see figure 13). The five tested LR-colonies are showing in order 1-5. The green arrows points to the expected band sizes. The bands showing are not correlated to the expected bands size. The bands are lower in size compared to the expected band sizes. All five colonies show similar results.

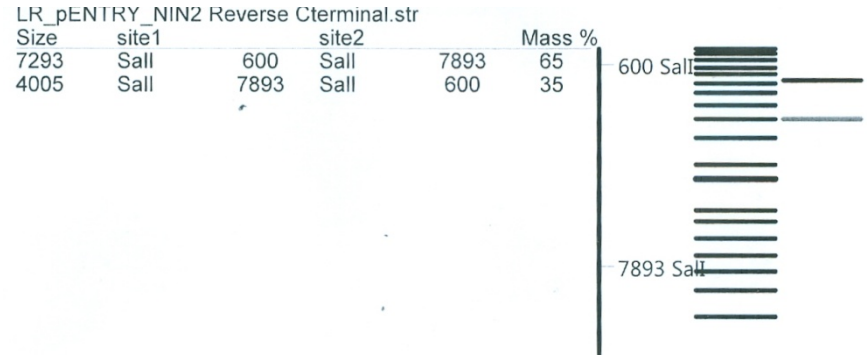


Figure 13. Above are expected band sizes for restriction digestion for the products from the LR reaction with the enzyme SalI, using a prediction from A plasmid editor® (figure 8, Materials and methods).

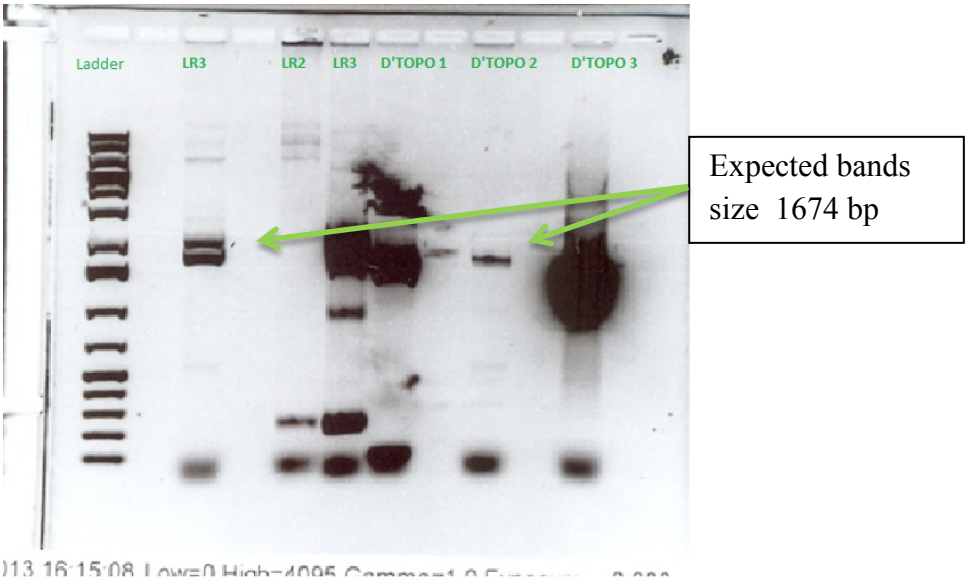


Figure 14. Picture of the gel from with LR-reaction products. The ladder used are 1 kb plus from Thermodynamics. LR 2 and LR 3 is the destination vector plasmids extracted from the Gateway LR reaction. There are two different LR-colonies tested and three D'TOPO colonies tested. The picture shows that there are bands at the correct size corresponding to the NIN-gene at 1674 bp, from both the LR and D'TOPO reaction products. The bands on the other hand are unclear in shape and there are artifacts in many of the lanes. The PCR results are therefore a bit ambiguous with some correct bands, but with poor quality of the shape of the bands and artifacts showing.

Confocal microscopy results

The fluorescent pattern from the neutral invertase (NIN) C-terminal YFP-construct showed an intervallic dotted pattern along the border of the leaf epidermal cells (figure 15). The border part would be in the cytosol in tobacco leaf cells (Brandizzi et al. 2002). The dotted expression indicated that the N-terminal YFP is located in some kind of cell compartment (figure 15). If the signaling part is intact at the N-terminal side of the protein, the C-terminal YFP-tag would be a good indicator of where the protein is located in native conditions.

The N-terminal YFP-NIN fusion protein looks to be localized in a fluorescent pattern in the cytoplasm in the tobacco leaf cell. The fluorescent signal is strong and it is spread all around the cytosol (see figure 18). It is also possible to see fluorescence in the canals in the middle of the cell between cytosolic parts (see figure 19). In the N-terminal fluorescence results it also looks like a pattern with yellow dots that fluorescence brighter, this pattern would be similar to the aggregated pattern found in the C-terminal construct results. Both the N- and C-terminal YFP-signal can be seen in the cytoplasm, but the N-terminal signal seems more widespread, while the C-terminal construct looks more aggregated (figure 15). The absence of a fluorescent pattern seen in the non-infiltrated samples (see figure 17), confirms the findings in the infiltrated samples, where it is possible to see a fluorescent pattern. The red chlorophyll autofluorescence signal does not correlate with the yellow YFP-signal in the C- or N-terminal YFP-tags (see figure 16). This means that it is likely that the NIN-protein is not localized in the chloroplasts.

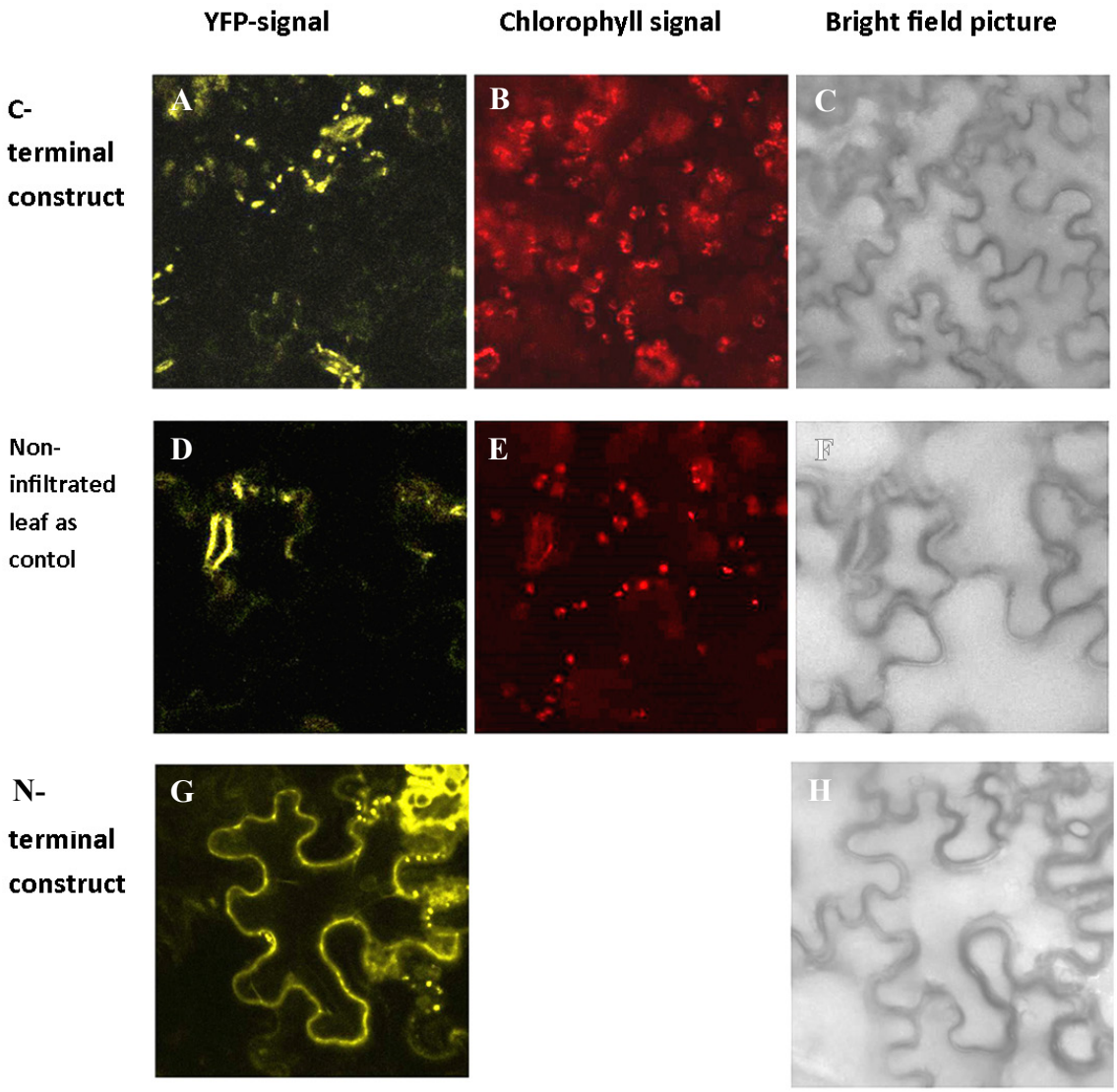


Figure 15. Representative pictures of the tobacco epidermis leaf cells infiltrated with the C and N-terminal YFP-tags and control. Pictures (A, B and C) are the C-terminal YFP-construct; (A) YFP-signal, (B) chlorophyll signal, (C) Bright field picture. (D, E, F) show a non-infiltrated leaf as control. (G) YFP-signal from the N-terminal tag, and (H) bright field picture of the same cell. The intervallic pattern with yellow bright dots can be seen in the C-terminal YFP-picture (A). The non-infiltrated sample show absence of the fluorescence seen in the infiltrated samples (D,E,F). The N-terminal construct show a widespread signal, while it is also possible to distinguish an intervallic pattern with brighter dots (G).

Pictures NIN C-Terminal YFP-tag

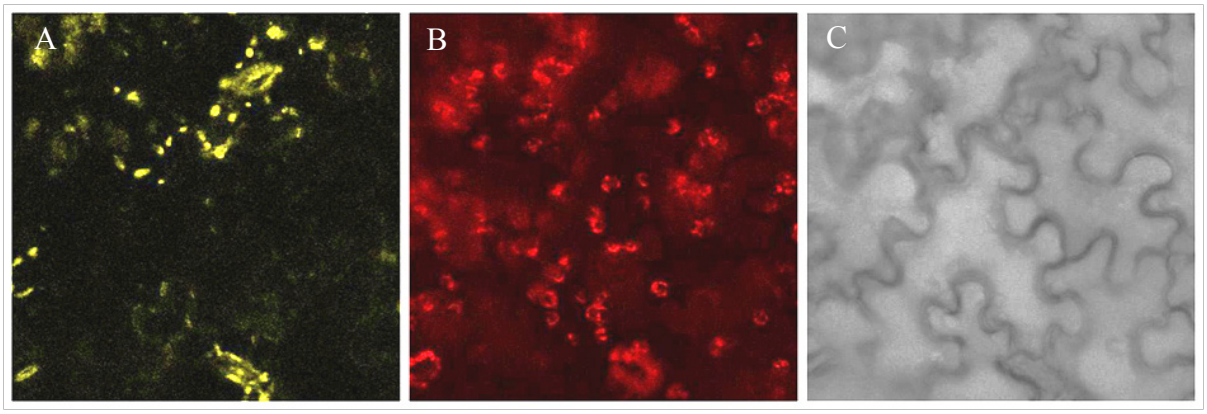


Figure 16. Tobacco leaf infiltrated with C-terminal YFP-tag. YFP-signal (A), chlorophyll signal (B), bright field picture (C). In this picture the yellow fluorescence is clearly concentrated in dots distributed along the upper border of a tobacco leaf cell. The red chlorophyll signal is not at the same location at the yellow YFP-signal (B). This makes it likely that that the YFP-signal does not come from the chlorophyll containing chloroplasts.

Non-infiltrated tobacco leaf as control

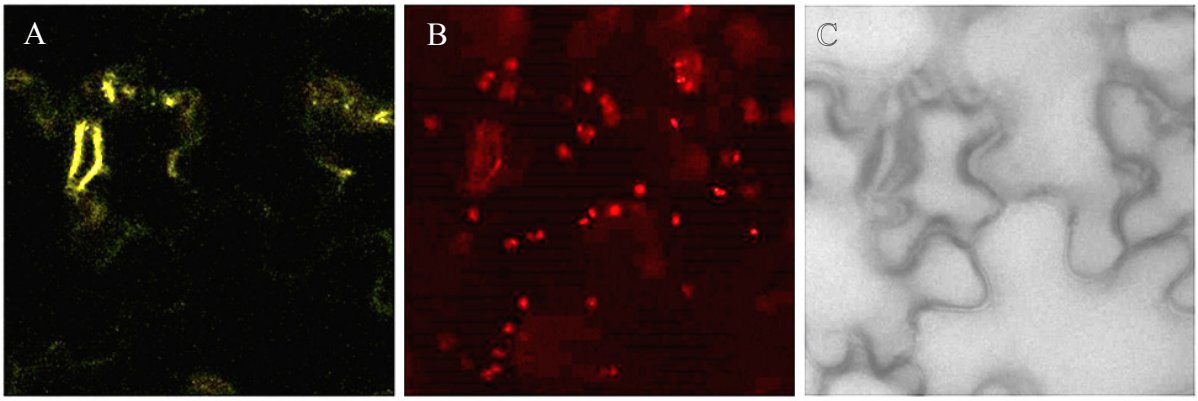


Figure 17. A non-infiltrated tobacco leaf as control sample. YFP-signal (A), chlorophyll signal (B), bright field picture (C). Compared to the florescence in other infiltrated samples, an absence of the fluorescent signal can be seen in this picture. There are not any signs of a pattern other than the background florescence, the yellow fluorescence seen at the upper left of picture (A) show what looks a stomata. The non-infiltrated control pictures confirm the findings with the infiltrated samples, where there is a significant amount of yellow florescence shown.

Pictures NIN N-Terminal YFP-tag

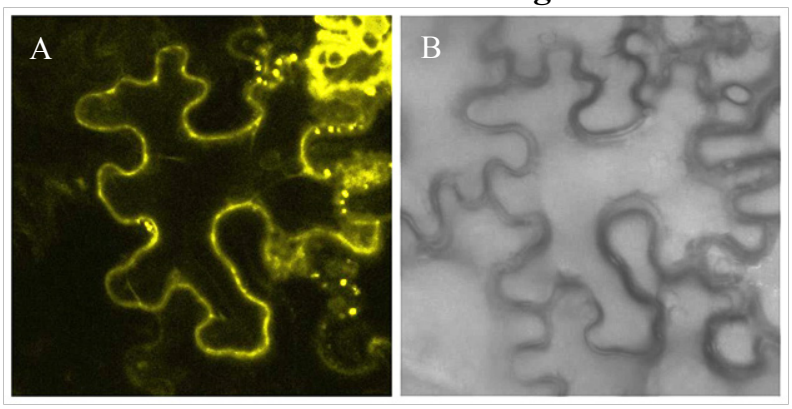


Figure 18. Tobacco leaf infiltrated with an N-terminal YFP-tag. YFP-signal (A), Bright field picture (B). In this picture the yellow fluorescence is spread all around the border of the tobacco leaf cell. There are some dots along the border that fluoresce more brightly.

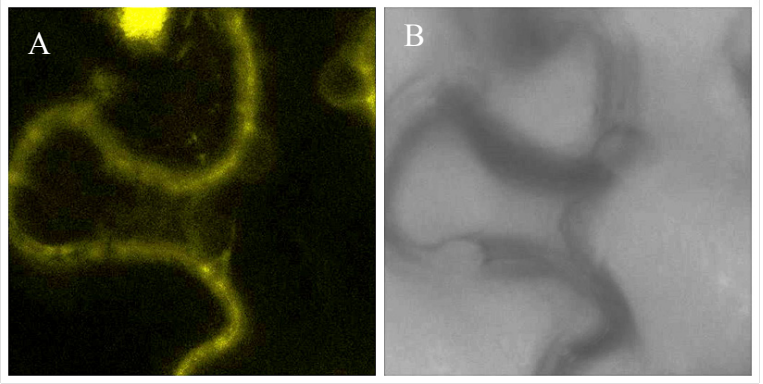


Figure 19. Tobacco leaf infiltrated with an N-terminal YFP-tag. YFP-signal (A). Bright field picture (B). It is possible to distinguish canals crossing in the middle of the cell between brighter dots in the border area. These fluorescent “canals” might be cytoplasmic canals (see discussion).

Prediction of the subcellular localization

I analyzed the NIN-protein sequence using prediction programs for subcellular localization. General prediction programs predicts that the protein is not localized in the mitochondria, chloroplast and that the protein does not contain a signal peptide (CELLO, Bannai et al 2002; IPSORT, Su et al. 2006), one of the programs indicated that the NIN-protein is likely to be cytoplasmic protein (CELLO, Bannai et al 2002). Another prediction program indicates that the protein is localized in the Nuclear Lamina (SCPS, Lei et al.2005). When the protein sequence was tested in a specific Golgi protein localization prediction program (Yuan & Teasdale 2002 http://ccb.imb.uq.edu.au/golgi/golgi_predictor.shtml), the sequence tested positive as a Golgi type 2 transmembrane protein. The positive prediction result was significant with a value of 24, clearly above the threshold value 20 marking significance. The prediction as a Golgi type 2 membrane protein stands out as being a clear significant result among the predictions from the prediction programs.

In summary the results from the confocal microscope indicate that the NIN-protein is located in the cytoplasm. The signal is more aggregated for the C-terminal tag, and more widespread in the cytoplasm for the N-terminal tag. The aggregation seen could indicate a subcellular compartment localization. Combined with the results from the Golgi prediction program (Yuan & Teasdale 2002 http://ccb.imb.uq.edu.au/golgi/golgi_predictor.shtml), it gives a hint of a possible localization.

Discussion

NIN N- and C-terminal construct results

Results from the neutral invertase (NIN) N-terminal YFP-tag show fluorescence in the cytoplasmic region of the tobacco leaf epidermis cells. The fluorescence is clear in the N-terminal pictures and the fluorescence can be seen all around the cytoplasm of the tobacco leaf cell (see figure 15). The pictures from the N-terminal YFP-NIN also show fluorescence in the cytosolic canals in the middle of the cell connecting the cytoplasm (see figure 19). If the signaling part of the protein has been disrupted by the N-terminal YFP-NIN construct, the N-terminal construct would say little of the native location of the NIN protein. There is also a pattern of more bright dots that fluoresces in an intervallic pattern, similar to the pattern that can be seen in fluorescence from the C-terminal NIN YFP-construct. This similarity indicates that the N-terminal fusion protein to some extent show aggregation in a similar way as the C-terminal YFP-NIN construct.

The results from the NIN C-terminal YFP-tag show fluorescence in the cytoplasmic area of the tobacco leaf cell. The fluorescence from the C-terminal YFP-tag can be seen in a dotted intervallic pattern along the border of the leaf cells (see figure 16). The dotted pattern would indicate that the C-terminal YFP-tag is aggregated in some way. The aggregation in an intervallic pattern could mean that the NIN gene is targeted to a compartment or perhaps membrane. Assuming that the C-terminal tag is less likely to interrupt the protein targeting, it is likely that the fluorescence from the C-terminal give a better hint of where the NIN protein natively is located in tobacco leaf cells. The pictures show that the signal from the yellow fluorescent protein wavelength is not correlated with the signal from the chlorophyll autofluorescence wavelength (see figure 16-17). This finding makes it probable that the C- or N-terminal YFP do not aggregate in or nearby chloroplasts. The pictures from the non-infiltrated leaf show little fluorescence in the YFP-wavelength spectrum, and indicate that the results seen with the C-terminal YFP-tag are different from control.

There are a number of different locations the NIN-protein could be targeted, including compartments such as: plastids, mitochondria, Golgi apparatus and the ER. Since the cytoplasm in tobacco leafs cells is seen in an area near the border, the YFP might also be in connection with the plasma membrane. When comparing the intervallic pattern with other studies with tobacco leaves, the results shows some resemblance to structures labeled as the Golgi apparatus (Hanton and Brandizzi 2006; Brandizzi et al. 2002; Stefano et al. 2006)

Combined with the conclusions from the prediction results the Golgi apparatus is a candidate for explaining the aggregated compartment-like pattern showing from the fluorescence results. The Golgi apparatus is where hemicelluloses and pectin are synthesized to become part of the cell-wall matrix during cell expansion in developing wood (Cooper 2000). If the NIN-protein somehow is associated with the Golgi apparatus it could support the view that neutral invertase is contributing to the cell wall biosynthesis (Figure 3 UPSC) (Gerber et al. 2014).

It should also be noted that the experiment is done with a hybrid aspen gene in tobacco, and the results could be different with the same experiment done natively in hybrid aspen. A similar study made with the NIN-protein in aspen protoplasts would be illuminating to show if the YFP-signal correlates with those compartments. Co-localization studies are in progress in aspen with markers for Tonoplast, ER, Golgi and plastids (Takata & Eriksson 2012). These experiments would make it possible to see if the NIN-YFP signal match with the compartment tags or not.

Prediction results

Results from the prediction programs for subcellular localization indicate that the peptide sequence is matching a Golgi localization. Some general prediction programs claimed the protein to be either cytoplasmic or Nuclear Lamina (CELLO, Bannai et al. 2002; IPSORT, Su et al. 2006; SCPS, Lei and Dai 2005), but the clearest significant prediction came from a Golgi-protein prediction program (Yuan and Teasdale 2002). The program

predicted that the protein sequence as a Golgi Type II transmembrane protein.

The prediction result was significant with a value of 24, clearly above the threshold value marking significance. Golgi Type II membrane proteins are responsible for synthesizing the pectin, xylan and adding side chains to polysaccharides (Bacic 2006; Persson et al.2007; Pena et al. 2007). Golgi Type II membrane proteins are therefore important in the assembly of the cell wall components hemicelluloses and polysaccharides. Since invertase is not a typical Golgi type II membrane protein such as glycosyltransferases (Cooper 2000), it raises the question of the function of invertase at the same possible localization as Golgi Type II membrane proteins.

Considering the results, literature and the up-regulation of the NIN-gene in the developing wood in hybrid aspen; the prediction results gives reason to suspect that the NIN-protein could be involved in supplying the Golgi cell wall biosynthesis machinery with sucrose derived hexose sugars.

Cell wall properties

The Golgi apparatus is important for sorting, modifying and packaging proteins to different locations in the plant cell. Golgi apparatus enzymes are able to modify proteins by both phosphorylation and glycosylation (Pavelka and Mironov 2008). The Golgi apparatus is where the biosynthesis for the glycosaminoglycans takes place and where the hemicelluloses in the cell wall are modified (Cooper 2000). If the NIN is located in the Golgi apparatus, an absence of the NIN in the *NIN RNAi*-null mutants would supposedly affect synthesizing proteins in the Golgi-apparatus. It would therefore be advisable to look for the effects of an absence of the supplier of sucrose derived hexose sugars possibly would cause. For example if the NIN feeds one of the glycosyltransferases in the Golgi-apparatus that affect the synthesis of hemicelluloses, it would be worthwhile to test for changes in the hemicellulose composition (Cooper 2000) in the *NIN RNAi*-mutant lines.

But there are many possible glycosyltransferases and many have different functions. A localization study made with isotope tagging found 197 Golgi apparatus proteins, of which 41 of are glycosyltransferases described in the CAZy database (Nikolovski et al. 2012) (http://www.cazy.org/GlycosylTransferases.html). A way to further investigate is by comparing the phenotype of the *NIN RNAi*-mutant lines, with *RNAi* null mutants of previously investigated Golgi-glycosyltransferases, affecting the cell wall biosynthesis (Scheible et al.2004; Nikolovski et al. 2012).

After this study was finished very preliminary evidence suggests (Rende, U, Unpublished) that the pectin levels may have changed in mutant *NIN RNAi*-lines. A previous described glycosyltransferase mutant "QUASIMODO1" have been found to be associated with pectin synthesis (Bouton et. al 2002), and an isoform have been located at a Golgi-localized type II integral membrane protein location (Miao et al. 2011). It is an unproven association, but a possibility of using previous studies when looking at the cell wall properties in the *NIN RNAi*-mutant lines.

According to (Scheible et al.2004) many *RNAi*-null mutants of glycosyltransferases that affects the cell wall biosynthesis, show little or no phenotypical variation compared to control. It could then be even more difficult to find changes in the phenotype of an *NIN RNAi* null-mutant, possibly feeding carbon to a glycosyltransferase affecting the cell wall biosynthesis. If the NIN supplies carbon from sucrose to the Golgi apparatus, it would also be surprising that only one aspect of the Golgi-machinery is affected, and other Golgi functions that require sucrose derived hexose sugars would remain relatively unaffected.

Conclusion

The results from the neutral invertase investigation look promising. My hypothesis considering the results is that it is plausible that the neutral invertase protein is localized in the Golgi apparatus supplying the Golgi Type II membrane proteins with carbon; thus affecting the secondary cell wall growth. The Golgi Type II membrane proteins would need sugars to synthesize pectin and hemicelluloses (Bacic 2006 ; Persson et al.2007 ; Pena et al. 2007), and the neutral invertase protein could perhaps be the link to supply the needed carbon from sucrose. The hypothesis that the neutral invertase protein is near and is part of the supply pathway to the glycosyltransferases is based on very preliminary results and would need much testing and experimenting to be verified. Such tests could be localization studies made in hybrid aspen with fluorescence tags for each of the compartments (Takata and Eriksson 2012), Western-blot to confirm the fusion protein size, and also testing the properties of hybrid aspen mutants to see how the cell-wall properties change.

If the NIN-protein is shown to be inside one compartment such as Golgi, it could also be interesting to investigate if there is any connection to the osmotic regulation such as theorized by (Sergeeva et al. 2006; Roitsch & González 2004; Ruan et al.2010). In previous studies and characterization of the Neutral and Alkaline invertases in *Populus trichocarpa*, it is predicted that all 16 Neutral and Alkaline invertases are localized in the cytosol (Bocock et al.2008). If the neutral invertase protein is localized in the Golgi apparatus, it would also mean that the way of thinking about neutral and alkaline invertases in hybrid aspen would have to be rewritten.

Further studies also could confirm the importance of invertase versus sucrose synthase in hybrid aspen. If the neutral invertase protein proves to be involved in the cell wall biosynthesis pathway, it would support studies that claim that sucrose synthase can be by-passed in the cell wall biosynthesis pathway (Gerber et al. 2014) by invertase.

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