DIFFERENTIAL GENE EXPRESSION OF PNCES A4 IN *POPULUS NIGRA*

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ABSTRACT

DIFFERENTIAL GENE EXPRESSION OF PNCES A4 IN *POPULUS NIGRA*

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Populus nigra L. owes to be the most critical pioneer tree species in riparian ecosystems and is considered an ecologically and economically-wise imperative fast-growing species. Moreover, it is a great tree species for biomass and bioenergy utilization and cellulose content manipulation. The limited energy resources from fossil fuels demand an alternative efficient biomass crop, where *Populus nigra* shows to be a great candidate. In this study, the CesA (cellulose synthase) secondary cell wall-related CesA4 subunit gene expression (PnCESA4) and its relation to cellulose were investigated in *Populus nigra* L. The cellulose synthase complex (CSC) involving the CESA subunits, PnCesA4/ 7/ and 8, for secondary cell wall regulation and involvement, was not yet fully discovered. Thus, from the CesA secondary cell wall-related subunit genes the expression of the PnCesA4 subunit gene, which is the only single-copy cellulose synthase gene, was investigated in *Populus nigra* trees. In addition to PnCesA4 gene expression, its relation to UGPase and SuSy activity, and growth data which were provided by Taşkıran (2020) were explored to understand the relationship among cellulose-related traits. The results of the study surprisingly are in contrary to what was expected that is, there were negative correlations between PnCesA4 gene expression levels and growth traits (Height and Diameter). Moreover, interestingly PnCesA4 gene expression was not correlated with cellulose content or cellulose synthesizing enzymes. These outcomes taken altogether, indicate in owing

to a more complicated regulation in the pathway that is taking place, and a more unique functional role for the PnCesA4 subunit that is yet to be known.

Keywords: Cellulose synthase, PnCesA4, *Populus nigra*, Cellulose, and Gene expression

ÖZ

POPULUS NIGRA'DA PNCES A4' ÜN DİFERANSİYEL GEN İFADE EDİLMESİ

Akkad, Raitha
Yüksek Lisans, Moleküler Biyoloji ve Genetik
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Populus nigra L., nehir kıyısı ekosistemlerinde en kritik öncü ağaç türü olmaya borçludur ve ekolojik ve ekonomik olarak zorunlu hızlı büyüyen türler olarak kabul edilir. Ayrıca, biyokütle ve biyoenerji kullanımı ve "selüloz içeriği" özelliğinin manipülasyonu için harika bir ağaç türüdür. Fosil yakıtlardan elde edilen sınırlı enerji kaynakları, *Populus nigra*'nın büyük bir aday olduğunu gösterdiği alternatif verimli biyokütle mahsulleri gerektirir. Bu çalışmada, *Populus nigra* L'de selüloz ile ilgili CESA (selüloz sentaz) ikincil hücre duvarı ile ilgili "PnCesA4" alt birim gen ifadesi araştırıldı. CESA alt birimlerini içeren selüloz sentaz kompleksi (CSC), "CesA4/ 7 ve 8" ikincil hücre duvarı regülasyonu ve tutulumu için henüz tam olarak keşfedilmemiştir. Böylece, CESA ikincil hücre duvarı ile ilgili alt birim genlerinden *Populus nigra*'da tek kopya selülaz sentaz geni olan PnCesA4 alt birim geninin ifade edimesi araştırıldı. PnCesA4 gen ifadesine ek olarak, Taşkıran (2020) tarafından sağlanan veriler kullanılarak bu genin UGPase ve SuSy aktivitesi, büyüme ve selüloz karakterleriyle olan ilişkileride etraflıca araştırıldı. Çalışmanın sonuçları şaşırtıcı bir şekilde beklenenin aksine PnCesA4 gen ifade seviyeleri ile büyüme özellikleri (boy ve Çap) arasında negatif korelasyonlar bulundu. Ayrıca ilginç bir şekilde PnCesA4 gen ifadesi, selüloz içeriği veya selüloz sentezleyen enzimler ile ilişkili olmadığı görüldü. Buluan bu sonuçlar birlikte değerlendirildiğinde, bu genin bulunduğu biyokimyasal yolakda daha karmaşık bir düzenleme ve CesA4 alt birimi için henüz bilinmeyen daha benzersiz bir işlevi olduğu düşünülmektedir.

Anahtar Kelimeler: Selüloz syntaze, PnCesA4, *Populus nigra*, Selüloz, ve Gen ifadesi

To my family

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LIST OF ABBREVIATIONS

ANOVA: Analysis of Variance

BcsA: Bacterial cellulose synthase subunit A

Bp: Base pairs

cDNA: complementary DNA

CESA: Cellulose synthase

CesA: Cellulose Synthase

CSC: Cellulose synthase complex

DNase I: Deoxyribonuclease I

EST: Expressed sequence Tag

ETOH: Ethanol

EUFORGEN: European Forest Genetic Resources Program

Fwd: Forward

GTs: Glycotransferases

GUS: β-glucuronidase

Ha: hectares

INV: Invertase

Kor: Korrigan

Oligo: Oligonucleotides

PnCesA4: Cellulose Synthase gene in *Populus nigra*

PCD: programmed cell death

PCW: primary cell wall

PPI: Pyrophosphate

PRI: Poplar research institute

qPCR: quantitative polymerase chain reaction

Rev: Reverse

RNAi: RNA interference

rpm: revolutions per minute

RT-qPCR: Quantitative reverse transcription PCR

SCW: Secondary cell wall

SPSS: Statistical Product and Service solutions

SuSy: Sucrose synthase

UDP-Glu: Uridine diphosphate glucose

UDP: Uridine diphosphate

UGPase: Uridine diphosphate glucose pyrophosphorylase

WGD: Whole-genome duplication

CHAPTER 1

INTRODUCTION

1.1 *Populus Nigra* L.

Populus nigra L. also known as European black poplar is a specie belonging to the *Populus* genus. This tree species is native to areas in Europe, northwest Africa, central and southwest Asia, (Vanden Broeck, 2003), including Turkey. European black poplar trees are large deciduous trees, with many economical uses; one is, being utilized as a parent pool for breeding programs in many areas across the world. Moreover, they are recognized for their rapid growth and vegetative regeneration. Therefore, they are great alternative crops for bioenergy use and paper and pulp production. Aside from the fact that *Populus nigra* L. is a vital indicator of an environment's diversity, *Populus nigra* L also possesses adaptive plasticity. Which indicates an organism's ability to respond to environmental changes and improve its chances of survival. Hence, *Populus nigra* L showed that they can be used in polluted industrial zones for afforestation, soil protection, and phytoremediation purposes.

Populus nigra L. species also retains ecological importance. It is considered a vital specie in an ecosystem, due to the dependence of a large number of insect and animal species survival on them. *Populus nigra* L. supports ecosystem services, like soil stabilization and watershed protection. Moreover, they possess great tolerance for increased water levels and therefore are good pioneer tree species in riparian woodlands. Nevertheless, they are at the risk of becoming the most endangered tree species in the European lands due to constant degradation of the habitat, demographic pressure, and genetic diversity scarcity (Vanden Broeck, 2003; EUFORGEN, 2015).

1.1.1 Biology, Systematics, and Distribution

As *Populus nigra* L. is native European tree species, it forms floodplain forests along river sides in riparian ecosystems. It is known to be pioneer species, due to its critical role in initiating and developing riparian forests. European black poplars are monumental trees in managing water floods and water quality. However, they act as a natural corridor, where they accelerate the gene flow for numerous other riparian species. European black poplar maintains the survival and specificity of the ecosystem of the river flow. European black poplars were previously widespread, but are now considered threatened. Hence, European black poplar requires special attention and conservation strategies (Siler et al., 2014). *Populus nigra* L. belongs to the *Salicaceae* family (the willow family). It is a species of cottonwood Poplar (Vanden Broeck, 2003). European black poplar are strictly heliophiles, and form metapopulations in open areas, by colonizing through seeds and propagules. They are known for having diverse population types; such as isolated trees, mixed, or huge pure stands (Lefèvre et al., 1998).

Poplars cover a widespread natural distribution area, stretching areas of Europe to Siberia (Vanden Broeck, 2003; EUFORGEN, 2015). More specifically, from North of the British Isles to the Mediterranean coast, Africa, the Middle East, Kazakstan, and China. They are cultivated in India at nearly 29°N latitude and naturalized in southern and northern America (de Rigo et al., 2016). Their distribution area also encompasses the Caucasus region and many different countries in the Middle East (Vanden Broeck, 2003; EUFORGEN, 2015).

Populus nigra L. being a deciduous tree has a fast-growing rate. They can reach a height of up to 40 meters and a diameter of 2-2.5 meters. They possess a dark brown or black bark, with numerous fissures. Their leaves display a cuneate base and serrated margins (Figure 1.1 D). Moreover, flowers develop from specialized buds containing preformed inflorescences and appear before foliage development. Their fruits are made up of capsules, grouped in catkins (Figure 1.1 B, C).

Populus nigra L propagation occurs in generative ways (via wind or water-dispersed seeds), and correspondingly in a vegetative way (as in cuttings) (de Rigo et al., 2016). Their seeds have a short viability characteristic. When dispersed, they require adequate water and soil conditions to germinate (Lefèvre et al.,1998). Mature *Populus nigra* L. trees live up to 100 years, and occasionally up to 300 to 400 years (de Rigo et al., 2016). European black poplar trees are dioecious species, meaning they have an individual male and female trees (Vanden Broeck, 2003). Flowering plants that are dioecious account for a little amount, compared to the rest of the flowering plants (Renner, 2014). European black poplar trees can reach maturity and reproductive cycle after 10-15 years if favorable conditions were met (Vanden Broeck, 2003). *P. nigra* belongs to the Aigeiros section from the *Populus* genus. They have a diploid 38 chromosome number (2n) (Gaudet et al., 2007), and a sex-determining system of XY (Geraldes et al., 2015). *P. nigra* L. flowering occurs one to three weeks before leaves initiate, at the timing of early spring between March and April (Vanden Broeck, 2003). This timing coincides with the duration of the flood peak period in Europe along rivers (Vanden Broeck, 2003). *P. nigra* L. female catkins are yellowish-green in color, and male catkins are reddish in color (Figure 1.1 B, C) (de Rigo et al., 2016). *P. nigra* L. mainly depends on wind for pollination (Vanden Broeck, 2003).

When mature capsules break, which is approximately four to six weeks following fertilization, they detach their seeds with great amounts of pappus, a white long and silky collection of hairs enclosed to the seeds (Figure 1.1 E). The seed pappus aids in wind dispersal over long distances, increasing migration, genetic diversity, and gene flow in the forests. *Populus* are known as abundant makers of seeds, where old trees produce seeds that can reach up to 50 million or more in one season. (Siler et al., 2014).

In naturally occurring poplar stands in flood plains, a robust age structure can be deducted, reflecting the flood history, where tree age correlates with the hydrological record and flood magnitude. Thus, they can be used as good species indicators for successful regeneration. Moreover, poplars can undergo vegetative propagation by

root-borne shoots, and can moreover regenerate deriving out of pieces, after breakage (Siler et al., 2014).

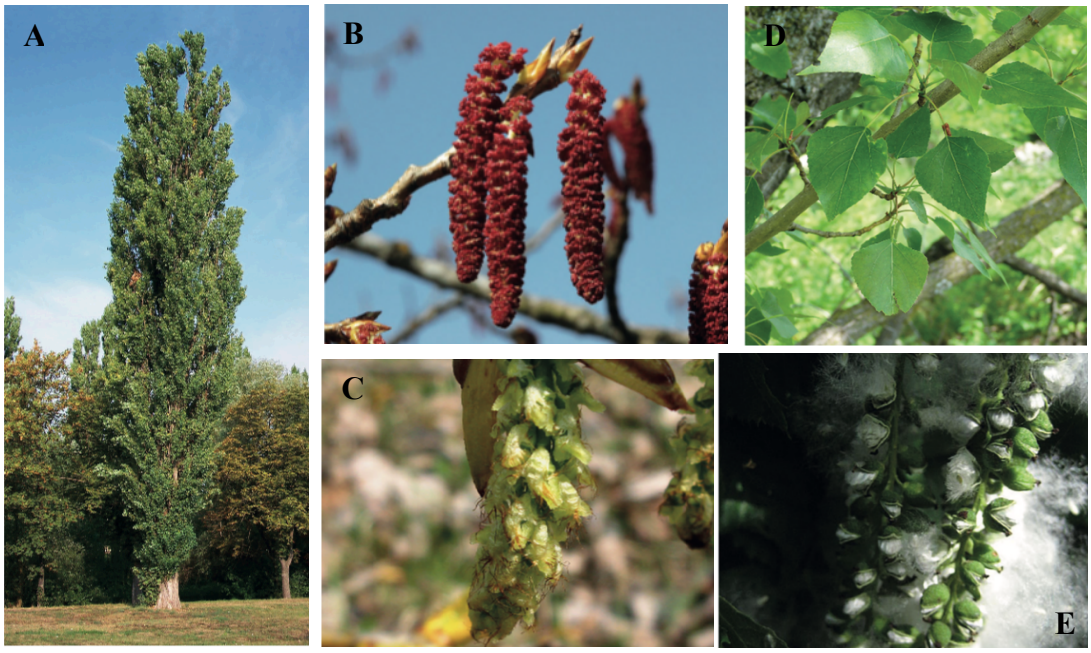


Figure 1. 1. A. European Black Poplar tree (*P. nigra* L.), B. Male catkins (reddish color) during pollination, C. Female catkins (greenish flowers) before pollination, D. Poplar leaves, with cuneate base and acuminate apex, E. Fluffy seeds coming out of capsules (spread mostly via wind). de Rigo et al., (2016) Retrieved on April 8, 2022, from (https://forest.jrc.ec.europa.eu/media/atlas/Populus_nigra.pdf)

As European black poplars grow in mixed riparian forests, they grow with White Poplar (*Populus alba.* L.), willow (*Salix* spp.), alder (*Alnus* spp.), maple (*Acer* spp.), Elms (*Ulmus* spp.), and occasionally Oak trees (*Quercus* spp.). Poplar species cannot withstand shade and drought. They are known as opportunistic species, where they are capable of spreading easily and colonize into new sites after undergoing many disturbances (de Rigo et al., 2016).

1.1.2 Economic and Ecological Value

Almost every country in Europe has a national conservation program for the *P. nigra* species. They were chosen as pilot species in the EUFORGEN program (Lefèvre et

al., 1998). *Populus nigra* L. has demonstrated its importance as a forest tree species. Due to its vital economic value, when it comes to breeding programs, they are used as parent pools in many areas across Europe and the world. Particularly, the *Populus x euramericana* (*P. deltoides* x *P. nigra*.) hybrid. Additionally, European black poplar has a role in mitigating pollution effects, just like other *Salicaceae* species. These species have multifunctional roles. Areas where European black poplars are found overlay with areal distribution regions in Europe with known high erosion rates, moist slopes, and increased drainage areas within a mountain system. The ecological value of *P. nigra* L. is recognized in riparian floodplain ecosystems, where they act as a windbreaker, and as a controlling factor of erosion alongside riverbanks. In some areas in the Mediterranean region which are under soil erosion risk, *P. nigra* L. can be used as a silvo-arable agroforestry species, considering its cover-management effectiveness on erosion rates (de Rigo et al., 2016). The narrow variety of *Lombardy* poplar and European black poplar trees are usually used as ornamental trees. Moreover, *P. nigra* wood has many desirable unique characteristics, such as having moderate fire resistance, shock-proof, and soft and fine wood texture. Earlier on, *P. nigra* wood was utilized for carts, clogs, furniture, and flooring near fireplaces. Now, its wood is being utilized for paper and pulp manufacture. With their fast-growing characteristic, they are well-matched as an appropriate bioenergy crop. *P. nigra* extracts include antioxidant and anti-inflammatory effects. *P. nigra* belongs to a group of plant species that possess remarkable intense isoprene emission. Isoprene is an organic biogenic volatile compound. An increase in isoprene concomitantly increases atmospheric concentrations of greenhouse gases, thus acting as positive feedback to global warming. Its effect and role in climate change and the world's biosphere are yet not fully clear (de Rigo et al., 2016).

1.1.3 Importance of Poplar in Turkey

As recorded by the General Directorate of Forestry of Turkey, also called as OGM institution, forest areas in Turkey cover 29% of the country (Velioğlu et al., 2020). *P. nigra* has been labeled among one of the most threatened tree species in Turkey and Europe, due to the long-term effects of human impact (Çiftçi and Kaya, 2019). Moreover, bestowing to 2020 data, 19 million cubic meters of wood is manufactured from 22.6 million ha as reported by the OGM in Turkey. The natural stands in Turkey from fast-growing species make up around 25% of the 22.6 million hectares of forest in Turkey. The leading species of fast-growing species naturally occurring in Turkey are *Pinus brutia* and *Populus tremula* (Velioğlu et al., 2020). Moreover, some of the naturally occurring poplar stands belong to the *Populus euphratica* and are found in south and south eastern Anatolia. Different species, such as "*P.nigra*, *P. alba,* and *P. x canescens"* are found in different areas in Turkey. They occur in minor groups or as individual trees (Velioğlu and Akgül, 2016). Most cultivated poplars cultivated in Turkey belong to the *Aigeiros* section of the *Populus* genus (Velioğlu et al., 2020). The timber production of fast-growing species in Turkey makes around 9.3 million cubic meters annually. Yet, it was reported by the forest industry that there has been a major shortage in raw-wood-material to ample the required demand in Turkey. This led to wood import initiation. Consequently, fast-growing species of Poplar were given priority in industrial plantations to meet the required demand and fill the gap of raw wood material shortage (Velioğlu et al., 2020).

According to the nursery and propagation practices, the planting material is of great importance for a plantation to be successful. Poplar plantations are done by the usage of stem cuttings or one-to-two-year-old rooted and unrooted plants. Usage of unrooted samplings is cheaper and more practical. Research has shown that unrooted and rooted plant samplings had corresponding results concerning the rate of survival, and diameter and height levels of increment. Thus, an economical advantage can be achieved in the usage of poplar plantations. Main climatological zones were determined during the last years. Turkey was sectioned into two main zones

regarding its climatic descriptions. The first zone was; moderate or coastal zones and the second was; continental zones. In the first zone, *P. euramericana* and *P. deltoides* clones were planted, while *P. nigra* clones were grown in the second zone (Velioğlu and Akgül, 2016).

There are five naturally occurring poplar species in Turkey. 1) *P. nigra* L. (European black poplar), 2) *P. alba* L. (White poplar), 3) Grey poplar (Hybrid *tremula x Alba* called *Populus x canescens* Smith), 4) *P. tremula* L. (Aspen), 5) *Populus euphratica* Oliv. (Euphrates Poplar). Additionally, *Populus Tremula* L. accounts to be the pioneer species in the poplar natural distribution in Turkey. Moreover, *Populus nigra* L. (European black poplar) is the main tree species in the central and east areas of Turkey. Thus, breeding *P.nigra* is concentrated in these regions in Turkey. Anatolia's traditionally practiced poplar cultivations are done with the European black poplar plantations. Centuries ago until now, farmers have made European black poplar plantations and have established them along rivers, stream sides, fields, and roadsides. In the reported time average of 2016-2019 years, fast-growing species of industrial plantations have contributed to wood production of 3.6 million m3 in a year. From the industrial wood production, on average, poplar plantations were responsible for the production of 3 385 154 m3, which makes 94% of the total wood production in a year (Velioğlu, et al., 2020)

As of 1965, indigenous European black poplar "Gazi (TR-56/52) and Anadolu (TR-56/75)" have been grown intensely in Central, Eastern, and South-eastern Turkey, which complement the areas where continental climatic conditions are met. A recent research study conducted by PFGFTRI (Poplar and Fast Growing Forest Trees Research Institute), indicated that 3 new clones of Indigenous European black poplar were added. The added clone names were; "Geyve (TR-67/1), Behicbey (TR-62/154), and Kocabey (TR-77/10)". These clones can be also utilized in commercial plantations successfully, as done by the earlier clones (Velioğlu and Akgül, 2016).

According to the harvesting and utilization report of Turkey's poplars and willows, two-thirds of total poplar wood production is fiber chip wood. The installed capacity

of Turkey's fiberboard and particleboard is recorded to have increased at a major level, making it a leading country in the world, concerning its capacity. Moreover, in line with this growth, the necessity for fine-scale poplar wood has also increased. "Anadolu (TR-56/75) and Gazi (TR-56/52)" clones are cultivated in central, eastern, and south eastern Anatolia and were registered by The International Poplar Commission of Food and Agriculture Organization of the United Nations (FAO). These registered clones were cultivated in areas where the forest cover was poor. Poplar leaves were traditionally used in feeding livestock (as supplementary fodder) from branches. Poplars were used also for recreational purposes, and in improving the environment. In the eastern Mediterranean basin of Turkey, different *P. nigra* cultivars were grown for ornamental purposes, due to their extraordinary beautiful well-designed cylindrical leaves and their long-lasting intense color, and for serving as a windbreak as well. Moreover, poplar was accepted as a model for perennial wood species in studying forest tree-related genetics, and in understanding the molecular processes of their development, growth, adaptations, biotic interactions, and responses to environmental stresses (Velioğlu and Akgül, 2016).

1.2 Wood

It is apparent that the increase in the world's population, feeding capacity, humans health, intense climate change, the loss of biodiversity, and limited energy resource from fossil fuels, mandate an important alternative, like; biomass crops, and agriculture improvements for the well-being of humans and the coming future. Wood accounts as one of the most important natural resources, representing a valuable renewable energy reserve in the world. It has a great effect on the terrestrial ecosystem affecting the world's carbon cycle (Weih and Polle, 2016). Moreover, the developing wood cells account for one of the most important sinks of the overloaded CO_2 in the world. This reduces the risk of increasing global warming (Plomion et al, 2001). Wood is mostly used as a resource for pulp, paper, and timber industries and the production of energy. The cells of wood, such as fibers, are critical cells where

their morphology and chemical composition affect the wood quality characteristic (Takahashi and Shmidt, 2008).

Our understanding of the whole wood formation process is not yet complete, with little known about its complicated cellular, molecular, and development processes that underly the development of wood (Plomion et al, 2001). Moreover, wood formation, also called "Xylogenesis", is a critical process for biological and economical aspects. Wood quality is assessed from many criteria like cellulose and lignin content, fiber length, and solidity. To improve these criteria, a more in-depth understanding of the wood formation mechanism in trees, biosynthesis, and cell wall modifications, (during wood formation that determines the fiber cells' morphology and chemistry), should be inferred more deeply (Takahashi and Shmidt, 2008).

1.2.1 Development

Increased plant body thickness is referred to as secondary growth. This growth occurs from the lateral meristems present in the plant; the vascular cambium and the cork cambium (Takahashi and Shmidt, 2008). Cambium is made of initials which make xylem and phloem mother cells, by division. During Spring, the cambium first starts producing phloem cells, and then xylem cells (Déjardin et al., 2010). The secondary xylem is generally known as "wood", and begins from inside of the vascular cambium. It is developed in the direction of the middle of the stem. Meanwhile, toward the outer cross of the vascular cambium, secondary phloem is developed. The development of wood, or the xylogenesis process, includes the formation of xylem cells by major distinctive developmental stages, which are: 1) Cell division (of xylem mother cells), 2) Cell expansion and elongation (radially), 3) Secondary cell wall thickening and secondary wall synthesis (followed by lignification), 4) Programmed cell death (Takahashi and Schmidt, 2008), and 5) Heartwood formation (Déjardin et al., 2010). Most trees pass through the process of heartwood formation, in which wood turns to inactive form as in conduction, water, and nutrients repository, where it resembles the tree's age.

There are two types of wood: softwood and hardwood. Poplars are hardwoods since they are angiosperms. The wood consists of elongated cells, fibers, vessel elements, and axial parenchyma cells. During cell division, mother cells divide periclinal with intrusive tip growth in the cambial meristem (Takahashi and Schmidt, 2008). The primary cell wall (PCW) is formed. The PCW is made up of cellulose microfibrils rooted in a matrix of hemicelluloses and pectins (Sjostrom, 1993). Then subsequently, the expansion phase starts, which involves growth (Takahashi and Schmidt, 2008). Angiosperms' vessels and gymnosperms' tracheids (cells that conduct water) undergo radial expansion, while angiosperms' fiber cells undergo intrusive elongation (Abedini, 2014). Once completed, the cell stretches to its ultimate size at the end of the cell expansion phase, and the secondary cell wall is deposited inside the primary cell wall called the: Secondary cell wall formation. A three-layered structure is formed; S1, S2, and S3, which are the outer, middle, and inner layers respectively (Figure 1.2 B). As the secondary cell wall is developed, rigidity increases (Takahashi and Schmidt, 2008). Each layer of the three-layered structure has a different microfibril angle arrangement. Lignin deposition arises in all layers at the end of xylem differentiation. When lignification is completed, the cell wall development formation process reaches the programmed cell death (PCD) stage (Plomion et al., 2001). PCD is the final stage of the development of secondary xylem cell differentiation. During this process, the content of the living cells is autodigested by released nucleases and proteases in the cytoplasm, associated with morphological and nuclear characteristic changes in fiber and vessel elements. (Takahashi and Schmidt, 2008).

Secondary walls are most critical for strength in fiber cells, and water conduction in vessel elements (Takahashi and Schmidt, 2008). The secondary cell wall formation is directed by the gene expression of many different genes. Those genes are involved in the assembly and biosynthesis of four major compounds: cellulose, hemicellulose, lignin, and proteins in the cell wall (Plomion et al., 2001)

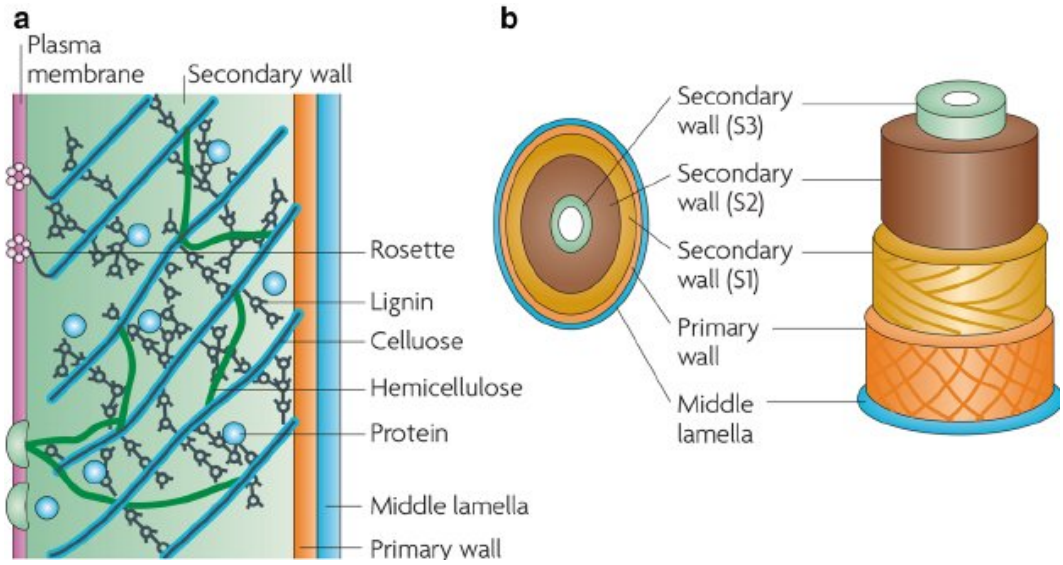


Figure 1. 2 Illustration of wood cell component. A) Cellulose fibers, lignin, hemicellulose, along with cell wall rosette structures, and proteins are illustrated. B) The structure of the wood cell wall, its Middle lamella, primary cell wall, secondary cell wall, and S1, S2, and S3 layers. (Adapted from Mathews et al., 2015)

1.2.2 Component

Mother cells of xylem divide more excessively than phloem mother cells do. This elucidates the disproportion between xylem and phloem tissues (xylem more than phloem). Cellulose makes up 40-50% of wood (Plomion et al, 2001), and is the main structural component of primary and secondary cell walls. It is immensely produced during wood formation in SCW (Takahashi and Schmidt, 2008). Hemicellulose is a noncellulosic soluble polysaccharide and accounts for almost 25% of wood. The third major component of the wood is lignin, making 15-35% of the wood. Lignin is a phenolic polymer, resulting from three hydroxycinnamyl alcohols, where lignin content and monomeric composition differ greatly among distinct plant taxa, individuals, cell types, tissues, and cell wall layers (Plomion et al., 2001). Pectin comprises less than 10% of wood (Takahashi and Schmidt, 2008). Cell's pectin and cell wall proteins make up the minor compounds that are found in the cell wall. These

proteins have a critical role in determining xylem cell wall morphology and composition (Plomion et al., 2001).

Wood cells mainly consist of 3 distinct layers; the middle lamella, primary cell wall, and secondary cell wall (Figure 1.2). The duration of the primary cell wall (PCW) determines the cell wall's morphology, while the secondary cell wall (scw*)* determines the mechanical and chemical properties of the wood. The primary cell wall is deposited along with the outermost layer; the middle lamella, which consists of proteins and pectic compounds. The primary cell wall is composed of indiscriminately arranged cellulose microfibril layers cross-linked with hemicellulose making it a rigid skeleton (Takahashi and Schmidt, 2008).

The main function of the secondary cell wall is strengthening, which is critical for fibers and water-conducting cells. The strength comes from the abundant cellulose which is found more in secondary cell walls than in primary cell walls. Moreover, secondary cell walls lack pectin. In Poplars, the xylem secondary wall has 43-48% cellulose, while the primary wall has only 22% cellulose. The secondary wall consists of a matrix of hemicellulose and lignin. The main difference between primary and secondary walls is in the wall's chemical constituents and the organization of cellulose microfibrils. PCW has randomly distributed cellulose microfibrils, aligning parallelly with the longitudinal axis. While the microfibrils of the secondary cell wall (scw) are immensely ordered, being laid in a helicoidal pattern parallel to one another. In the three-secondary cell wall layers, S1, S2, and S3, the microfibril angle differ from layer to layer. The S1 outermost layer's microfibrils are nearly perpendicular to the cell axis, 50-90° angle. The S2 middle layer, (the thickest layer), has more longitudinally organized microfibrils, with a 5-30° angle, impacting the wood elasticity the most. The S3 innermost layer has a more perpendicular angle to the microfibril (Takahashi and Schmidt, 2008).

1.2.3 Importance

Throughout the history of civilization, wood has always had a vital role. Humans have been using wood as fuel, and for furniture, building materials, paper, tools, weapons, and many more. Concurrently, wood demand is intensely continuing to increase annually, initiating many conflicts between areas for control over limited shared tree resources. The relationship between wood to us has not changed for a long time. And methods for developing and managing woodlands are still somehow dependent on old tried methods made in early civilizations (D'Costa, 2015). Besides plant cell wall having a fundamental role in providing strength against turgor pressure, cell wall has many more important functions. Thick-walled cells in a plant supply shield and defense against insects and pathogens. Moreover, defense responses are stimulated by polysaccharides of the cell wall as latent signal molecules. They are released through the degradation of the cell wall by pathogenesis. Moreover, polysaccharide fragments and proteoglycans (in the cell wall), have a critical role in cell-to-cell communication during the developmental stages (Takahashi and Schmidt, 2008).

Wood cell elaboration is done by well-coordinated biosynthesis and chemical components modification, and by many dynamic actions of certain carbohydrate-active hydrolases. Thus, the apprehension of cell wall biosynthesis and enzyme actions will lead to the advancement of wood characteristics (Takahashi and Schmidt, 2008).

As the world is running out of fossil fuels, efforts have been made in switching to renewable energy resources: such as cellulose. Cellulosic ethanol is a product of cellulose and has been of great interest and research area, as it is a great alternative to fossil fuel (Kumar and Turner, 2014). Even though cellulose's societal and biological importance has been indicated, the exact molecular mechanism underlying its synthesis is just now beginning to emerge (McNamara et al., 2015).

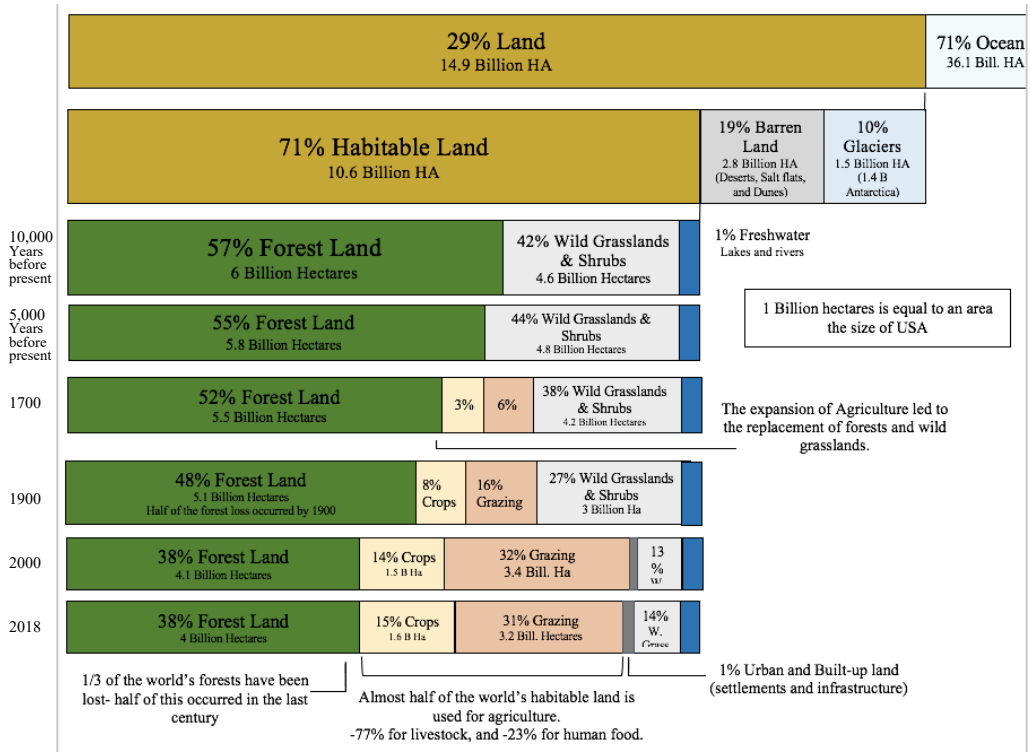


Figure 1. 3. Deforestation of Forest land statistics over time in years. (Adapted from Ritchie and Roser, 2021)

Over the past 10,000 Millenium years, the earth's surface has changed after the last ice age until our present-day today. Out of 14.9 billion hectares of land on the planet, 71% of it is habitable (10.6 billion hectares). Of that habitable land, 57% is covered by forests, which is around 6 billion hectares. Now only 4 billion hectares of forest land remained. This means that one-third of the world's forests have been lost due to accelerated deforestation in the past century. This was caused by the constant increase in land use for agriculture and many other factors. The global population will continue to increase reaching 10.8 billion by 2100, and land per person needed will consequently increase (Ritchie, 2021).

1.3 Cellulose

Cellulose makes up the main structural component of primary and secondary cell walls. It is greatly produced during wood formation in the secondary cell wall.

Cellulose biosynthesis was a major research focus area for nearly 40 years, yet molecular methods for studying woody perennial plant species were only recently developed. Cellulose accounts for being the most abundant biopolymer in the world, making up more than 50% of the biosphere's carbon (Takahashi and Schmidt, 2008). Cellulose is the main component of cell walls (Nobles et al., 2001; McNamara et al., 2015). Making up almost 50% of wood's dry matter, cellulose mainly comes from secondary cell walls (Delmer and Haigler, 2002). Cellulose has a critical function in cell wall organization; determining the cell's shape by regulating growth and elongation (McNamara et al., 2015). Wood is mainly made up of cellulose (35-50%), lignin (15-35%), pectin (<10%), hemicellulose (15-35%), and different integrated proteins (Takahashi and Schmidt, 2008).

Improving the genetics of cellulose biosynthesis in commercially important trees (such as Poplars) has become one of the most challenging objectives in forest biotechnology research. Thus, a more in-depth understanding of the complex cellulose biosynthesis process in trees should be understood. The availability of poplar's rich genomic resource makes them good species for genetic augmentation of cellulose. Studies on the poplar cellulose biosynthesis process have a critical economic impact on the world forest product industry (Joshi et al., 2004)

Cellulose is always released outside of the cell into the extracellular matrix or cell wall. Cell walls contain cellulose chains organized into cellulose microfibrils and macro fibrils (cable-like para crystalline structures) embedded in a rich polysaccharides and glycoproteins matrix. Cellulose polymeric structure was determined in the research of Hermann Staudinger, where he showed that cellulose is composed of glucose units, covalently linked, giving cellulose its remarkable stability. Cellulose is an amphipathic macromolecule, self-associating, becoming insoluble in water. Cellulose is assembled into supramolecular structures by Van der Waals forces causing aggregation (McNamara et al., 2015). The chemical structure of cellulose consists of covalently linked ß-1,4-D-glucan residues making a linear polymer (Takahashi and Schmidt, 2008; Delmer and Amor, 1995). Cellulose is an unbranched glucan (Vincken et al., 1997). The degree of polymerization differs

between primary and secondary cell walls, where it's lower in the primary cell wall (PCW*)*, and higher in the secondary cell wall (scw*)*. Thus, they have different cellulose features (Takahashi and Schmidt, 2008).

Individual glucose units are linked by acetal linkages amid the C1–C4 carbons of the glucose ring (Rojas et al., 2015). Cellulose synthases (CeSs) are generally recognized as inverting GTs (glycosyltransferases) due to their capability in inverting the configuration of C1 anomeric carbon of the UDP-glucose donor sugar from alpha - α (when attached to UDP) to beta (when attached to cellulose polymer) (McNamara et al., 2015). Thus, the anomeric C1 carbon has the ß- configuration (unlike starch and glycan units that possess α bond), where every secondly neighboring glucose unit is rotated 180° compared to the unit before, forming a cellobiose disaccharide repeating unit (Rojas et al., 2015; Delmer and Amor, 1995).

Cellulose fibers are made up of microfibrils, which are made up of elementary fibrils (nanofibers), which are made up of around 30 -100 aggregated cellulose chains. During cellulose biosynthesis, the elementary fibrils (nanofibrils) are formed (Rojas et al., 2015). Cellulose chains are held together in a crystalline structure by hydrogen bonds and Van der Waals forces making microfibrils (Nishiyama et al., 2003; Somerville, 2006). Cellulose consisted of glucan chains arranged in parallel forms (Cousins and Brown, 1995). The exact mechanism controlling the length of the cellulose chain yet remains unknown. Although, it is known that cellulose polymers can reach up to great lengths where studies have shown they can reach up to 15,000 glucose units (McNamara et al., 2015).

1.3.1 Cellulose Biosynthesis

Regulation of plants' cellulose biosynthesis during growth development and in response to environmental distresses remains to be a central question in plant biology (Polko and Kieber, 2019). Cellulose synthesis occurs by localized plasma-membrane cellulose synthase complexes (CSCs), which are made up of cellulose synthase (CESA) catalytic subunits. These cellulose synthase catalytic subunits are known as

CESAs. (Li et al., 2022). CSC refers to membrane-integrated cellulose synthase A (CesA) protein complex (Somerville, 2006). CSC has a characteristic rosette morphology (Li et al., 2022). Thus, the remarkable CSC is a multi-subunit complex containing cellulose synthase (CESA) proteins and is called a rosette due to its six-lobed structure (Haigler and Roberts, 2018).

Each rosette is comprised of six-rosette subunits, in which each subunit is assumed to contain six CESA proteins. And each CESA is assumed to make a ß -1-4, glucan molecule from UDP-D-glucose, this makes a rosette structure mainly making 36 glucan chains into the apoplastic side of the plasma membrane (Takahashi and Schmidt, 2008). Each elementary fibril is synthesized by one CSC; hence it was claimed that the number of chains and number of CESA proteins in the CSC complex are linked (Kumar and Turner, 2014). Yet, these studies of the 36-chain model proposed by Herth (1985) of CSC have been recently considered uncertain, where different studies showed that the number 36-chain model is just too large and that the precise number of CESA proteins in a CSC is ambiguous. Many recent studies have made certain assumptions of an 18-24 chain model yet indicated that the specific number of chains and number of CESA proteins in a CSC complex is still uncertain (Kumar and Turner, 2014). Other proteins such as Korrigan (Kor) (membrane-bound) and Sucrose synthase (SuSy*)* can be involved in the CSC formation complex (Abbas et al., 2019).

Multiple CESA isoforms are needed to create a CSC complex (Meents et al., 2018). The functional CSC complex in any primary or secondary cell wall has three different CESA interacting isoforms (Abbas et al., 2019). These different CESA isoforms (drawn from different classes) and different CesA genes, involved in the CSC complexes, are responsible for the secondary and primary cell wall synthesis (Polko and Kieber, 2019). Different CESAs have different functions, location, and fit in the CSC complex (Meents et al., 2018). CESA proteins in the CSC complex synthesize linear ß -1-4, glucan from cytosolic UDP- activated glucose, which is the donor sugar. The enzyme furthermore translocates polysaccharides across the cell membrane by a channel made from its membrane-embedded domain. Plants organize

CESAs into CSC complexes (McNamara et al., 2015). UDP-Glucose may be produced from 3 different enzymes; SuSy (Sucrose synthase), INV (Invertase) which is cytosolic, and UGPase (UDP-glucose pyrophosphorylase) (Delmer and Haigler, 2002), as shown in Figure 1.4. Moreover, UDP-glucose can be used as a precursor in callose, starch, and pectin synthesis (Fujii et al., 2010).

1.3.2 Cellulose Synthase

1.3.2.1 CesA Genes, their Gene family and all genes involved, and CSC Complex:

As mentioned earlier, the specific molecular mechanism of cellulose synthesis is not yet well understood despite efforts in the literature done in the past years in trying to identify the genes responsible for coding the catalytic subunits of the CSC, and the possible proteins that might be conceivably involved in cellulose synthesis. A major difficulty commonly faced in understanding cellulose synthase was the intense instability of the CSC complex, and its localization in the plasma membrane (Bessueille and Bulone, 2008). Luckily, due to the structural conservation of the cellulose biosynthesis genes between *Arabidopsis* and Poplar genomes, understanding *Arabidopsis* gene function can be applied to poplar genomes. Yet, the regulation of these genes might remain distinct, due to differences in the model systems (Joshi et al., 2004). CesA genes encode a glycosyltransferase (the CSC complex), which belongs to the GT2 family and has a crucial role in cellulose biosynthesis (Su et al., 2021). Analysis of genomes and EST sequencing on plants studies such as *Arabidopsis*, barley, rice, and poplars, have revealed the identification of 18 different CesA genes in each of the species. Thereafter, each gene's function question was raised (Bessueille and Bulone, 2008).

CesA genes of vascular plants were firstly identified in the 1990s by the work of Arioli et al. (1998) and Pear et al. (1996). Moreover, Pear et al. (1996) discovered two ESTs in rice and cotton plants by checking similarity sequences from bacterial BcsA genes. Now, these higher plant genes came to be known and defined as the;

CesA gene family. Later on, Arioli et al. (1998) showed that CESA proteins are involved in cellulose biosynthesis (Kumar and Turner, 2014).

The angiosperm genome has undergone many ancient duplication events, including whole-genome duplication (WGD), which initiated the diversity of phenotypic and developmental features in plant species. A long time ago, almost 65 million years, the *Populus* species has undergone a polyploidy event which was later called: the *Salicoid* WGD. This duplication affected almost 92% of the Populus genome. The catalytic subunit responsible for cellulose biosynthesis, *CSC*, makes up a multigene family in land plants (Takata and Taniguchi, 2014). *Arabidopsis thaliana* genome has 10 CesA genes, three of them, CesA4, CesA7, and CesA8, are responsible for secondary cell wall (scw) cellulose formation and synthesis (Takata and Taniguchi, 2014; Kumar and Turner, 2014; Su et al., 2021; Joshi et al., 2004; Kumar et al., 2009). Another study implied that mainly CesA4 and CesA8 are associated the most with the scw cellulose synthesis (Su et al., 2021). The three CesA scw-associated genes make up a triple subunit. They are conserved genes and are considered one-to-one orthologue genes to monocotyledonous and eudicotyledons (Takata and Taniguchi, 2014). The three secondary cell wall genes are co-expressed, and their proteins all create part of the same complex (Kumar and Turner, 2014). Primary cell wall (pcw) cellulose synthesis is associated with CesA1, CesA3, and CesA6 genes. (Kumar and Turner, 2014; Abbas et al., 2019; Joshi et al., 2004; Kumar et al., 2009). CesA genes, CesA4/7/8, associated with the scw formation in poplars, correspond with *Arabidopsis* scw associated-CesA genes, indicating that the functional feature is conserved within angiosperms (Takata and Taniguchi, 2014). CesA6 is partly redundant with CesA2, CesA5, and CesA9 (Kumar and Turner, 2014; Abbas et al., 2019; Kumar et al., 2009). CesA10 subunit's role remains unclear (Kumar and Turner, 2014; Kumar et al., 2009). PCW CesA expression patterns are way less correlated to one another than secondary cell walls (Kumar and Turner, 2014). The results of a study done by Abbas et al. (2019), are consistent with the same claim, which indicated that PtrCesA4, PtrCesA7-A, PtrCesA7-B, and PtrCesA8-A, PtrCesA8-B in *Populus* genome are homologs of the AtCesA4, AtCesA7 and

AtCesA8 CesAs *in Arabidopsis,* respectively. This also confirms the conservation of CesA genes, associated with cellulose biosynthesis in scw, between *Arabidopsis* and poplars (Abbas et al., 2019).

1.3.2.2 The five Genes that have a function in Secondary Xylem Development:

Cellulose synthase (CesA) genes that are associated with the scw*,* PnCesA4, PnCesA7, and PnCesA8, are duplicated in Poplars due to the past whole-genome duplication (WGD) occurrence. As a result of this duplication, the copy number of CesA-scw-associated genes has increased to five in *Populus.* The 5 PnCesA genes; PnCesA4, PnCesA7-A, PnCesA7-B, PnCesA8-A, and PnCesA8-B, might constitute the CSC which plays a central role in the synthesis of the secondary cell wall in developing xylem tissues. The two PnCesA7 and PnCesA8 duplicate gene pairs; CesA7-A, CesA7-B, CesA8-A, and CesA8-B, first emerged in the *Salicoid* WGD. Despite that the primal CesA4 was likewise duplicated into two genes during WGD, nevertheless, only one member of the gene pair of genes remained and the other was deleted during many chromosomal changes and rearrangements. This made Poplars left with five genes; PnCesA4, PnCesA7-A, PnCesA7-B, PnCesA8-A, and PnCesA8-B, that are officially associated with scw. It was implied that the triplet CesA scw-related members are considered an essential set of genes that are needed for scw biosynthesis. It was suggested that the expression patterns of tissue dependent CesA genes associated with scw are regulated by transcription factors (TFs), which may be master genes controlling the development of fiber and vessel cells in xylem tissues. The increased number of CesA genes in *Populus* plants after the WGD was indicated in phylogenetic analysis (Takata and Taniguchi, 2014).

1.3.2.3 Cellulose Synthase A4 (PnCesA4):

PnCesA4 gene in the *Populus trichocarpa* genome remains as the only gene member of the scw-associated PnCesA genes that were initially duplicated in the *Salicoid* WGD and was later deleted by chromosomal rearrangement. Making it the only single CesA member that is associated with the secondary cell wall development, unlike PnCesA7 and PnCesA8 genes which have PnCesA7-A/B and PnCesA8-A/B duplicates. Studies showed that expression was mostly observed in CesA4 and CesA8 genes, were GUS staining procedures in Takata et al., (2014)'s work, revealed that CesA4 had 50% GUS staining in root cap (indicating expression level) in Pt x tCesA4 line. Nevertheless, results have shown that the CesA8 gene had more expression levels than CesA4 gene. CesA8-A had more staining results than CesA8-B in root cap expression level. This supports the hypothesis of scw-associated PnCesA genes having differential tissue-dependent expression levels in poplars (Takata and Taniguchi, 2014).

Studies have proposed that dimerization of CESA proteins occurs prior to higher assembly of the rosette structure, requiring CesA4/ 7 /or 8 subunits. In regular conditions, these subunits multimerize, nevertheless in the case of deficiency of one of the subunits, the complex tends to form only dimers. It is not yet proven whether the dimers form hetero- or homodimers but results from split ubiquitin screen in yeast and bimolecular fluorescence method analysis have shown that only CesA4 was capable of homodimerizing meanwhile CesA4, 7, and 8 only form heterodimers in many combinations. This demonstrated a unique significant feature of the CesA4 subunit among all the other CesA- scw-associated gene subunits (Kumar and Turner, 2014). Mutation of AtCesA4, 7, and 8 genes resulted in collapsed xylem cells which illustrated their role in scw cellulose synthesis. Additionally, cellulose content declined in CesA RNAi knockdown transgenic plants when compared to wild type. Cellulose reduction was observed in PtrCesA4, PtrCesA7, and PtrCesA8 CesA-associated genes (Abbas et al., 2019).

When the AtCesA-4 gene was studied in *Arabidopsis*, transformed with the ß-glucuronidase (GUS) gene, and undergone in situ assays of GUS activity to determine expression patterns, its gene expression pattern showed that they were exclusively in cells undergoing SCW cellulose deposition. Moreover, AtCesA8 was predicted to have a similar function to that of AtCesA4 (Holland et al., 2000).

1.4 UGPase and SuSY Enzyme

UDP-Glucose pyrophosphorylase (UGPase) enzyme has an imperative role in carbohydrate metabolism (Kleczkowski et al., 2004). UGPase enzyme belongs to the nucleotidyltransferase class of enzymes (Kleczkowski 1994a; Becker et al., 1995). It catalyzes a reversible reaction using Glucose 1-phosphate (Glc 1-P) and Uridine triphosphate (UTP**)** to form UDP- Glc (Uridine diphosphate Glucose) and PPi (Pyrophosphate). UDP-Glucose is mainly generated by the two enzymes UGPase and SuSy action. Where UGPase forms the product of UDP-Glc and pyrophosphate (PPi), and the SuSy enzyme catalyzes a reversible reaction utilizing sucrose and uridine diphosphate (UDP), to form UDP-Glucose and fructose. (Kleczkowski et al., 2004; Jong-In Park et al., 2010).

UGPase is accepted to be involved in the sucrose biosynthesis pathway in young and mature leaves. Moreover, in other tissues (mostly the ones that are dependent on imported carbon) UGPase takes part in sucrose breakdown by using UDP-Glu provided by the SuSy enzyme. This makes it the only enzyme having an immediate precursor source of starch in all plants. Several studies have shown that UGPase is regulated and involved mainly at the gene and protein levels (Kleczkowski et al., 2004). Susy enzyme directly moves the UDPG substrate to the CSC complex that forms the glucan chain (Joshi et al., 2004). Studies have shown that SuSy is an important enzyme with a critical role in the biosynthesis of the cell wall. Its phosphorylation/dephosphorylation actions are of importance for the binding of SuSy to the plasmalemma. The phosphorylated form of the SuSy enzyme is fully

soluble and dephosphorylation enables the binding of SuSy to the membrane (Kleczkowski et al., 2004).

Moreover, other than that UGPase product, UDPG, is a key metabolite in the carbohydrate metabolism and has a role in the sucrose pathway, it is additionally important for having UDPG as a precursor in the biogenesis of the cell, where it is utilized in the biosynthesis of the cell wall polysaccharides. UGPase is localized mainly in the cytosol. Although, some studies showed its existence at the membrane fraction, and this association of UGPase is quite similar to SuSy enzyme properties owing to the action of providing UDPG to the cellulose synthase. (Kleczkowski et al., 2004).

There are two homologous UGP genes found in *Arabidopsis* and poplar genomes. Even though no doubt SuSy and UGPase enzymes are greatly involved in cell wall biosynthesis, the specific role of the enzymes still needs to be considered more intensely. The direction of the reaction that both two enzymes undergo, depends greatly on the difference in the tissue type: source or sink. In sink tissues, for UGPase to be effective enough Glc-1-P should be present. This is indirectly related to the SuSy reaction, which produces fructose as a product. For every one UDP-G, fructose is released. This could also be involved with the interaction of the Invertase (Inv) enzyme (Kleczkowski et al., 2004).

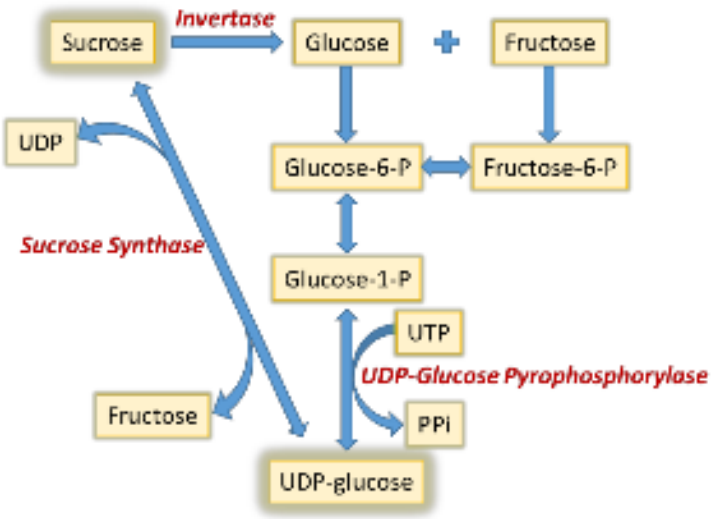


Figure 1. 4 Illustration of UDP-Glucose formation pathway (Adapted from Taşkıran, 2020)

CHAPTER 2

JUSTIFICATION AND OBJECTIVE OF THE STUDY

Despite that cellulose accounts for the most abundant biopolymer on earth, and has various biomass, bioenergy, and economical advantages, little is yet known about its molecular biology composition and regulation at the genetic level. The exact mechanism of cellulose regulation and how it is transcribed, translated, and deposited in the cell wall of woody tree species is yet to be known. Many studies have reported that Poplars show to be a great crop species for biomass and bioenergy utilization and for the manipulation of their cellulose content in the woody trees, where they could be massively taken advantage of for their whole known genome sequence and a great amount of cellulose. The cellulose synthase, UGPase, and SuSy enzymes are known to be the three main enzymes responsible for the deposition of cellulose in woody trees. Nevertheless, the exact activation and regulation of CesA complex subunit genes and their mechanism in making cellulose in wood in the past recent years was yet not been completely discovered. On this account, the current study conducted covers the investigation of PnCesA4 gene expression and cellulose content, along with other cell wall-related components. They were chosen to further understand the effect of PnCesA gene expression level on cellulose, cellulose-related enzymes, their relation to each other, and the effect of their roles on the cellulose biosynthesis pathway in plants. Moreover, from the 5 CesA genes in Poplars that are responsible for the secondary xylem-related cellulose deposition, the PnCesA4 subunit gene was chosen as a study gene of interest due to its known unique importance and being the only gene from the five scw xylem-related CesA genes not having a duplicate pair. Thus, this gene was chosen in the current study to broaden the understanding of its relation to cellulose, its function, and cellulose enzymes (UGPase and SuSy), along with tree growth traits such as; height and diameter.

According to the literature, the current study manifests to be a novel study such that, it investigates the relationship between the genetic expression of PnCesA4 subunit, related to the scw-related CesA, and its relation to cellulose. Moreover, the study also aimed to investigate the relation of the PnCesA4 subunit to SuSy and UGPase enzyme activity, which is responsible for the same cellulosic pathway. The study was carried out by using samples from *P. nigra* trees to investigate the contribution of the PnCesA4 gene expression level and possible different factors to the variation. The current study also aimed to determine the relationship between PnCesA4 gene expression, cellulose, components of cell wall studied traits and growth parameters. These criteria are of great importance in understanding the cellulosic cell wall biosynthesis in woody trees. The established outcome from this study will further aid in understanding the unknown function of the PnCesA4 subunit, its involvement in the general CSC complex, its genetic mechanism in the woody tree species, and their importance in the relation to cellulose of the studied trees.

Succeeding the analysis of gene expression and estimation of the relationship between the studied traits, the main interrogation that remained to be answered is how is cellulose regulated and deposited regarding the CESA subunits' gene expression, and their relation with other the cell wall-related components? Is there a possibility of self-regulation of the PnCesA4 subunit gene? Is the activity of a single CesA subunit dependent on the activity of other related CesA subunits for cellulose deposition? As mentioned earlier, *P.nigra* has a great potential for biomass and bioenergy making, and the samples used in the current study were chosen from a natural range of species across Turkey and placed in a clone bank in Ankara. The clonal collection in this study used was similar to the earlier studies that originated from our laboratory (Yıldırım, 2013; Taşkıran, 2020), where numerous researches were conducted on *P. nigra*, adding imperative information regarding the current knowledge of the *P. nigra* species in Turkey.

Moreover, the current study it was aimed to investigate the difference between commercial and non-commercial trees of *P. nigra* clones in Turkey. In addition, the

current study conducted covered the investigation of clone trees with the highest cellulose amounts among Commercial and Non-commercial clones. Clones with the highest cellulose and PnCesA4 gene expression levels were additionally affirmed. Clones of *P. nigra* with a possibility of being commercial tree candidates were moreover proposed in the current study conducted.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Materials and Sampling

Through the collaboration of METU, Central Anatolia Forest Research Institute in Ankara, Ministry of Forestry and Water Affairs in Turkey, and the Poplar and Fast-Growing Forest Trees Research institute in Izmit, study materials were available as an ex-situ conservation program which was established as *Populus nigra* clonal bank, having 297 clones (as in genotypes) collected all across Turkey. The clone bank is located in Ankara Behiçbey Nursery. The clones in the clone bank represented 7 geographical regions in Turkey and were available for the studies conducted since 2009. All clones subjected to the current study were grown under identical growth and environmental conditions and harvested at the same time and physical stage. The samples were taken from 5-year-old trees with the help and collaboration of Dr. Bircan Taşkıran. The number of trees that were utilized for the study sampling is listed in (Appendix A.Table.1), and samples were stored at – 80°C freezer.

Approximately 44 European black poplar genotypes were determined to sample for the study from Behicbey Nursery at the end of June 2015 with the kind collaboration of Taşkıran (2020). The wood samples provided for the study were sized around (7 mm x 5 cm x 10 cm) and were taken 130 cm from above the ground (bark was removed). The samples with the best RNA isolated yield were chosen to be continued in the study. Moreover, for further research and study, additional samples were taken from 1-year-old trees with the same measurements in Behicbey Nursery at the end

of May 2021 to further investigate the differences and mainly aimed for usage in future studies.

3.1.2 Sample wood Homogenization

After samples were obtained, they were immediately frozen in liquid nitrogen and stored at -80°C until used. The frozen tissues were homogenized later by grinding in a mortar chilled with liquid nitrogen. More specifically, to study the PnCesA gene expression isolation of RNA and synthesizing cDNA, the homogenization of the samples was done by removing the bark and grinding the wood sections with a mortar and pestle in the liquid nitrogen. The mortar and pestle were autoclaved before usage. Moreover, around 100 mg (milligrams) were used for the RNA isolation method. Additional wood samples were saved in a -80° C freezer for further studies and later usage.

3.2 Methods

3.2.1 RNA Isolation

Favori Prep Plant Total RNA Purification (FAVORGEN) mini kit was firstly used according to the manufacturer's protocol to isolate total RNA in the grinded wood samples. RNase-free water was used to purify the RNA, and to increase purity they were later subjected to DNase I treatment. Nanodrop biological analyzer was used to detect the integrity of the RNA and to determine the concentration where results showed mostly values between ~ 1.6 and 2.0 but showed little yield. To further improve RNA quality and yield. several different RNA isolation methods and protocols were modified and done. One manual protocol (involving Phenol and Trizol) which showed the most effective results in yield and integrity of RNA was therefore selected. The rest of the samples were conducted with the following RNA

isolation method, as the following; Initially, wood samples were ground by mortar and pestle in liquid nitrogen and stored at – 80 °C freezer. Then samples were ready for RNA isolation method usage. Each sample tube contained a range of 100 ~ 200 mg of ground wood sample. Firstly, 1 ml of Trizol was added to each sample. Moreover, sample suspensions were homogenized by shaking moderately. Next, they were incubated for 10 minutes at room temperature. Later, 250 µL of chloroform was added for every 1 ml of Trizol. Samples were shaken for 15 seconds and underwent a 10-minute incubation period at room temperature. Then, they were centrifuged for 10 minutes at 14000 rpm speed. The aqueous phase of each sample was removed and 550 µL was obtained and transferred into 1.5 µL Eppendorf. Then, 600 µL (1 volume) of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the aqueous phase and mixed by vertexing. Then, again centrifuged for 10 minutes at 14 000 rpm speed. After centrifugation, the aqueous phase was removed and transferred into 1.5 µL Eppendorf. A 300 µL of isopropanol, 250 µL (1.2 M Na-Citrate), and 250 µL of (0.8M NaCl) were added to each Eppendorf and mixed by inverting. Then samples were let to rest for 2-3 minutes at room temperature. Then, incubation could be achieved by one-hour incubation at room temperature or incubation at – 20 °C freezer overnight, the latter was chosen in the study to achieve better results. The following day, samples were centrifuged at 14000 rpm speed for 10 minutes. After centrifugation, the supernatant was discarded, and the pellet was washed with 1 ml of 70% Ethanol (ETOH). Next, samples were centrifuged again at 7500 speed for 5 minutes at 4 °C. The pellet was left for air-drying under a filtered-laminar flow hood. Getting the pellet completely dry was avoided not to decrease the solubility of RNA, nevertheless, no droplets were left. Consequently, RNA was dissolved in 50 µL RNase-free water. After recovery of the aqueous phase, samples were kept on ice or were stored at -80 °C freezer.

3.2.1.1 Trizol and Phenol Usage Basis

Accurate and reliable analysis of gene expression relies greatly on the isolated RNA purity and quality. Nevertheless, increasing RNA yield increases the possibility of RNA contamination. To avoid RNA contamination chloroform extraction was recommended, and done in the study, with RNA washing steps. During phase removal, removing extra contaminants such as proteins or phenol was avoided. To increase RNA purity, ethanol washes (and precipitation) were included in the RNA isolation method to remove any residual salts left from the added isopropanol and residual phenol. These procedures aimed to increase the accuracy of RNA quantification and purity (Toni et al., 2018).

Trizol reagent is designated to supply a high-quality RNA isolation from all kinds of cells. It is a monophasic solution of phenol and guanidine isothiocyanate and has related propriety components that accelerate RNA isolation (TRIzol Reagent*,* 2016). Trizol maintains RNA stability as it has an effective RNase activity inhibition along with its ability to disrupt cells and cell components. Usage of Trizol and chloroform allow homogenate specimens to separate into phases, causing the RNA to accumulate in the clear upper layer. The addition of isopropanol allows RNA to precipitate from the aqueous layer. RNA was washed several times to remove impurities and was then re-suspended for downstream application usage. Moreover, chloroform promotes phase separation to accelerate RNA isolation from samples. Phenol solubilizes biological materials and gets proteins denatured. When chloroform is added with phenol RNA isolation is eased. ("aatbio.com | AAT Bioquest", 2022).

3.2.1.2 DNase I Treatment

In order to improve RNA purity of samples DNase I treatment was done. (M03035 DNase I / Bio-Rad PureZOL RNA Isolation Reagent Cat: # 732-6890) DNase I was used. DNase I is an endonuclease that cleaves DNA nonspecifically aiding in RNA purity. The method used for DNase I treatment was, as follows.

100 µL reaction mixture was prepared including 48 µL RNA, 2 µL DNase I, 10 µL DNase buffer, and 40 µL of ddH20. The reaction was let to sit and underwent 1-hour incubation at 37 °C. Later on, 100 µL of Phenol: Chloroform: Isopropanol in (25:24:1) at pH 4 was added. Samples were mixed for 30 seconds and incubated on ice for 10 minutes. Then, centrifuged at 14000 g speed for 20 minutes at 4°C. An 80 µL was transferred from the upper phase to 1.5 µL Eppendorf. Then, 8 µL 3M Na-Acetate and 200 µL of absolute 100% ethanol (ETOH) were added. The mixture was mixed and incubated at -20 °C overnight. The next day, ethanol precipitation was performed. Samples were centrifuged at 14000 g speed for 20 minutes at 4 °C. Next, the supernatant was discarded, and samples were washed with 70% Ethanol (600 µL was added). Then, samples were centrifuged at 7500 g speed for 5 minutes at 4°C. The supernatant was discarded, and RNA samples were air-dried under a laminar flow hood. Then, samples were resuspended in 25 µL in RNase-free water. RNA samples were measured with a Nanodrop device and then left to store at -80°C.

3.2.2 cDNA Synthesis

Bio-Rad I-Script cDNA Synthesis Kit 1708891 (cDNA synthesis kit) was used according to the manufacturer's protocol to synthesize the cDNA library from isolated RNA samples. The kit provided a solution for reverse transcription Q PCR. The enzyme included in the kit possesses an RNase inhibition feature. Oligo (dt) and random hexamer primers in the reaction mix combined work efficiently well with many targets. In the current study, the gene of interest was the PnCesA4 gene and the reference gene was Ubiquitin. Components of the cDNA synthesis protocol included: a total of 15 µL RNA template, 4 µL (5x iScript reaction mix), 1 µL iScript Reverse Transcriptase enzyme, and a varied amount of Nuclease-free water according to the RNA content in each of the samples. Next, the reaction mixture was incubated in the PCR thermal cycler with the following reaction protocol: *Priming* at 25 °C for 5 minutes, *reverse transcription* at 46 °C for 20 minutes, *RT inactivation*

at 95 °C for 1 minute, and then *hold* at 4 °C. Later for Q RT-PCR, 2 µL of cDNA was used in the reaction mixture (iScript cDNA Synthesis Kit., 2000).

3.2.2.1 Primers preparation

Primers were designed to be amplified by PCR, a fragment of DNA that spans the PtCesA4 gene in the *P. nigra* tree. Based on the identified PtCesA gene model of *P. trichocarpa*, the PCR primers were designed such that the sequence would complement the PtCesA4 sequence of PnCesA4, Primers used in the study were (shown in table 3.2) as the following: Interest gene PnCesA4 oligo sequence: (Fwd) BT_CesA4_104F: TGCTGGAGAGGCTTGCTTAC, and (Rev) BT_CesA4_105R: CCTGGATGCTGACTCCACTC. Ubiquitin for reference gene oligo sequence PtUBQ.RT.forward: GGTTGATTTTGCTGGGAAGC, and PtUBQ.RT. reverse: GATCTTGGCCTTCACGTTGT. The designed primer set was expected to amplify a PCR product of 216 base pairs (bp) in size for PnCesA4, and 200 bp in length for Ubiquitin. To check the optimum oligo annealing temperature of the oligo sequences, the PCR amplification was tested with several different temperatures. Additionally, a 3% agarose gel was run on PCR products to further check the amplification and size of bands. Consequently, a 55°C temperature showed the best annealing temperature result from the tested temperatures. Moreover, 5 U/ µL of the *Taq* DNA Polymerase (labeled as Invitrogen, Lot: 2236785, Ref: 10342-020) was used in the PCR run.

Table 3.2.1 Primer sequence, temperature, and product size of the genes.

Gene	Forward Primer	Reverse Primer	Annealing Temperature	Product Size
	(5' → 3')	(5' → 3')	(°C)	(bp)
CesA4	TGCTGGAGAGGCTTGCTTAC	CCTGGATGCTGACTCCACTC	60	216
Ubiquitin	GGTTGATTTTGCTGGGAAGC	GATCTTGGCCTTCACGTTGT	60	200

3.2.3 Real-Time PCR

To determine the Differential Gene Expression Q RT-PCR was carried out using the synthesized cDNA library and the designed specific primers based on the PnCesA4 gene across different tree clones of the *P. nigra* species. The earlier mentioned forward and reversed primers were used (Table 3.2). After RNA isolation, and resuspension with RNase-free water and DNase I treatment and purification, the RNA was further reverse transcribed using *SYBER Green* PCR master mix and reverse transcription PCR according to the manufacturer's manual protocol. Reverse-transcription Real-time PCR was conducted using the (C1000 Touch Thermal Cycler – Bio RAD Q RT-PCR) system. In order to detect if any contamination exists, no template control (NTC) was used. As an internal standard, the UBIQ (Ubiquitin) gene was used. The DNA amplification carried out in a reaction mixture containing a specific nucleotide sequence for the related genes was given in Table 3.2.1. The final reaction mixture volume was 10 µL were 5 µL SYBER Green PCR Master Mix, 0.5 µL (5 pmol/ µL) primer (for each ubiquitin and PnCesA4 forward and reverse primers), 2 µL cDNA (50 ng), and 2 µL of ddH2O were used (as shown in Table 3.2.1). Each reaction was repeated 3 times. The amplification program cycling profile and PCR conditions were 30 seconds at 95 °C, followed by an additional 39 cycles of 10 seconds at 95 °C, 20 seconds at 60 °C, and for Melt Curve 65°C to 95 °C in increments of 0.5 °C for 05 seconds. The melting curve was used to confirm the expected amplification product without any non-specific products. Moreover, the products were separated on a 3% agarose gel and stained with ethidium bromide. Furthermore, the bands were visualized on gel electrophoresis and showed results at the expected base pair lengths around (~200/216) bp for ubiquitin and PnCesA4 gene oligos respectively, on the gel.

Table 3.2.3 Reaction mixture components

Component	*Volume*	
cDNA	2	µL (50 ng)
SYBR Master Mixture	5	µL
Forward primer	0.5	µL (5 pmol/ µL)
Reverse primer	0.5	µL (5 pmol/ µL)
RNase-free water	2	µL
Total Volume	10	µL

Table 3.2.4 All traits, units, and sources

Variables	Units
UGPASE Activity SuSY Activity (Taşkıran, 2020)	Units / mg protein
Cellulose Content Glucose Content (Taşkıran, 2020)	µg / ml
Height and Diameter (Yıldırım, 2013; Taşkıran, 2020)	cm
PnCesA4 Gene Expression	RPKM

.

3.2.4 Growth Parameters

3.2.4.1 Height and Diameter

Growth parameters such as height and diameter measurements of clones were kindly provided by the previous studies carried out with the same *P. nigra* clones (Yıldırım,

2013; Taşkıran, 2020). The height and diameter measurements were obtained from the 5–year old clones of *P. nigra*.

3.2.5 UGPase and SuSy Enzyme Activities

The UGPase and SuSy enzyme activity measurements, cellulose, and glucose content of clones were kindly provided by Taşkıran, (2020) in the previous studies carried out and given in detail with the same *P. nigra* clones (Taşkıran, 2020).

3.3 Data Analysis

Data from this study were analyzed using SPSS 11.5 (Statistical Product and Service Solutions) software. Consequently, bivariate Correlations between PnCesA4 gene expression, cellulose, SuSy, and UGPase/ cellulose-related traits, Glucose, and cell wall growth parameters (diameter and height) were carried out with Pearson's coefficient. Tests among studied traits and PnCesA4 gene expression level were done by One-way ANOVA. Normality and descriptive tests, along with frequency test approaches were used for understanding the nature of the data and the relationship between them. All data on the traits were evaluated and the results were provided as means along their standard deviation (mean ± SD), and $p < 0.05$ was chosen as the significance level.

CHAPTER 4

RESULTS

In order to calculate and measure the central tendency and dispersion accurately, SPSS software was used in data analysis. The data in the current study is quantitative data since it is measured and is numerical. Before any further analysis, normality, homogeneity of variances, and outliers were checked for all the traits using SPSS software. Results showed that all traits were normally distributed except for cellulose content which did not meet the normality criteria. Hence, in order to normalize the data, a log transformation was performed on cellulose content data while the other traits were not changed. The results indicated that the samples had equal variance, and thus ANOVA and other statistical tests were done by the SPSS software to partition total variance to the components for the studied traits.

In the current study, cellulose synthase PnCesA4 gene expression level was analyzed along with cellulose, glucose content cell wall-traits, and growth parameters (such as height and diameter) were obtained from previous studies (Taşkıran 2020). This sampling approach allowed the implementation of proper statistical analysis to elucidate the relationship among the traits, gene expression, and growth-related cell wall characteristics.

4.1 The variation in PnCesA4 gene level, cellulose, cellulose related-enzymes, and growth traits

The Descriptive statistical analysis results of the traits indicated that all data was normal and significant, except for cellulose content data that violated the normality assumption, which as mentioned earlier, later underwent log transformation. The results from descriptive statistics were provided in Table 4.1. The minimum, maximum, mean, standard deviation, variance, skewness, and kurtosis were given in

detail for each trait. Such as, the PnCesA4 gene expression ranged from 0.33 to 3.35 with a mean of 1.59. However, the UGPase Activity had its minimum value of 22.94, its maximum of 90.05 with a mean of 52.87. Thus, the mean values for SuSy Activity were much higher (245.48 ±7.33) with a higher standard deviation. Moreover, Log10 Cellulose content ranged from 0.81 to 1.93 with a mean of 1.33 (Table 4.1.1).

Table 4.1.1 Descriptive Statistics of All clones

	All Clones			
Variable	*N*	*Mean ± SD*	*Minimum*	*Maximum*
UGPase Activity	117	52.87 ± 1.36	22.94	90.05
SuSy Activity	114	245.48 ± 7.33	99.72	418.9
Log10 Cellulose Cont.	117	1.33 ± .023	.81	1.93
Glucose Content	117	35.16 ± .846	14.12	59.33
Height (m)	117	4.26 ± .039	3.08	5.11
Diameter (m)	117	.327 ± .005	.250	.46
PnCesA4 Expression	113	1.59 ± .081	.330	3.35

Moreover, commercial clones were separated from the rest of the clones which are "Gazi, Anadolu, Kocabey, Geyve, Ata1, Çubuk1, Çubuk2". Their descriptive statistics are shown below (Table.4.1.2). In the commercial clones, we can clearly see the difference in their descriptive statistics compared to the rest of the clones above (Figure.4.1.1). In which, PnCesA4 gene expression had an average of 1.59 in both commercial and non-commercial clones, but with a different range (from 0.67 to 3.198) and a higher standard deviation compared to the non-commercial clones. However, the commercial clones' Log10 Cellulose content had a minimum of 1.15 compared to .81 in Non-commercial clones. Additionally, the mean of commercial

clones Log10 Cellulose content had a slightly lower mean of (1.27) than the non-commercial clones (1.33).

Table 4.1.2 Descriptive Statistics of Commercial clones

	Commercial Clones			
Variable	*N*	*Mean ± SD*	*Minimum*	*Maximum*
UGPase Activity	7	46.93 ± 9.27	33.62	62.17
SuSy Activity	7	200.71 ± 95.15	64.35	328.14
Log10 Cellulose Cont.	7	1.27 ± 0.096	1.15	1.43
Glucose Content	7	30.78 ± 6.33	23.05	41.45
Height (m)	7	4.58 ± 0.28	4.36	5.11
Diameter (m)	7	0.39 ± 0.05	0.297	0.46
PnCesA4 Expression	7	1.59 ± 0.89	0.67	3.198

Next, from the studied data, trait data were analyzed to check if the data were normally distributed by using frequency histogram graphs. For each of the given trait diameter, height, log10Cellulose, and PnCesA4 Gene Expression levels, the frequency of distributions was provided in Figure 4.1.

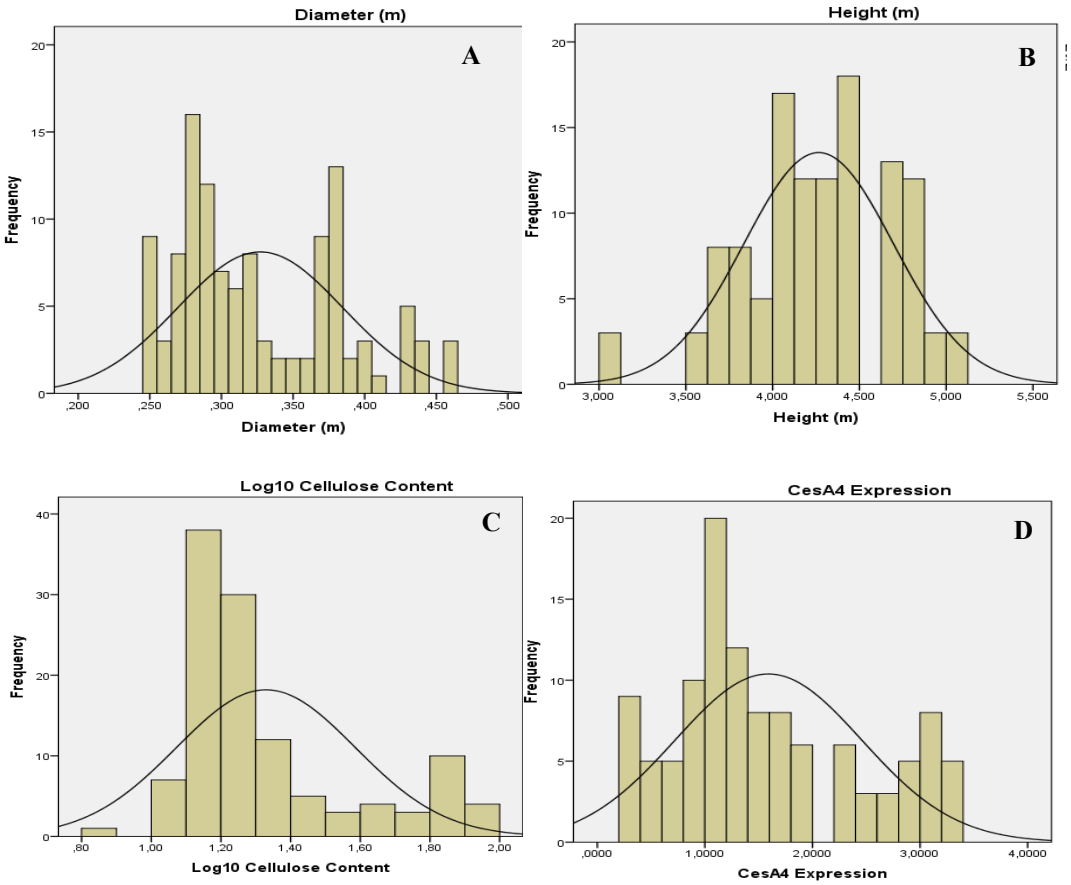


Figure 4.1 Frequency distributions as histograms for the studied traits in black poplar clones. A. Diameter, B. Height, C. Log10 Cellulose content, D. PnCesA4 Gene expression, traits.

4.2 Genetic variations in cell wall-related enzymes, cell wall components and growth traits

The results of the analysis of variance (ANOVA) revealed that there was significant variation among clones concerning PnCesA4 gene expression level as well as the other traits as seen in (Table 4.2.1). A significant P-value suggests that at least one group mean is significantly different from the others. All of the traits were significantly varied, indicating that there were great variations among clones. With

respect to PnCesA4 gene expression (Table 4.2.1), there is a significant difference in PnCesA4 gene expression level among clones. The significant

Table 4.2.1 Analysis of variances (ANOVA) for the studied traits

		df	Mean Square	F
UGPase Activity	Between Clones	43	278.595	1.540*
	Within Clones	73	180.964	
SuSy Activity	Between clones	42	10194.971	2.736**
	Within clones	71	3725.797	
Log10 Cellulose content	Between Clones	43	.152	10.157**
	Within clones	73	.015	
Glucose Content	Between clones	43	164.247	4.517**
	Within clones	73	36.366	
PnCesA4 Expression	Between clones	43	1.759	13.791**
	Within clones	69	.128	

*Significant at P<0.05; ** significant at P<0.01

Moreover, in order to check if there is a significant mean variation between commercial and non-commercial (or the rest of the clones), ANOVAs were carried out, and the results of ANOVA and means of traits were given in Tables 4.2.2 and 4.2.3. The results of the analysis of variance (ANOVA) revealed that UGPase and Glucose content were significant varied among commercial and non-commercial clone groups. Thus, there is a difference between the mean of the commercial and the mean of non-commercial clones in UGPase and Glucose content variables (Table 4.2.2, Table 4.2.3). Nevertheless, commercial and non-commercial clones did not significantly vary for the remaining the variables (SuSy activity, Log10 Cellulose, and PnCesA4 gene expression level). This suggests that there is no variation between Commercial and non-commercial clones.

Table 4.2.2 Means of Commercial clones vs. the non-commercial clones.

Groups		*N*	*Mean*	*SD*
Commercial Clones	Cellulose	7	1.2651	0.095
Rest Of Clones		37	1.339	0.255
Commercial Clones	PnCesA4 Gene Expression	7	1.591	0.898
Rest Of Clones		37	1.591	0.811
Commercial Clones	UGPase Activity	7	46.935	9.274
Rest Of Clones		37	53.699	10.738
Commercial Clones	SuSY Activity	7	200.707	95.147
Rest Of Clones		37	254.219	64.893
Commercial Clones	Glucose	7	30.783	6.330
Rest Of Clones		37	36.054	8.045

Table 4.2.3 ANOVA for Commercial and Non-commercial Clones

ANOVA

		df	Mean Square	F
UGPase Activity	Between Clones	1	957.309	4.543*
	Within Clones	115	210.719	
SuSY Activity	Between Clones	1	4873.221	0.793
	Within Clones	112	6141.492	
Log10 Cellulose content	Between Clones	1	0.091	1.394
	Within clones	115	0.066	
Glucose Content	Between Clones	1	628.535	7.953**
	Within clones	115	79.033	
PnCesA4 Expression	Between Clones	1	0.085	0.112
	Within clones	111	0.760	

*Significant at P<0.05; ** significant at P<0.01

4.3 Relationship between PnCesA4 gene expression and growth as well as cellulose traits

In order to examine the relationship among the traits, *c*orrelation coefficients between PnCesA4 gene expression level and all the traits were calculated. Thus, the bivariate correlation was done with the option of *Pearson* correlation coefficient in the SPSS program. The main purpose for checking the correlation and performing a scatterplot matrix in a correlation analysis is to verify which variables will have a linear relationship. The fitted Linear line characterizes the linear association represented by Pearson's bivariate correlation. A correlation conveys the relationship or how strong the correlation or co-varying occurrence exists between the variables in a value between 1 and -1. This value measures the strength of the relation and linkage between the variables and is called the correlation coefficient. The correlation coefficient between continuous variable levels can also be referred to as Pearson's *r* correlation coefficient. Accordingly, a positive value denotes a positive relationship between the variables and a negative value conveys a negative linkage among the variables. A zero-correlation coefficient implies no relationship at all between the traits.

The results of the Pearson correlation analysis were given in Table 4.3 and Figure 4.3. It was observed that there was a strong positive correlation between cellulose synthase-related enzyme activity (UGPase and SuSy activity) and cellulose content. Yet, there was no significant correlation observed between cellulose synthase PnCesA4 gene expression level and cellulose content. The most unpredicted result in our study was the cellulose synthase PnCesA4 gene expression being significantly and negatively correlated with height and diameter, which was an unexpected outcome. Moreover, as expected correlation between diameter and height was strong and positive since the volume was calculated based on them.

Moreover, it was clearly observed that cellulose content was significantly and positively correlated with UGPase activity, SuSy activity, and Glucose content (Where UGPase activity showed a stronger positive correlation with cellulose

content than did SuSy activity). Moreover, diameter had a significant positive correlation with SuSy activity and a low, but significant and negative correlation with the PnCesA4 gene expression level.

Table 4.3 Correlations between PnCesA4 gene expression and all the studied trait

	SuSy Activity	Log10 Cellulose Content	Glucose Content	Height (m)	Diameter (m)	PnCesA4 Expression
UGPase Activity	**.407****	**.373****	-.045	-.041	-.072	-.054
SuSy Activity		**.191***	-.139	.136	**.198***	-.025
Log10 Cellulose Content			**.199***	.036	.025	-.100
Glucose Content				-.087	-.149	-.150
Height (m)					**.800****	**-.331****
Diameter (m)						**-.196***

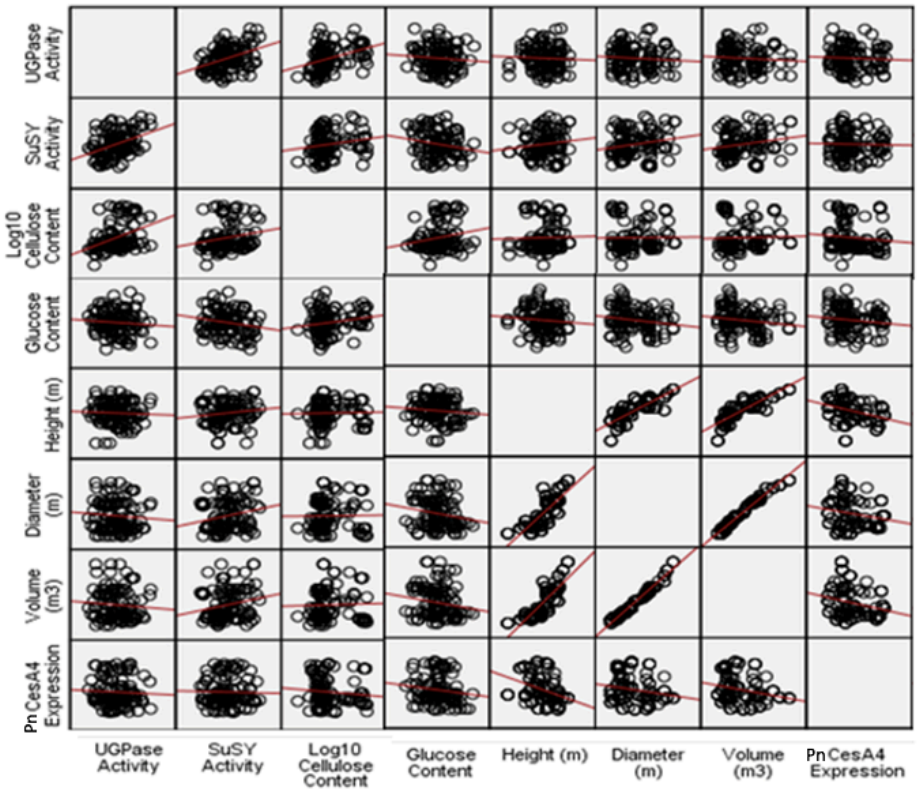


Figure 4.3 Scatterplot matrix of all studied traits.

4.4 PnCesA4 gene expression of *P. nigra* clones from wood samples

The PnCesA4 gene expression levels were determined by Q-RT- PCR using gene-specific primers. Specific primers and annealing temperatures for PnCesA4 and Ubiquitin genes were used as shown in Table 3.2.1. The ubiquitin gene was used as an internal standard. To verify PCR amplification and specificity, the amplification curve and melt curve were plotted, respectively (Figures 4.4.1 and 4.4.2). Relative mRNA expressions were determined by the formula below. As shown in (Figure 4.4.3), there was only one peak which means one amplicon without primer dimer or non-specific PCR product.

The Formula used:

ΔCt (Control) = Ct (Target) - Ct (reference)

ΔCt (PnCesA4 Gene) = Ct (Target) - Ct (UBIQ)

$\Delta\Delta Ct = \Delta Ct$ (PnCesA4 Gene) - ΔCt (Control)

$2^{-\Delta\Delta Ct}$ = Relative mRNA expression

Statistical analysis was done with SPSS software by checking the correlation relations, and one-way ANOVA method, and some descriptive statistical analysis.

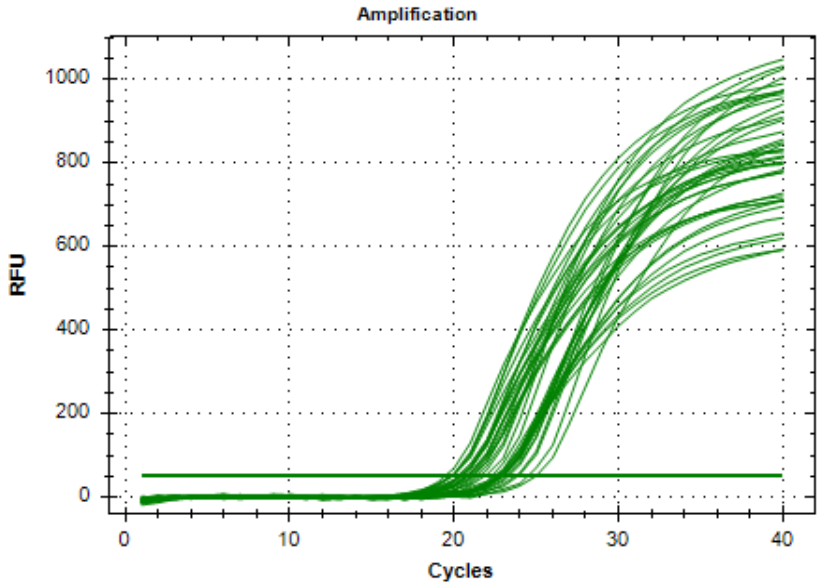


Figure 4.4.4 Amplification curve of Q RT-PCR of PnCesA4 gene showing fluorescence accumulation at each cycle.

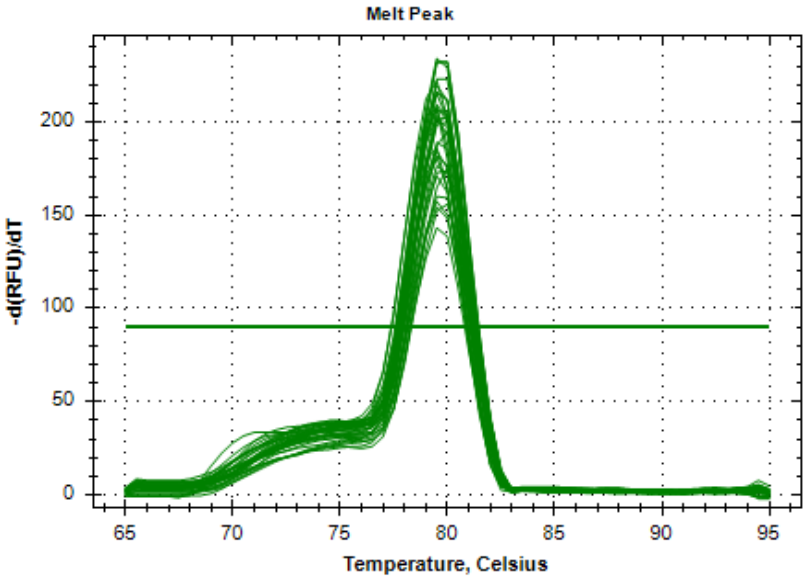
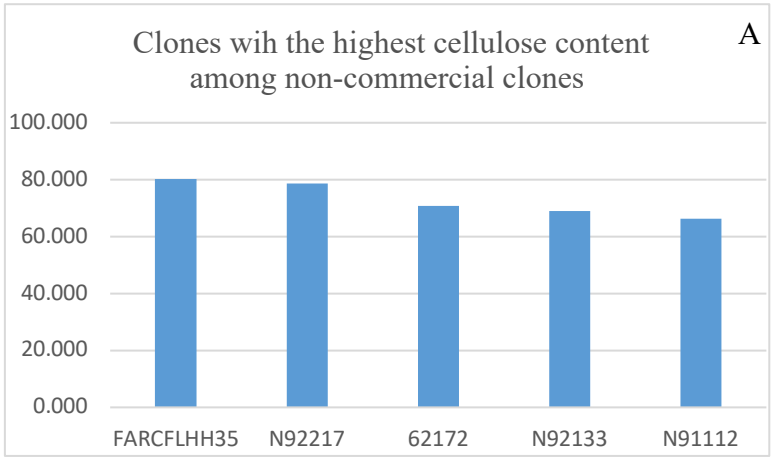
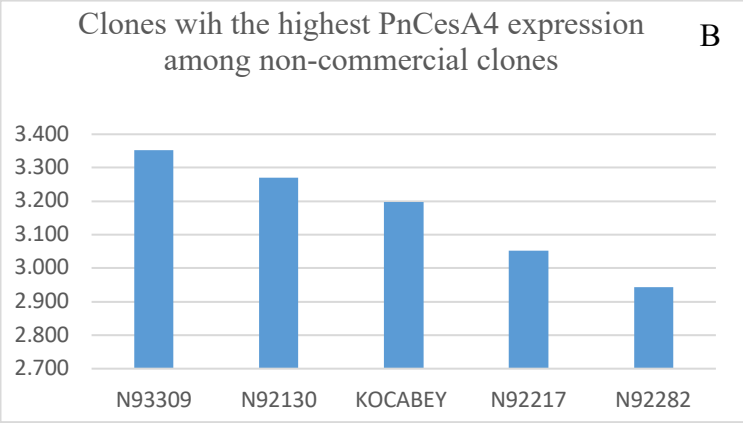


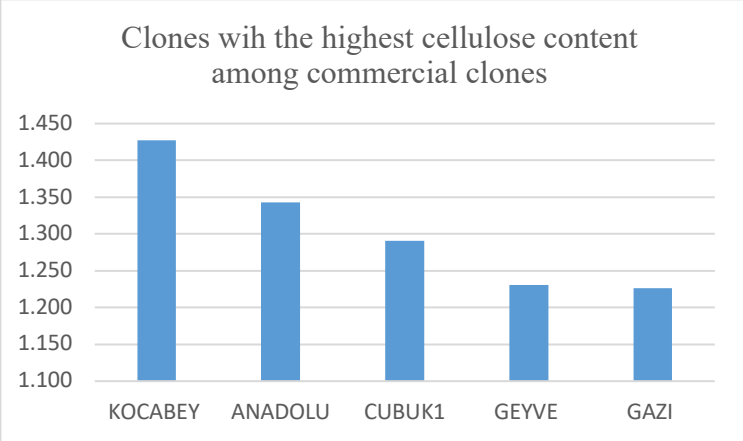
Figure 4.4.5 Melting curve of Q RT-PCR of PnCesA gene showing fluorescence emission change versus temperature.

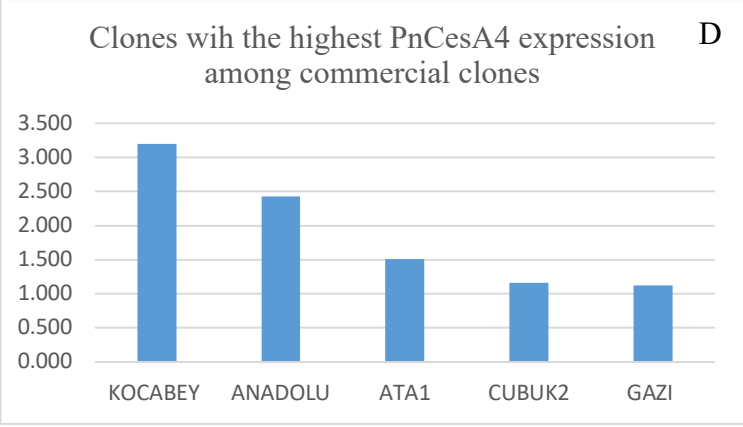
4.5 Results of highest Cellulose and PnCesA4 Gene expression in Commercial and Non-commercial containing clones

In order to determine from the studied clones, the clones with the most cellulosic potential, average mean values of clones were arranged for the first five maximum clones for each cellulose and PnCesA4 Gene expression variables between non-commercial and commercial clones. The first five clones representing the highest cellulose and PnCesA4 Gene expression levels were analyzed (as shown in Figure 4.4) to investigate any possible relationship between those traits. All these results led us to examine the maximum five clones in each of the cellulose and PnCesA4 Gene expression levels. The clone labeled as "N92217" was the only clone from All clones among the highest 5 clones (2nd highest clone) in respect to cellulose content and from also among the highest 5 clones (4'th clone) in respect to PnCesA4 gene expression level from all clones. Thus, this clone shows to be a potential candidate as a cellulosic commercial clone for future poplar breeding programs and long-term strategies. Moreover, the FARCFLH35 clone was the clone with the highest cellulose content among all clones. Meanwhile, from among the commercial clones, the "Kocabey" clone showed to have the most cellulose content level. Interestingly, the "Kocabey" clone was also the clone with the highest PnCesA4 gene expression level in the commercial clone section.




Clones wih the highest PnCesA4 expression among non-commercial clones
B
3.400
3.300
3.200
3.100
3.000
2.900
2.800
2.700
N93309
N92130
KOCABEY
N92217
N92282


Clones wih the highest cellulose content among commercial clones
1.450
1.400
1.350
1.300
1.250
1.200
1.150
1.100
KOCABEY
ANADOLU
CUBUK1
GEYVE
GAZI


Clones wih the highest PnCesA4 expression among commercial clones
D
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
KOCABEY
ANADOLU
ATA1
CUBUK2
GAZI

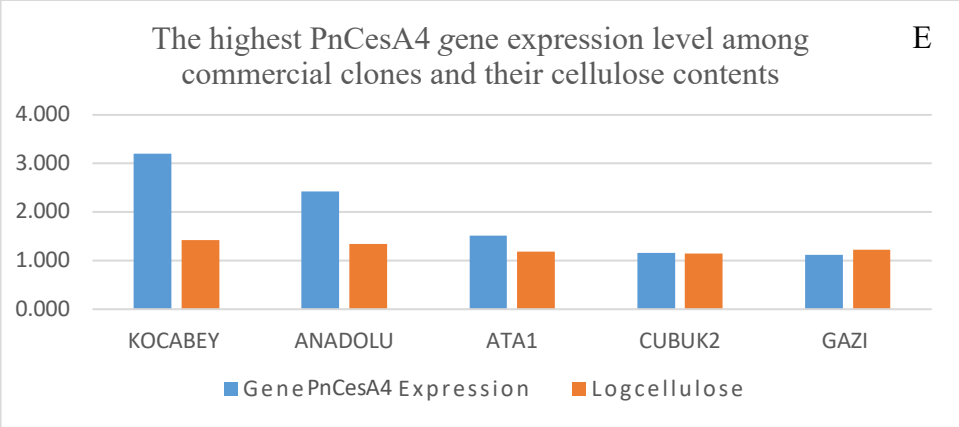


Figure 4.5 The clones among non-commercialwith A) the highest cellulose content, B) the highest PnCesA4 gene expression level, the clones among commercial clones with C) the highest cellulose content, D) the highest PnCesA4 gene expression level, and E) the clones among commercial clones with highest PnCesA4 gene expression level and cellulose contents.

CHAPTER 5

DISCUSSION

As it was shown in our results section, PnCesA4 gene expression level results had no correlation with cellulose content and the cellulose-related enzymes. The reason for this is not clear yet. UGPase (UDP-Glucose pyrophosphorylase) and SuSy (Sucrose synthase) enzymes are two of the three enzymes that are related to the formation of UDP-Glucose. The three enzymes being UGPase, SuSY, and Inv (Invertase) enzyme (as shown in Figure 1.4). Nevertheless, in the current study only UGPase and SuSy, two enzymes that were available from the previous studies, were analyzed. In the literature, these two enzymes were asserted to be the main provider enzymes of UDP-Glucose to cellulose synthase. As mentioned earlier, five genes are generally accepted to have critical roles in the secondary xylem development and the CesA4 gene is one of those genes. Likewise, it is the only gene from the *scw*-related CesA genes not having copies. While CesA7 and CesA8 have gene duplicates; CesA7-A/B and CesA8-A/B, respectively. However, in the literature and work of Takata et al., (2015) it was designated that CesA7 and CesA8 are shown to be more accountable for cellulose deposition in the cell wall than CesA4 does.

Additionally, unexpectedly the results from the PnCesA4 gene expression were shown that the gene expression was negatively correlated with height and diameter. Moreover, PnCesA4 gene expression was expected to be positively correlated with cellulose, but the results from the current study indicated that there was no significant relationship exists between these two traits.

It has not been yet reported a clear relationship between PnCesA4 subunit gene expression alone and cellulose. Nevertheless, to justify further why PnCesA4 gene expression level had an insignificant correlation with cellulose content in the current study, a detailed literature review was done. One study conducted by Mazarei et al.

(2018) illustrated that overexpression of PvCesA4 resulted in decreased levels of cellulose content and crystallinity, and increased level of xylan content. The increase in xylan content was suggested to be related to the amount of non-cellulosic cell wall polysaccharides in the plants. Moreover, in a study done by Timmers et al. (2009), it was shown that the CesA4 gene was the only CesA gene able to self-interact with itself compared to CesA7 and CesA8 genes which had no self-interaction. Nevertheless, they were only able to have multiple interactions with the other CESAs in the rosette. This could elucidate the reason why PnCesA4 gene expression level did not have a significant correlation with cellulose content. The PnCesA4 could not have an effective role in the cellulose deposition by itself, its action with PnCesA7 and PnCesA8 gene subunits altogether indicates that the three genes altogether may play a stronger effect on cellulose deposition in the plant's cell wall. In a previous study by Timmers et al. (2009), they showed that when one CESA was absent, remaining isoforms tend to form mono- or di- mers. This indicates the importance of the three subunits working altogether. Moreover, this could emphasize a dependency of CesA4 to be more active in relation to another CesA subunit. Moreover, in their study, they showed that subunits themselves are not symmetric, yet the overall complex is. Thus., this could imply that each of the subunits has a unique, and different function and effect on the cellulose deposition. Moreover, it was indicated that the existence altogether could be critical and required for cellulose deposition to occur completely. Consequently, Timmers et al. (2009) suggested that due to the interaction being so specific, it is proposed that the CESA proteins' incorporation into the rosette complex is non-random and implies that each single CESA protein might have a specific unique function in the CSC rosette complex. This could further aid in the interpretation of the current study's results to clarify why PnCesA4 subunit gene expression had an insignificant correlation with the cellulose content and could have a possible regulatory role in the complex.

The PnCesA4 gene among the other CesA genes was further chosen as the study's gene of interest due to its critical role in the CesA complex and its unknown proposed

function in the cell wall biosynthesis. Interactions between scw-related CESA proteins are of great importance, and the structure of the rosette complex implies that the assembly is highly regulated. Consequently, the interaction between blocks of the complex is critical. A study done by Timmers et al. (2009) showed that CesA4 was the only subunit that was able to homodimerize and showed strong interaction with itself, along with interaction with CesA8 and CesA7 (less strongly, but yet significant). Meanwhile, CesA8 and CesA7 were unable to homodimerize, but had strong interactions with the other CESAs. This is one of the reasons that indicate the importance of choosing the PnCesA4 gene as a gene of interest in the current research study. Along with its distinctive characteristic of being the only gene from the secondary xylem-related CESA subunits (CesA4, CesA7, and CesA8) that does not possess duplicated genes. Moreover, a study done by Endler and Persson (2011) suggested that because CesA4 is the only subunit capable of homodimerizing, it could have a possible role in the linkage of the different complexes altogether in the CSC rosette structure.

Additionally, the differential gene expression levels between leaf and wood samples from the same genotype trees should be inspected. Many studies have indicated the importance of CesA genes having different expression levels among different tissue types. Thus, further study objectives should focus to study PnCesA4 gene expression levels in leaf tissues with the same *P. nigra* L. clone samples that were used in the current study. Furthermore, the results from leaf PnCesA4 gene expression levels would be compared to the PnCesA4 gene expression results of the current study. Moreover, a study done by Mazarei et al. (2018) revealed that CesA4 gene expression levels in switchgrass (*Panicum virgatum* L.) varied among different roots, leaves, stems, and tissues related to reproduction (Mazarei et al. 2018), which aids in the objective of further studying the PnCesA4 gene expression level among different tissue types. In the literature, it was shown that there is a significant and positive correlation between cellulose synthesizing enzymes (UGPase and SuSy) and cellulose (Taskiran 2020, Konishi et al. 2004, Endler & Persson, 2011, Hertzberg et al. 2001 Coleman et al..2006). However, there was no correlation between these

enzymes and the PnCesA4 gene expression level. Moreover, in the current study, the PnCesA4 gene expression was significantly and negatively correlated with height and diameter growths.

For future research and study, in addition to PnCesA4 gene expression, PnCesA8 gene expression level can be additionally included in studies. Depending on the literature review, a study conducted by Nayeri et al. (2022) affirmed that CesA4- and CesA8 heterodimerize which is another robust reason that further triggers the investigation of CesA8 gene expression level just as done with the PnCesA4 gene in the current study. The PnCesA8 gene expression level could be studied and compared to the current study's PnCesA4 gene expression results in order to further understand the scw associated-CESA relation and which CesA subunit is responsible for the cellulose deposition. In many literature reviews, it was proposed that CesA8-B gene duplicate is expected to have higher gene expression results than PnCesA4 did.

Hence, for future studies, PnCesA4 gene expression of primary xylem tissues can be furthermore investigated and compared to the current study results. Additionally, gene expression levels could be studied among different tissue types, such as leaf and wood tissue samples, to check if there is a different PnCesA4 gene expression level among them. Moreover, PnCesA4 gene expression levels could be further investigated among different ages of trees. Comparing *P. nigra* PnCesA4 gene expression levels between 1-year-old and 5-year-old trees.

In conclusion, Firstly, for future objectives and research additional PnCesA8/B subunit gene expression levels can be investigated and compared with PnCesA4 gene expression levels. Secondly, gene expression levels of PnCesA4 indifferent-tissue source types (leaf and wood) and aged trees (1-year and 5-year-old) can be investigated. These future study objectives taken altogether could further improve the understanding of PnCesA genes' role and function more precisely. Moreover, these objectives will further facilitate an in-depth understanding of the complicated process underlying cellulose deposition in cell walls of European black poplar trees.

CHAPTER 6

CONCLUSION

In conclusion, the European black poplar is one of the most important tree species in riparian forests, owing to its major economic and ecological value, and for accounting as the most potential biomass crop species having great bioenergy production potential and vast growing rate among other tree forests. According to the literature, the current study is a strong novel study where it was exceptionally conducted to study one of the essential CesA subunits (PnCesA4) gene expression to analyze the relationship between cellulose content trait and cell wall components. The methodology and design aided in studying the effect of different factors within each other and with the PnCesA4 gene expression in European black poplar.

As expected, it was determined that cellulose-related enzymes (UGPase and SuSy enzymes) were significantly positively correlated with one another. Moreover, the SuSy enzyme was significantly and positively correlated with the growth traits, as claimed in previously done research studies and the literature. In the current study, the purpose was to gain a better understanding of cellulose deposition and its relation to the PnCesA gene expression level. Cellulose traits were compared to the PnCesA4 gene expression level. Cellulose content of clones was significantly correlated with UGPase (UDP-Glucose pyrophosphorylase) and SuSy (Sucrose synthase) enzymes and glucose content. Moreover, the glucose content and cellulose content were significantly and positively correlated. In consistency with our findings, various previous studies showed that UGPase and SuSy are strongly related to cellulose.

Surprisingly, on the contrary to what was expected, a correlation was significantly negative between PnCesA4 gene expression with growth traits. The PnCesA4 gene expression, moreover, showed an insignificant correlation to cellulose content, to our surprise. These findings could owe to a more complicated regulation in the pathway

that is taking place during cellulose synthesis. According to the results of the current study, among the clones with the highest PnCesA4 gene expression level/ cellulose content, the "N**93309"** clone had the highest PnCesA4 gene expression. Yet, this was not consistent with cellulose levels as demonstrated in this study.

Further studies in investigating the PnCesA7 and PnCesA8 gene expressions are necessary to have a better understanding of how cellulose synthesis is regulated at the gene expression level and which specific subunits from the CESA scw–related CesAs are mostly responsible for the cellulose deposition and yield. The PnCesA8 gene expression level is not investigated yet, and it was proposed to be more accountable for cellulose deposition in the cell wall. Thus, it would be auspicious to further study PnCesA8 gene expression levels and compare it to the current study of the PnCesA4 gene expression results to further decipher the relationship between CESA subunits gene expression and the main cause of cellulose deposition.

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APPENDICES

A. Appendix A (Table.A.1)

Table A. 1. Clones of Poplar trees that are used in the current study taken from Behiçbey Clone Bank

Clone Identity	Region	City
62/172	Central Anatolia	Ankara
7740/ 77/40	Central Anatolia	Ankara
82/1	Central Anatolia	Kırşehir
82/4	Central Anatolia	Kırşehir
87/1	Central Anatolia	Kırşehir
88/1	Central Anatolia	Ankara
88/3	Central Anatolia	Ankara
ANADOLU	Central Anatolia	Ankara
ATA1	Central Anatolia	Ankara
ÇUBUK 1	Central Anatolia	Ankara
ÇUBUK 2	Central Anatolia	Ankara
FARCFLHH 35	Foreign	Foreign
GAZI	Central Anatolia	Ankara
GEYVE	Central Anatolia	Ankara
KOCABEY	Mediterranean	Adana
N.02.05.06	Open pollination	Open pollination
N.02.07.05	Open pollination	Open pollination
N.03.324	Unknown	Unknown
N.03.355	Eastern Anatolia	Erzincan
N.03.377	Central Anatolia	Ankara
N.03.399	Unknown	Unknown

Table A.1 (Continued)

N.90.013	Eastern Anatolia	Muş
N.90.065	Unknown	Unknown
N.90.102	Unknown	Unknown
N.91.021	Unknown	Unknown
N.91.058	Central Anatolia	Yozgat
N.91.075	Mediterranean	Kahramanmaraş
N.91.089	Central Anatolia	Niğde
N.91.112	Mediterranean	Hatay
N.91.117	unknown	unkown
N.92.130	Marmara	Bilecik
N.92.131	Marmara	Bilecik
N.92.132	Marmara	Bilecik
N.92.133	Central Anatolia	Eskişehir
N.92.142	Central Anatolia	Çankırı
N.92.171	Blacksea	Amasya
N.92.208	Aegean	Kütahya
N.92.217	Central Anatolia	Konya
N.92.219	Central Anatolia	Konya
N.92.239	Central Anatolia	Niğde
N.92.282	Aegean	Afyon
N.93.304	Southeastern	Gaziantep
N.93.309	Central Anatolia	Sivas
N.96.320	Eastern Anatolia	Bitlis