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Overexpression and gene profiling of asparagine synthetase in hybrid poplar

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Overexpression and Gene Profiling of Asparagine Synthetase
in Hybrid Poplar

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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Science
And Renée Crown University Honors
May 2015

Honors Capstone Project in Biochemistry

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Date: December 11, 15

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Abstract

Plants with polysaccharide-rich secondary cell walls have great potential as a source of carbohydrates for bioethanol production. However, the presence of phenolic lignin inhibits the isolation of bioethanol precursors cellulose and hemicellulose from the secondary cell wall. Recent studies have linked nitrogen availability to secondary cell wall development and composition, making nitrogen metabolism genes an interesting target in the improvement of plant cell walls for biofuels production. Plants use a nitrogen assimilation pathway to convert inorganic nitrogen sources into organic sources, including amino acids, which play key roles in metabolism and cell wall development. Asparagine synthetase (AS), a key enzyme involved in the nitrogen assimilation pathway, produces asparagine from aspartate and either glutamine or ammonium. As asparagine is one of the major translocatable forms of nitrogen in poplar, AS has been selected for analysis in this study. Initially, gene profiling of the AS gene family will be conducted using hybrid poplar as a model organism to elucidate AS expression patterns throughout the plant tissues. Primers for RT-PCR have been generated and optimized for analysis of relative expression levels of different tissues at different developmental stages to localize where and when AS is most highly expressed. Transgenic poplar harboring AS are being regenerated and will be assessed for changes in development, growth and cell wall chemistry and structure. Nitrogen fertilization resulted in a significant difference in growth between no nitrogen and low nitrogen treatments in P39 hybrid poplar, but not between any other treatments. There was a difference in growth between 717 and P39 poplar hybrids, where the 717 hybrids grew to be statistically significantly taller. The results of this project will be used in the development of a model of how nitrogen assimilation impacts cell wall formation in poplar.

Executive summary

As the world's population increases and resources have become more in demand, the need for renewable energy sources has never been greater. Billions of dollars have been invested worldwide to develop sustainable and clean alternatives to energy sources like coal and crude oil, and biofuels represent one of the most promising alternatives. Much research is focused around cellulosic biofuels, as they represent a higher potential energy source than the more commonly used cornstarch-based alternative, but are currently less prevalent due primarily to economic factors. The expense of breaking down and isolating the cellulose polysaccharides from these tissues is too high to make it a viable source of the energy fuel bioethanol. Cellulosic biofuels are formed from long chain carbohydrate molecules called cellulose, which can be broken down into individual glucose molecules and fermented into bioethanol to be used as fuel is a major component of the plant cell wall.

While all plants have cell walls, certain plant species like trees have carbohydrate rich secondary cell walls that act as an extra structural support and are a primary component of wood. Plants with secondary cell walls have great potential as biofuel feedstocks, as they have significant amounts of polysaccharide-rich biomass that can be hydrolyzed and fermented into bioethanol. However, the presence of phenolic lignin, which cannot be degraded into bioethanol precursors and interferes with the isolation of the compounds that can, has made the use of these high potential plants expensive and ineffective. The focus of my research is on cellulosic biofuels, as they represent one of the

most promising ways to decrease use of fossil fuels and decrease humankind's carbon footprint.

An industrial process is utilized to generate bioethanol, but two major issues exist that make using cellulosic sources financially untenable. Treatment of the cell wall with compounds that interrupt the linking of the cell wall to isolate the individual components is difficult and expensive, and finding ways to interrupt and weaken the interactions of the different cell wall components would alleviate this. Generating plants with more polysaccharides that are packed less tightly is ideal for bioethanol production. Heavy nitrogen fertilization is a necessary step for many crops, but the high cost of nitrogen fertilizers and the potential for nitrogen runoff to contaminate water sources has created a need for plants that metabolize nitrogen efficiently. Improving nitrogen use efficiency of bioethanol crops would greatly reduce costs of producing these plants on an industrial scale.

One way to bring about these changes is to modify the plant cell wall is to alter its nitrogen content, as proper plant development is heavily dependent on nitrogen. Plants take up inorganic nitrogen from soil and employ a nitrogen assimilation pathway to generate organic nitrogen containing compounds, including amino acids, to be utilized throughout the plant in many metabolic and developmental pathways. Asparagine synthetase (AS), a gene vital to nitrogen transport in plants, acts by generating the amino acid asparagine, which plays many diverse roles in metabolism. Previous studies have established that nitrogen availability is heavily linked to secondary cell wall formation, but the specific

impact of genes like AS on cell wall formation are still poorly understood. By examining the expression of AS in the model organism poplar, my research is aimed at generating a better understanding of how this gene and nitrogen in general affects both the overall and secondary cell wall development of trees.

The role of AS has been examined in many plant model organisms, but has not been studied in woody plants like the poplar tree, leaving a serious gap in knowledge of how AS and Asn affect wood formation. Nitrogen availability has been shown to have a significant impact on wood formation and cell wall composition, but how and why it has this impact is poorly understood. Observation and modification of the transcription of genes like AS could give significant insight on how nitrogen affects wood formation and cell wall composition, and a better understanding of the development of these polysaccharide-rich areas of the plant. To determine if and how much AS and the resultant production of Asparagine affects wood formation and cell wall composition, this project will consist of two parts: overexpression of AS transcription, and gene profiling of all native AS genes in hybrid poplar.

By overexpressing the asparagine synthetase gene, more AS will be generated in transgenic trees than wild types, magnifying the phenotypic effects of AS on poplar development. To generate transgenic trees, expression plasmids containing the gene under the control of promoters that will amplify the expression of the gene in key tissues were transformed into hybrid poplar. Four individual transgenic events have been generated. The transgenic trees will be grown under nitrogen luxuriant and limited conditions and assessed for changes in

biomass quantity and quality. Using ion chromatography to measure soluble carbohydrate, amino acid, and starch content, these transgenic trees will be examined for changes in cell wall composition and AS expression. Any changes in levels and location of expression can be examined to further understand the roles of AS and asparagine in overall plant development, and specifically in wood and cell wall development. Based on previous work, we have hypothesized that AS overexpression will result in an increase in cellulose polysaccharides, making these plants more efficient feedstocks for the bioethanol.

Gene profiling of the AS gene family, will examine and quantify the transcription levels of three AS genes in different key tissues of the tree to understand where and when the gene is most highly expressed. Each individual asparagine synthetase gene was identified, allowing all AS to be examined. Hybrid poplar trees grown in a controlled greenhouse environment were harvested for key developmental tissues to be analyzed for AS expression using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and primers specific to each gene. This will elucidate where each gene is most strongly expressed, which could be utilized to focus areas of research of AS's role in development and to understand how high and low levels of AS transcription affect the development of key fibers in the plant.

With a clearer knowledge of tree fiber development and the role of AS in cell wall development, adjustments in nitrogen availability and gene transcription could make trees more effective at producing cell walls with qualities tailored towards efficient biofuel production.

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Introduction

Generating reliable and inexpensive sources of renewable fuels is vital to the future of the world's energy. Renewable energy sources like biofuels can limit the need for fossil fuel use and reduce atmospheric CO₂ levels, and generating biofuels from plants has the added benefit of CO₂ uptake during plant growth and the associated metabolic processes (Novaes *et al.* 2009). The carbohydrate rich cell wall of woody plants make them an ideal source for producing bioethanol, which can be formed by fermenting glucose monomers isolated from the cell wall and used as a source of liquid transport fuel (Li *et al.* 2014).

There are two primary sources of glucose for bioethanol from plants: noncellulosic and cellulosic tissues. Noncellulosic tissues composed of starch and soluble sugars are the most prevalent source of bioethanol today made primarily in the US from corn, but cellulosic sources of bioethanol could be another effective source if able to reach their full potential. There is a need for an increase in cellulosic biofuel production from plant biomass, as corn is a vital food and feed crop, requiring significant nitrogen fertilization, and is inefficient due to the small amount of kernel biomass used for bioethanol production (Patzek 2008). Difficulties in efficiently transporting the quantities of feedstock necessary for bioethanol production, as well as finding a viable amount of land, among other issues, hold back large-scale bioethanol production from cellulosic sources. Cellulosic biofuels are an energy source that could revolutionize the way the world is powered and represent an environmental benign option to decrease dependence on fossil fuels.

One source of lignocellulose that has great potential as a bioethanol feedstock is the secondary cell wall of dedicated fuel crops such as poplar, which contain high quantities of fermentable sugars. The secondary cell wall, a thick layer inside the primary cell walls of some

plant species and the primary component of wood, acts as extra support to the plant by increasing the thickness and strength of the cell wall (Wang *et al.* 2014). The secondary cell wall is composed primarily of phenolic lignin and the structural polysaccharides cellulose and hemicellulose. Cellulose and hemicellulose can be hydrolyzed into fermentable sugar monomers and fermented into the biofuel ethanol, and low lignin content makes the separation of these sugars easier and more efficient (Pitre *et al.* 2010). The overall chemical composition of the cell wall make the costs of isolating these sugars too expensive for them to currently be viable as a source for bioethanol. The recalcitrance of the secondary cell wall drives up the cost of isolating cellulose and hemicellulose for bioethanol production, limiting their use as renewable energy sources.

Lowering the cost of glucose recovery from the cell wall would allow for cellulosic sources to be an affordable and feasible way to produce bioethanol, but two major obstacles must be overcome to make this process more economically viable. First, the interactions of the different cell wall components make it difficult to isolate the polysaccharide components. In the cell wall, the various structural components interact in a tight network, with branched elements extending from the polysaccharide network to prevent penetration of the cell wall from and the cell itself from outside threats such as viruses and bacteria (Buckeridge *et al.* 2014). The variety of hemicellulose polysaccharide side chains creates a complex mixture that is difficult to alter by chemical treatments, but weakening the interactions between the cell wall components through genetic manipulations would allow for the isolation of the individual polysaccharides before hydrolysis and fermentation. Generating plants with more polysaccharides that are less tightly packed is ideal for bioethanol production.

Second, reduction of the need for nitrogen fertilization of crops would drastically reduce costs and prevent nitrogen runoff into groundwater (Sharpley 1997). Many crops currently require significant amounts of nitrogen fertilization to be viably produced on a major scale, but finding ways to reduce the need for fertilization would be both economically and environmentally favorable. A close examination of the factors controlling nitrogen's influence on the development of the secondary cell wall and its components could lead to the improvement of trees as a source of bioethanol.

Plant growth and development is heavily dependent on nitrogen, which plays a key role in many metabolic pathways (Canales *et al.* 2010). Nitrogen's impact on the development of the secondary cell wall is not well understood, but it has been established that it plays an important role (Pitre *et al.* 2007, 2010). Plants acquire two main inorganic sources of nitrogen from their environment: nitrate (NO_3^-) and ammonium (NH_4^+), and utilize a nitrogen assimilation pathway to synthesize nitrogen containing compounds from these inorganic sources for metabolic processes (Figure 1; Giannino *et al.* 2007). Nitrate reductase and nitrite reductase reduce NO_3^- to nitrite (NO_2^-), and NO_2^- to NH_4^+ , respectively, which can then be transferred from the chloroplast to the cytosol to be used by asparagine synthetase (AS). AS, a key enzyme in the plant nitrogen assimilation pathway, catalyzes the synthesis of the amino acid asparagine (Asn) (Gaufichon *et al.* 2010). AS uses aspartate and either ammonium or glutamine formed from glutamine-oxaloacetate transaminase (GOGAT) as an amino donor in its reaction to form Asn (Gaufichon *et al.* 2010). Asn is then transferred from the cytosol to the xylem, where it has been shown to be a key translocatable nitrogen source and to play a key role in nitrogen metabolism throughout the plant (Giannino 2007). The roles of the enzymes and products of the nitrogen assimilation pathway are well understood, however, the full effects of nitrogen and the enzymes

involved in this pathway on plant growth and development is not as clear (Wilson *et al.* 2012), particularly in woody species.

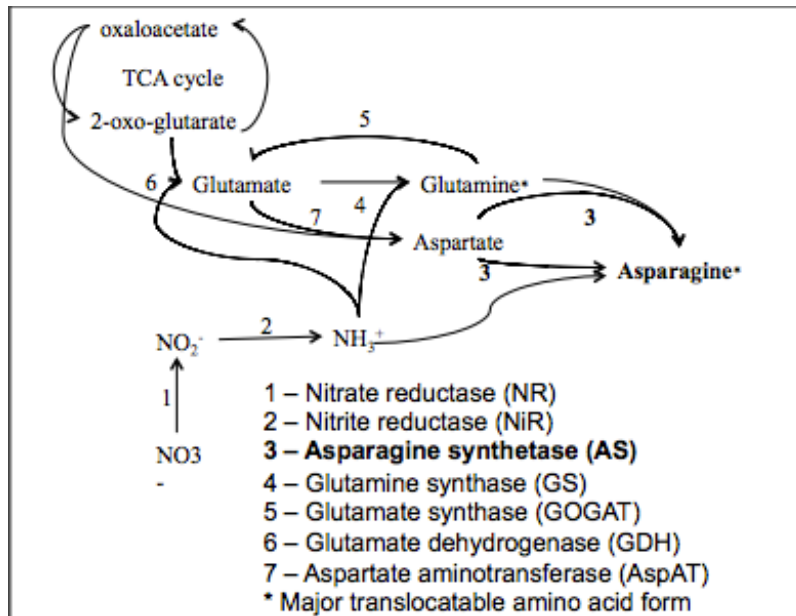


Figure 1. Plant nitrogen uptake pathway, enzymes identified from Miñin *et al.* 1976

Numerous studies have established a close link between plant nitrogen metabolism and cell wall development. Nitrogen typically is the limiting nutrient in tree growth, and nitrogen fertilization in poplar has been shown to increase wood cellulose content (Pitre *et al.* 2007). Nitrogen fertilization also changes allocation of biomass in favor of leaves and shoots over roots (Cooke *et al.* 2005). Another study examining the relationship between nitrogen fertilization and biomass development found a significant increase in all above ground tissues in response to nitrogen fertilization, with no changes in root development (Novaes *et al.* 2009). Using a genetic linkage map, Novaes *et al.* found 51 quantitative trait loci that are dependent on the presence of nitrogen fertilization. By studying the impact on genes involved in the nitrogen assimilation pathway, a greater understanding of the links between nitrogen fertilization, nitrogen metabolism, and cell wall development can be made.

Other genes in the nitrogen assimilation pathway have been found to have significant impacts on poplar development and cell wall composition. Glutamine synthetase overexpression in hybrid poplar caused a significant increase in galactose, mannose, xylose and glucose sugars, and improved ease of delignification (Coleman *et al.* 2012). Nitrogen reductase was found to have a 10-fold increase in expression in leaves compared to shoots and roots, and had increased expression in all tissues with increased nitrogen fertilization (Black *et al.* 2002). Modification of the expression of genes like AS could give significant insight on how nitrogen affects wood formation and cell wall composition, and provide a better understanding of the development of these fibrous areas of the plant. With a clearer knowledge of tree fiber development, adjustments in nitrogen availability and gene transcription will result in trees with cell walls with properties tailored to efficient biofuel production.

Profiling the expression levels of genes in hybrid poplar and other plant species have provided a framework of how gene families impact plant development. Characterization of the galactinol synthase genes in poplar revealed a temperature regulation of transcription for the PaxgGolSII gene that is involved in seasonal carbohydrate mobility, which was not seen in PaxgGolSI, which was found to have basic metabolic functions (Unda *et al.* 2012). An analysis of the amino acid transporter (AAT) gene family in rice, which identified that the OsAAT genes play key roles in response to abiotic stress signaling pathways (Zhao *et al.* 2012). Of the 85 OsAAT genes, many have been generated in duplication events to conduct specific functions in different tissues. The expression of each member of the glutamine synthetase (GS) and GOGAT gene families were altered by increased N fertilization, where expression of most genes were increased in leaves while there was either no change or a decrease in expression in roots (Zhao *et al.* 2006). The glutamine synthetase family in hybrid poplar was found to have specific seasonal

patterns of expression, and that GS duplicates were evolutionarily favored to increase enzyme levels in tissues needing significant N (Castro-Rodríguez *et al.* 2012). A similar study on the AS gene family would further characterize its role in N assimilation.

Poplar has become a common model organism for woody plant research and is a promising candidate as a bioethanol feedstock due to numerous factors. Poplar is characterized by rapid perennial growth and the ability to grow in many environments (Bradshaw *et al.* 2010). The poplar genome has been sequenced (Tuskan *et al.* 2006), and transformation and regeneration protocols are readily available. Traditional breeding techniques have been carried out to improve lines for experimentation. Hybridization improves growth rates, and hybrid lines such as 717 (*Populus tremula* x *alba*) and P39 (*P. grandidentata* x *alba*) are species that would potentially be used for commercial bioethanol production in North America.

Asn is a particularly effective nitrogen transport molecule to study, as it has the lowest carbon to nitrogen ratio of the nitrogen transport amino acids and would be the most efficient for the plant produce. The role of AS has been examined in many plant model organisms, but has not been studied in woody plants like the poplar tree, leaving a serious gap in knowledge of how AS and Asn affect wood formation. Experiments in non-woody plant species have shown that AS and Asn have significant effects on plant growth and development. In *Arabidopsis thaliana*, AS overexpression increased asparagine levels and led to improved growth in nitrogen limited environments (Gaufichon *et al.* 2013). In another study of *Arabidopsis* plants overexpressing AS had an increase in soluble seed and overall protein levels, along with improved Asn transport (Lam *et al.* 2003). Infection of transgenic *Arabidopsis* with *Xanthomonas campestris* lead to an increase in expression of pepper asparagine synthetase 1 (CaAS1), and asparagine production from aspartate is associated with enhanced resistance to pathogens (Hwang *et al.* 2011). In

lettuce (*Lactuca sativa*), AS overexpression caused a 30% increase in leaf area and dry weight (Giannino *et al.* 2007). In tobacco, overexpression of AS resulted in a 10 to 100 fold increase in free asparagine in leaves (Brears *et al.* 1993)

Two approaches were utilized in this study to better understand the role of AS in poplar development. First, gene profiling of the AS gene family examined and quantified the transcription levels of all copies of AS in different key tissues of the tree to understand where and when the gene is most highly expressed. Sequence analysis identified three unique AS genes in poplar. Using semi-quantitative PCR (qPCR), the expression of each of the three genes in poplar was quantified to clarify the expression patterns of the gene family. Trees for this experiment were grown under nitrogen luxuriant and limiting treatments and harvested for tissues expected to show AS expression when examined. Growth rates of each hybrid line were analyzed to study the impact of nitrogen fertilization on the overall plant phenotype. The data from this experiment will inform more targeted studies of poplar AS expression and function in the future.

Second, AS was overexpressed in poplar under the control of two different promoters. Based on previous work, we hypothesized that AS overexpression would result in an increase in cellulose polysaccharides, making these trees an improved feedstock for bioethanol. Transgenic plants were generated containing AS under the control of either the putative vascular specific subterranean clover stunt virus (SCSV) promoter or the constitutive cauliflower mosaic virus 35S promoter (CaMV 35s). The data from this experiment will determine the impact of AS on secondary cell wall development. Any changes in levels and location of expression can be examined to further understand the roles of AS and asparagine in overall plant development, and

specifically in wood and cell wall development. Further work will characterize these plants for alterations in biomass production and cell wall properties.

Results

Plasmid production

Bacterial plasmids containing the AS gene under the control of either the subterranean clover stunt virus (SCSV) promoter or the Cauliflower mosaic virus 35s (CaMV) promoter paired with the nopaline synthase (tNOS) terminator from *A. tumefaciens* were generated (Figure 1). Constructs were confirmed using restriction digest and sequencing.

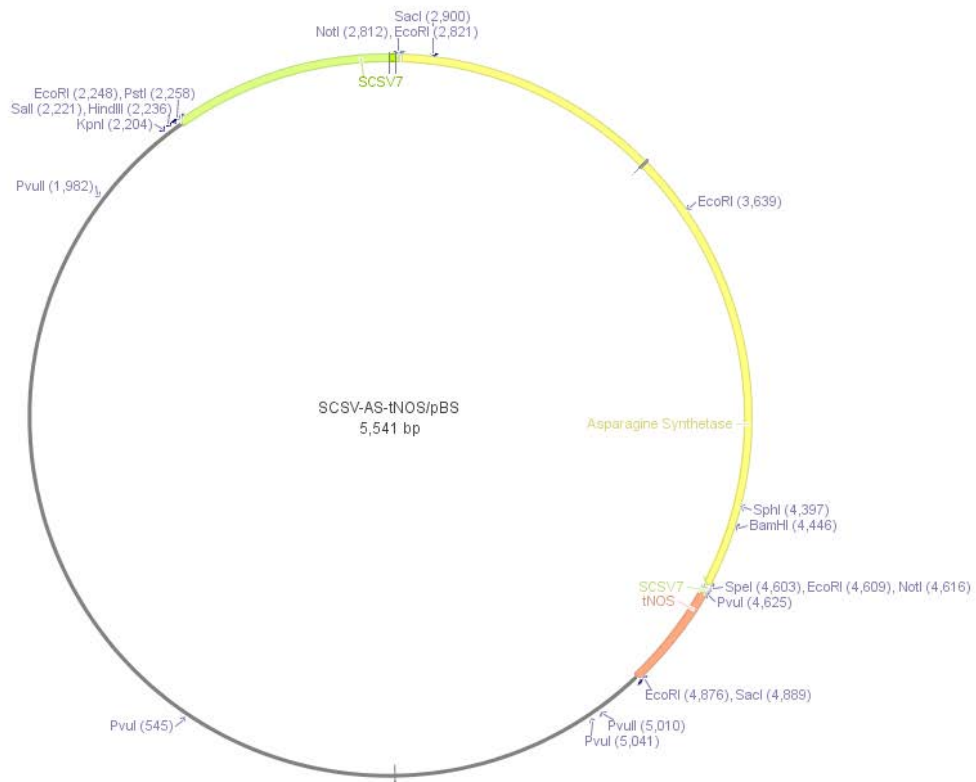


Figure 1. SCSV::AS::tNOS expression cassette in the pCAMBIA 1300 binary vector. The CAMV::AS::tNOS expression cassettes are identical with that with the SCSV promoter being replaced with the CAMV promoter. Adapted from Geneious program

Overexpression transgenic trees

Bacterial plasmids containing a copy of *P. trichocarpa* AS were transformed into *A. tumefaciens* and then into 717 hybrid poplar. Four individual transgenic events were generated under the control of the SCSV vascular tissue specific promoter. CAMV::AS::tNOS transformations are in progress One replicate of transgenic line 4 can be seen in Figure 2. Following the production of a minimum of three lines per construct, these lines will be grown in the greenhouse and analyzed for changes in biomass production, cell wall chemistry, and nitrogen metabolism properties



Figure 2. Transgenic poplar containing the SCSV::AS::tNOS construct

Identification of AS gene family

One gene (gi 566170058) had been previously identified as AS in poplar and was named AS1 for simplicity. Using sequence alignment programs from NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and HMMER (<http://hmmer.janelia.org/>), two other AS

genes were identified (gi 118488371 and 566186829), making three members of the poplar AS gene family. This number is consistent with the AS gene family in Arabidopsis. A strong homology is seen between the three AS genes. Each gene was given a shortened name for identification purposes, gi 118488371 corresponds to the gene identified as AS2 and gi 566186829 corresponds to the AS gene identified as AS3. Figure 3 shows the sequence alignment of each proteins amino acid sequence.


```

gi|566170058|ref|XP_002306310.      MCGILAVLGCADNSQAKRSRVIELSRRLKHRGPDNSGLHCHGDCYLAHQ 50
gi|118488371|gb|ABK96003.1|      MCGILAVLGCADNSQAKRSRVIELSRRLKHRGPDNSGLHCHGDCYLAHQ 50
gi|566186829|ref|XP_002313246.      MCGILAVLGCSDDSQAKRVRVLELSRRLKHRGPDNSGLYQCGDFYLAHQ 50
*****!;!*!***** **;*****!*****; ** *****

gi|566170058|ref|XP_002306310.      LAIVDPASGDQPLYNQDKTVVVTVNGE IYNHKLRAQLKSHFTFRGSDCE 100
gi|118488371|gb|ABK96003.1|      LAIVDPASGDQPLYNQDKTVVVTVNGE IYNHKLRAQLKSHFTFRGSDCE 100
gi|566186829|ref|XP_002313246.      LAIIDPASGDQPLFNEQAI VVTVNGE IYNHEELRKRLPNKHFRGSDCD 100
***;*****!;*!;!*!*****!***** !* .*.*****;

gi|566170058|ref|XP_002306310.      VIAHLYEEHGENFVDMLDGMF SFVLLDTRDKS FIAARDAIGITPLYIGWG 150
gi|118488371|gb|ABK96003.1|      VIAHLYEEHGENFVDMLDGMF SFVLLDTRDKS FIAARDAIGITPLYIGWG 150
gi|566186829|ref|XP_002313246.      VIAHLYEEYGENFVDMLDGMF SFVLLDTRD NSFIVARDAIGITPLYIGWG 150
*****;*****!*****;***.*****

gi|566170058|ref|XP_002306310.      LDGSVWFASEMKALSDDCERFMSFLPGHIYSSKQGALRRWYNPPWFSEQI 200
gi|118488371|gb|ABK96003.1|      LDGSVWFASEMKALSDDCERFMSFLPGHIYSSKQGALRRWYNPPWFSEQI 200
gi|566186829|ref|XP_002313246.      LDGSVWISSELKGLNDDCEHFECFPFGHLYSSKSGGLRRWYNPPWFSEAI 200
*****!;!*!*.*****! * . * *****!;!*!*****!*****

gi|566170058|ref|XP_002306310.      PSSPYDPLALRKAFAEKAVVKRLMTDVPFVGLLSGGLDSSLVAAVASRHLA 250
gi|118488371|gb|ABK96003.1|      PSSPYDPLALRKAFAEKAVVKRLMTDVPFVGLLSGGLDSSLVAAVASRHLA 250
gi|566186829|ref|XP_002313246.      PSTPYDPLVLRRAFEKAVIKRLMTDVPFVGLLSGGLDSSLVAAVTARHLA 250
**;*****.***;*****!*****!*****!*****!*****

gi|566170058|ref|XP_002306310.      DSEAAQQWGSQQLHTFCIGLKGSPDLKAAREVADYLRTRHHEFYFTVQEGI 300
gi|118488371|gb|ABK96003.1|      DSEAAQQWGSQQLHTFCIGLKGSPDLKAAREVADYLRTRHHEFYFTVQEGI 300
gi|566186829|ref|XP_002313246.      GTKAARQWGAQLHSFCVGLNSPDLKAAREVADYLGTVHHEFYFTVQDGI 300
.!!;***!***!***!***!***.***** * *****!**

gi|566170058|ref|XP_002306310.      DALEEVYIHIETYDVTTVRASTPMFLMSRLVPLCTMASVYNDGRLTVGTA 350
gi|118488371|gb|ABK96003.1|      DALEEVYIHIETYDVTTVRASTPMFLMSRKIKSLGVKNVIS-GEGSDEIF 349
gi|566186829|ref|XP_002313246.      DAIEDVYIHIETYDVTTIRASTPMFLMARKIKALGVKNVIS-GEGSDEIF 349
**;*****!*****!* : : * . * . !

gi|566170058|ref|XP_002306310.      TGYLYPHKAPNKEEFHQETCRKIKALHLYDCLRANKSTSAWGVEARVPFL 400
gi|118488371|gb|ABK96003.1|      CGYLYPHKAPNKEEFHQETCRKIKALHLYDCLRANKSTSAWGVEARVPFL 399
gi|566186829|ref|XP_002313246.      CGYLYPHKAPNKEELHRETCRKIKALHQYDCLRANKATSAWGLEARVPFL 399
*****!*****!;***** *****!*****!*****

gi|566170058|ref|XP_002306310.      DKEFINIAMSIDPEWKMI RPD LGRIEKWVLRNAPDDEKNPYLPKHILYRQ 450
gi|118488371|gb|ABK96003.1|      DKEFINIAMSIDPEWKMI RPD LGRIEKWVLRNAPDDEKNPYLPKHILYRQ 449
gi|566186829|ref|XP_002313246.      DKDFINVAMAIDPEWKMIKPGQGHIEKWVLRKAPDDEHPYLPKHILYRQ 449
**;***!***;*****!*. *;*****!*****!;*****

gi|566170058|ref|XP_002306310.      KEQFSDGVGYSWIDGLKD HANKQVTDANLMNASFIYPENTPTTKEAYYR 500
gi|118488371|gb|ABK96003.1|      KEQFSDGVGYSWIDGLKD HANKQVTDANLMNASFIYPENTPTTKEAYYR 499
gi|566186829|ref|XP_002313246.      KEQFSDGVGYSWIDGLKAAHAHQVTDKMQNAEHIFPHNTPTTKEAYYR 499
***** ***** ** !;*** *! **..*!* *****

gi|566170058|ref|XP_002306310.      TIFEKFFPKNSARSTVPGGPSVACSTAKAVEWDAAWSKNLDP SGRAALGV 550
gi|118488371|gb|ABK96003.1|      TIFEKFFPKNSARSTVPGGPSVACSTAKAVEWDAAWSKNLDP SGRAALGV 549
gi|566186829|ref|XP_002313246.      MIFERFFPKNSARLSVPGGPSVACSTAKAVEWDAAWSNNLDP SGRAALGV 549
*****!*****!;***** *****!*****!*****

gi|566170058|ref|XP_002306310.      HEAAAYEETVDTKAASQNNNGSPQKPKQCTLEKTATVA----- 585
gi|118488371|gb|ABK96003.1|      HEAAAYEETVDTKAASQNNNGSPQKPKQCTLEKTATVA----- 584
gi|566186829|ref|XP_002313246.      HLSDYDQQAALANAG---VVPKIIDTLPRMLEVSASGVAIHS 589
* ! *! : * . * * * .** ! *!

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Figure 3. Amino acid alignment of three identified poplar AS genes. Amino acids labeled with * indicate homology among all three genes, and there is strong sequence similarity among the three genes.

Greenhouse growth

Wild type 717 (*Populus tremula* x *alba*) and P39 (*P. grandidentata* x *alba*) hybrid poplar trees were grown under controlled greenhouse conditions. Five trees of each species were grown under each of three nitrogen treatments: nitrogen free (0 mM N), nitrogen limited (0.15 mM N),

and nitrogen excess (1.5 mM N) conditions. All plants were measured for height growth prior to harvest (Table 1). There was a significant difference between the 0.15N and 0N treatments for the P39 hybrid (p-value of approximately 0.0183), but all other treatments were not significant.

Following a period of 12 weeks, all trees were destructively harvested and used for RNA extraction.

Hybrid Line	0 mM N	0.15 mM N	1.5 mM N
717	69.75 ± 5.61 cm	72.5 ± 6.83 cm	67.7 ± 9.99 cm
P39	57.92 ± 4.83 cm	55.3 ± 7.97 cm	57.6 ± 2.10 cm

Table 1. Average height with standard deviations of each hybrid line under three nitrogen fertilization conditions.

Primer design

Primers to amplify each individual AS gene were designed using the NCBI primer-BLAST function (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Each primer was designed to exclusively amplify the specified AS sequence. The nucleotide sequences of each primer set can be seen in Table 1.

AS Gene Amplified	Nucleotide Sequence
AS1 - Forward	GAGTTCTTTTATCTGGAGG
AS1 - Reverse	GAAGGAATGGAGTTGTG
AS2 - Forward	AAGCCTTTGACGACGAGGAG
AS2 - Reverse	AACTGTAGCCAACGCCATCA
AS3 - Forward	ACCCAGAAAATACTCCTACAACC
AS3 - Reverse	CATCCATTCCACTGCTTTTG

Table 2. Nucleotide sequences of forward and reverse primers for each individual AS gene amplified

Discussion

Understanding how nitrogen fertilization influences both quantity and quality of poplar biomass is key to developing improved lines for use as a biofuels feedstock. In the greenhouse study, it was surprising that the height differences between the fertilization treatments was minimal, and there was a wide variety of heights for each treatment, as indicated by the large standard deviations. Were the plants to be grown longer, and with a larger sample size, a larger trend may be seen. There was a significant difference between the growth of the 0 N and 0.15 N treatments ($p \ll 0.01$) between hybrid species, and a trend towards an increase in growth for the 1.5 N treatments ($p \approx 0.0731$) according to a two tailed Students t-test. This implies that 717 hybrids grow to be taller than P39 regardless of N treatment. While height is an estimate of biomass, it would also be necessary to measure caliper of the stem to get a true understanding of the implication of this for biomass production.

Although the results of this experiment are expected to provide information about the expression patterns of AS in two poplar hybrids, studies in other species should be conducted to further characterize the homologous genes in other potential bioethanol feedstocks. Expression of 13,838 expressed sequence tag sequences from *Populus euphratica* were compared to the *P. trichocarpa* genome, and approximately 2,000 tags were unique to the species, showing that the regulation of gene expression varies in different subspecies of poplar (Brosché *et al.* 2005). Significant differences in expression levels of both gene families and individual genes in the xylem transcriptome has been seen between *Populus* and *Eucalyptus grandis* (Hefer *et al.* 2015). The availability of the genomic information of *E. grandis* provides a comparison point to determine whether trends seen in poplar are conserved in other woody species (Myburg *et al.* 2014).

One example of cell wall related genes that have been assessed for their expression profile are the invertase genes. Invertase has been identified to be heavily involved in sugar metabolism related to cell wall synthesis. Three genes encoding the sucrose cleaving cell wall invertase enzymes PaxgINV1, 2, and 3 were identified and found to have expression levels highly regulated to the plants developmental stage (Canam *et al.* 2008). PaxgINV1 was found to be exclusively involved in regulation of dormancy, while PaxgINV2 is involved in providing carbon resources necessary for cell wall synthesis and respiration in actively growing tissues, and PaxgINV3 is specific to floral processes. This information was able to inform future studies on the role of invertases on cell wall formation, and the information expected from a similar study in AS will inform further work on the impact of nitrogen metabolism on cell wall formation.

Understanding the variability of gene expression in nitrogen metabolism in woody species would clarify its role in secondary cell wall formation. Continued studies of the genes involved in the nitrogen assimilation pathway could provide a model of the impact nitrogen has on cell wall development. Studying the variability of expression of key genes involved in cell wall formation, particularly the cellulose synthase (CesA) genes in response to nitrogen fertilization would further clarify the overall process of cell wall development. Understanding how the genes that synthesize cellulose, hemicellulose, lignin, and the other minor components of the secondary cell wall function and are expressed under different nitrogen conditions would explain the relationship between nitrogen metabolism and carbon allocation.

Identifying genes in the nitrogen assimilation pathway with conserved functional impact between different woody species will clarify the impact of nitrogen metabolism on cell wall formation. Similar studies of the components of the nitrogen assimilation pathway in *Eucalyptis*

or *Salix* (willow), particularly AS, would confirm the role of AS in wood and secondary cell wall synthesis for all species.

The genes involved in cell wall formation are closely linked, and both the specific roles of each gene that have an impact and their roles in conjunction with each other are not well defined. All five members of the Cesa gene family in poplar that are highly expressed during wood formation were found to have at least one association with lignin formation (Wegryzn *et al.* 2010). The strong link between the major polysaccharide and lignin synthesis genes with lignin formation implies that the functions of these genes are highly connected and work closely in conjunction to generate fully formed secondary cell walls.

Nitrogen fertilization can have wide-ranging effects on plant development, and has been proven to impact developmental processes not directly linked to nitrogen metabolism. Nitrogen fertilization led to significant decreases in microbial biomass and soil respiration levels in cottonwood compared to lower treatments, establishing that it can have effects on a variety of characteristics not directly related to nitrogen metabolism (Lee *et al.* 2003). High nitrogen fertilization led to increased radial growth and vessel size in H11-11 hybrid poplar (*Populus trichocarpa x deltoides*), leading to xylem tissues with enhanced transport capacity (Plavcová *et al.* 2013). This fertilization led to changes in expression of 388 identified genes, many of which are directly or putatively involved in nitrogen and carbohydrate metabolism, including characterized secondary cell wall transcriptional regulators.

Once the overexpression experiment is finished, it is expected that there would be increased levels of both AS and Asn in shoot tissues (developing xylem, bark, and leaves relative to roots). The increased nitrogen allocation to asparagine and away from glutamate and the glutamine it was synthesized from would pull nitrogen from the roots to other tissue more

quickly. This increased availability of nitrogen made available by increased asparagine would then result in increased growth and higher cellulose content. This change would stimulate an increase in resource allocation to all above ground tissues, and thus an increase in carbon levels in the form of higher proportions of cellulose and hemicellulose in relation to wild type trees.

In the future, AS could be studied in poplar further by modifying its expression levels in ways other than overexpression. RNA interference (RNAi) of AS in particular would be an appropriate complement to AS overexpression by knocking down the expression of AS to establish how plants develop without AS. If knocking down AS via RNAi causes a favorable phenotype, CRISPR/Cas9 could be utilized to completely knock out the gene (Gao *et al.* 2015). The role of asparagine as a nitrogen fertilizer may also be examined, as little is known about the process of organic nitrogen uptake (Näsholm *et al.* 2009).

Methodology

Overexpression

Construct generation

To generate overexpression plasmids, AS was cloned and ligated into the CAMV 35S-tNos (cauliflower mosaic virus) and SCSV S7-tNos (subterranean clover stunt virus) entry vectors using T4 DNA ligase via the NotI restriction enzyme site (Schunmann et al., 2003). The expression cassettes were transformed into the binary vectors pCAMBIA 1300 and 2300 backbones. The vectors were transformed into DH5 α *E. coli* cells and confirmed using restriction digest and sent for sequencing using the M13 forward and reverse primers. The sequencing was carried out by GENEWIZ Inc.

To generate transgenic trees, these plasmids were transformed into *Agrobacterium tumefaciens* cells. 1 μ L plasmid DNA was added to 100 μ L of competent GV3101 *A. tumefaciens* cells and incubated at 37° C for 5 minutes. 1 mL of Luria Broth (LB) media was added and incubated for 2 hours at 28° C while shaking. The tubes were centrifuged for approximately 20 seconds, the supernatant was removed and the pellet was resuspended in the small amount of supernatant remaining. These cells were spread evenly on a LB plate with antibiotics appropriate for the strain, and left at 28° C to incubate for 2 -3 days. Colonies were picked and grown in liquid LB media with the appropriate antibiotics, and after DNA isolation using the Wizard[®] DNA purification kit the colonies were screened for presence of the transgene by restriction digest.

Poplar transformation

Transgenic plants were generated following protocols established previously (Coleman *et al.* 2007). Leaf tissue from hybrid poplar was cut into 1 cm by 1 cm pieces and incubated in liquid woody plant media (WPM, see Appendix 1) overnight. A WPM solution containing approximately 40 μ L of *A. tumefaciens* and 20 mg of acetosyringone was generated and shaken overnight to grow the bacteria. The leaf disks were incubated in the WPM/*A. tumefaciens* solution for 15 to 30 minutes to transfer the plasmid from the bacteria to the disks and plated onto solid WPM media.

Once transformed, these leafs were incubated on WPM solutions containing antibiotics to induce root and shoot formation to generate new plant lines containing the plasmid. These plants were screened using restriction digest to confirm the presence of the plasmid, and each individual transgenic line was propagated to generate clones.

Genomic screening

To screen for presence of the plasmid, DNA was extracted for PCR amplification. Leaf tissue was cut from each plant and placed in a 1.5 mL tube and frozen in liquid nitrogen. The frozen leaves were ground to a powder before adding a solution of 1mL CTAB and 2 μ L mercaptoethanol. Leaf samples were then incubated at 65° C for 30-60 minutes, and then centrifuged at 14000 rpm for 10 minutes. 900 μ L of the supernatant was removed and added to a 2 mL tube along with 900 μ L of phenol-chloroform and mixed. This solution was centrifuged at 14000 rpm for 10 minutes and 700 μ L of the upper aqueous layer was moved to a new 1.5 mL tube and mixed with 350mL isopropanol. This solution was centrifuged at 14000 rpm for 10 minutes and after removing the supernatant the pellet was washed with 1mL of 70% ethanol.

This mixture was centrifuged at 14000 rpm for 5 minutes and the supernatant was removed. The pellet was dried using the speedvac for 25 minutes at room temperature and resuspended in 50 μ L of water.

Gene Profiling

Gene identification and primer design

Each AS gene in poplar was found by comparing the nucleotide sequence of the known poplar AS with the *Populus trichocarpa* genome. Using the HMMER and BLAST homology databases, two additional AS genes were identified.

Greenhouse growth and harvest

Trees were grown in a controlled environment under no nitrogen (0 N), nitrogen limited (0.15 N) and luxuriant (1.5 N) environments (Appendix 2). Both 717 and P39 hybrid trees were planted, with 16 total trees for each hybrid species. Trees were grown under 16 hour light, 8 hour dark cycles at room temperature, watered twice a month with 500mL, and fertilized three times. Trees were grown for approximately 3 months. Before harvest, the height of each tree was measured in cm, and statistical tests on tree height were run using a one-sided t-test.

Each tree was harvested for relevant tissues for analysis. Approximately 3 cm of the apex was cut and saved (A). One newly developed leaf, the first leaf from the apex approximately 3 cm in diameter, was saved and marked as a developing leaf (TL). The largest leaf without any discoloration was saved and marked as a developed leaf (BL). Xylem tissue was isolated by cutting the portion of the tree separating the two isolated leaves in half, cutting open the bark, and scrapping the xylem tissue off of the stem. The bark from the top half of the shoot separating

the two isolated leaves was saved as new bark (TB), and the bottom half was saved as developed bark (BB) The top half of isolated xylem tissue was saved as developing xylem (TdX), the bottom saved as developed xylem (BdX). Roots were saved from each plant by isolating the newly developed fine roots (SR) and thick lateral roots (BR) from the soil of each plant. These tissues were stored on dry ice while harvesting, and transferred to -80°C for storage.

RNA isolation and cDNA synthesis

To isolate RNA for PCR analysis, approximately 100 to 150mg of each sample was ground in a mortar and pestle under liquid nitrogen. 1mL of Tri-reagent was added to each sample and incubated at room temperature for 5 minutes. 200 μL of chloroform was added to each sample, then shaken for 15 seconds. The samples were then incubated at room temperature for 5 minutes. The samples were centrifuged at 12000xg for 15 minutes at 4°C , and the aqueous phase was transferred to new 1.5mL tubes and mixed with 0.6mL isopropanol. The samples were then incubated at room temperature for 10 minutes. The samples were centrifuged at 12000xg for 10 minutes at 4°C . The supernatant was removed and the pellet was washed with 1 mL of 75% ethanol. The samples were vortexed and centrifuged at 7500xg for 5 minutes at 4°C . The supernatant was removed and the pellet was dried for 10 minutes and resuspended in 30 μL of RNase free water. The samples were incubated at 60°C for 10 minutes, checked for RNA concentration using the nanodrop, and frozen at -80°C .

To synthesize cDNA, RNA samples were first purified to remove DNA. 1 μg of RNA was diluted in 8 μL RNase free water. 1 μL of RQ DNase buffer and DNase were added to the solution and mixed. This solution was incubated for 1 hour at 37°C , and 1 μL of RQ DNase stop solution was added and mixed. This solution was incubated at 65°C for 10 minutes. 1 μg of

RNA from this solution was utilized to synthesize cDNA following the protocol for iScript cDNA synthesis.

Primer design and optimization

Using their nucleotide sequences, primers for selective amplification of each gene were developed and ordered based on the expected melting temperature and amplicon length.

Primers were optimized for maximum amplification efficiency by finding optimal amplification temperature, primer concentration, and cycle number for RT-PCR.

RT-PCR

cDNA was diluted into 1:10 dilutions. Each RT-PCR reaction contained 1 μL of the diluted cDNA, 1 μL of each 1 μM AS forward and reverse primer, 7 μL of water, and 10 μL of BIORAD SYBR Green. Elongation factor 1 (EF1) was used as a reference gene. Each reaction was run on a BIORAD thermal cycler under the protocol listed in Appendix 3. Relative expression levels were then checked by running the reaction on an agarose gel.

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Appendices

1.) WPM Media components

Liquid Woody Plant Media (WPM) mix per liter

20 mL WPM solution A

20 mL WPM solution B

5 mL WPM solution C

5 mL WPM solution D

5 mL WPM solution E

10 mL FeEDTA

2 mL WPM vitamins

1 mL Glycine

0.65 g Calcium gluconate

20 g Sucrose

deionized H₂O to one liter

Solid WPM mix per liter (0.1/0.1/0.1 Media)

Liquid WPM components

0.75 g Agar

0.275 g Phytogel

0.5 mL 6-Benzylaminopurine (BA)

0.5 mL Alpha-naphthalene acetic acid (NAA)

0.5 mL Thidiazuron (TDZ)

Solid WPM media mix with timentin

Solid WPM components

0.5 mL Timentin

CaMV 35S Shooting WPM media mix

Solid WPM components

0.5 mL Hygromycin

50 μ L BA

SCSV Shooting WPM media mix

Solid WPM components

0.5 mL 10mg/mL Kanamycin

50 μ L BA

CaMV 35S Rooting WPM Media

Solid WPM components

0.5 mL Hygromycin

50 μ L NAA

SCSV Rooting WPM Media

Solid WPM components

0.5 mL Hygromycin

50 μ L NAA

2.) Nitrogen Fertilization

No Nitrogen (0N) treatment, in 6 L diH₂O

60 mL 0.25M K₂SO₄

6 mL 2M MgSO₄

0.01M CaSO₄ (1.632 g)

0.005 M $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (0.078g)

6 mL Minors

60 mL FeEDTA

0.15 Nitrogen (0.15N) treatment, in 6 L diH₂O

60 mL 0.25M K_2SO_4

6 mL 2M MgSO_4

0.01M CaSO_4 (1.632 g)

0.005 M $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (0.078g)

6 mL Minors

60 mL FeEDTA

0.15 mL 1M NH_4NO_3

0.15 mL 2M $\text{Ca}(\text{NO}_3)_2$

1.5 Nitrogen (1.5N) treatment, in 6 L diH₂O

60 mL 0.25M K_2SO_4

6 mL 2M MgSO_4

0.01M CaSO_4 (1.632 g)

0.005 M $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (0.078g)

6 mL Minors

60 mL FeEDTA

1.5 mL 1M NH_4NO_3

1.5 mL 2M $\text{Ca}(\text{NO}_3)_2$

Minors

H_3BO_3 : 2.86g/L

$\text{MnCl}_2 \times 4\text{H}_2\text{O}$: 1.81g/L

$\text{ZnSO}_4 \times 7\text{H}_2\text{O}$: 0.22g/L

CuSO_4 : 0.051g/L

$\text{H}_2\text{MoO}_4 \times \text{H}_2\text{O}$: 0.02g/L

3.) PCR reaction procedure

- 1.) Heat to 95° C for 3 minutes
- 2.) Heat to 95° C for 15 seconds
- 3.) Heat to 53° C for 15 seconds for PS3, Heat to 53° C for 15 seconds for PS1, PS2, and EF1
- 4.) Heat to 72° C for 30 seconds
- 5.) Repeat steps 2 and 3 38 times
- 6.) Heat to 72° C for 5 minutes