

Wageningen University – Laboratory of Plant Breeding

M.Sc. Thesis Report – Bio-based Economy Research Group

*An investigation of gene expression of cell wall biosynthesis
and morphology in Miscanthus*



M.T. Klaassen
Wageningen, September 2010

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and morphology in Miscanthus***

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Cover image: close-up photo of *Miscanthus sinensis* stems at Wageningen University (photo by M.T. Klaassen 2010).

Preface

First of all thank you for reading my M.Sc. thesis report. I hope it is relevant and/or interesting to you. I must say that it was worthwhile spending my time and energy on this project for eight months. This was not only because I was able to acquire knowledge and practical skills in the field of plant breeding and molecular biology but also to discover the “world of energy”. In the future, many fossils fuels that our society currently depends on will be depleted. This brings forth a problem, yet also a challenge to cope with or solve this problem. This was my motivation for doing this research, which focuses mainly on cell wall biosynthesis relating to bio-fuel production. Cell walls provide a major source of biomass that may be used for producing bio-ethanol. By having done this research I hope to have contributed to this topic. I hope society may one day be fueled with sustainable energy sources and that the transition towards this state may occur in a peaceful manner without conflict.

I hope you enjoy reading my thesis report.

Michiel T. Klaassen

Wageningen, September 2010

Abstract

As the global reserves of fossil fuels run out, biofuels become an attractive alternative energy source. The high yielding biomass plant *Miscanthus* (also known as Elephant Grass), is considered a high potential second generation biofuel crop. Currently, recalcitrance to breakdown of the plant cell walls is considered a major drawback in the production process of bio-ethanol from *Miscanthus* biomass. The organic secondary cell wall polymer lignin is considered a major cause of this recalcitrance.

This descriptive study investigates the morphological characteristics of stems and internodes and expression of genes involved in cell wall biosynthesis at four different stages of development. The purpose of this study is to contribute new insights to the development of *Miscanthus* genotypes for bio-ethanol uses. A collection of twenty genotypes were assessed in terms of six morphological aspects of the stems and internodes. The results show that there is ample morphological variation within the *M. sinensis* genotypes. Three strong correlations (≥ 0.9) were found between stem mass and internodes mass, stem mass and internode diameter and between internode mass and internode diameter. Within six contrasting genotypes, gene expression and biochemical composition of the stem internodes was monitored over four stages of plant development. Gene expression patterns of PAL, 4CL, LAC and CESA differ across the stages of development. Differential expression of the genes was not observed between the top, middle and basal internode sections. In terms of biochemistry, lignin and cellulose content changes across the stages of development. However, a clear relationship between the levels of gene expression with lignin and cellulose contents was not observed.

Keywords: *Miscanthus sp.*, Bio-ethanol, Cell wall biosynthesis, Gene expression, qPCR, Biochemical analysis, Lignin, Cellulose.

Acknowledgments

Firstly, I want to dedicate this thesis report to my daughter Nina - who was born the most hectic time of this study - and to my beautiful and supportive wife Annegina.

Secondly, I would like to thank all the people who have participated in bringing forth this research and report. In particular I would like to thank my supervisors Luisa and Oene for their time and advice. Oene, I appreciated the fact that you were always willing to discuss complicated topics with me such as the statistical analyses in Genstat. Luisa, you have been a kind and pleasant supervisor to me. I am also grateful to Dianka and Heleen for helping me conduct my experiments in the lab. Dianka, thanks to your help and supervision I have gained new experience in my molecular lab skills. You were also a pleasant person to work with. Heleen, I appreciated your help in the biochemical lab and I enjoyed your jokes.

Finally, I would like to thank Prof. Richard Visser for granting me the possibility of conducting my thesis at his chair group.

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Abbreviations

4CL	4-coumarate: CoA ligase
ADF	acid detergent fibre
β -tub	β -tubulin
cDNA	complementary DNA
CESA	cellulose synthase
DM	dry matter
GADPH	glyceraldehyde 3-phosphate dehydrogenase
LAC	laccase
NCBI	National Centre for Biotechnology Information
PAL	phenylalanine ammonia-lyase
pLIG	permanganate lignin
qPCR	quantitative polymerase chain reaction

1. Introduction

Critical geopolitical implications may arise because our society is heavily dependent on a number of limited energy sources (e.g. petroleum), mainly produced in politically unstable countries and regions (Soetaert and Vandamme 2009). The World Energy Council states that fossil fuels (petroleum, natural gas and coal) account for approximately 82% of the global energy needs (World Energy Council 2010). Moreover, fossil fuels have disadvantageous consequences for the environment which entail greenhouse gas emissions, air pollution and acid rain (Wuebbles and Jain 2001; Soetaert and Vandamme 2006). Furthermore, the supply of fossil resources will not last forever. Soetaert and Vandamme (2009) state that at the present rate of energy consumption, it is generally agreed upon that petroleum will run out within 50 years, natural gas within 65 years and coal in approximately 200 years. It is predicted that the world production of petroleum will reach its maximum at around 2010 (Campbell and Laherrère 1998), from that moment onwards production levels will decrease (Figure 1). These manifestations occur in a global setting where energy use annually increases at a rate of 2-3 % due to industrial developments in South East Asia, Brazil, China and India (Hastings, Clifton-Brown et al. 2008).

Therefore, initiatives are undertaken to develop renewable energy sources that principally do not run out and are largely carbon neutral. Bio-fuel (e.g. bio-ethanol) from biomass is such an alternative. The U.S. Government intends to replace 30 % of petroleum use in 2005 (in the transportation sector) with U.S. produced renewable bio-ethanol in 2030 (Milliken, Joseck et al. 2007). The European Union aims to displace 5.75 % and 10 % of fossil fuels in the transportation sector with bio-fuels in 2010 and 2020 respectively (Fulton, Howes et al. 2004). Currently, bio-energy resources such as forestry, agricultural crops, biomass residues and wastes account for approximately 14 % of the world's primary energy supplies (IAE 2010). Furthermore, the International Energy Agency (IEA) stresses that bio-energy can potentially supply 50 % of world energy needs in the 21st century (IAE 2010).

An ideal biomass crop is one with a high energy output whilst requiring minimal inputs. The plant genus *Miscanthus* (including *Miscanthus x giganteus*) is a potential lignocellulosic biomass feedstock in Europe because it may produce up to 25 tonnes dry matter ha⁻¹ annually (Jones and Walsh 2001). *Miscanthus* has a higher energy yield per hectare than other bio-energy crops such as willow, poplar, oil and starch crops (Hastings, Clifton-Brown et al. 2008). *Miscanthus* has an efficient C-4 photosynthesis process, use of water and nitrogen (Heaton, Voigt et al. 2004; Yuan, Tiller et al. 2008). Furthermore, from a sustainability point of view the crop has the advantage of fixating atmospheric nitrogen (Christian, Riche et al. 2008).

However, a major obstacle in developing second generation lignocellulosic bio-fuels is the resistance to breakdown of plant cell walls (i.e. the recalcitrance problem). Increasing the efficiency of

process steps in converting lignocellulosic biomass into ethanol is necessary to decrease the production costs.

This thesis focuses mainly on gene expression of lignin (PAL, 4CL and LAC) and cellulose (CESA) biosynthesis genes. These genes are involved in the formation of plant cell walls. The aim is to gain insight into the process of cell wall biosynthesis, and so to get a handle on the improvement of the efficiency of processing lignocellulosic biomass into bio-ethanol. Furthermore, biochemical components that are related to the genes of interest will be investigated, along with multiple morphological characteristics.

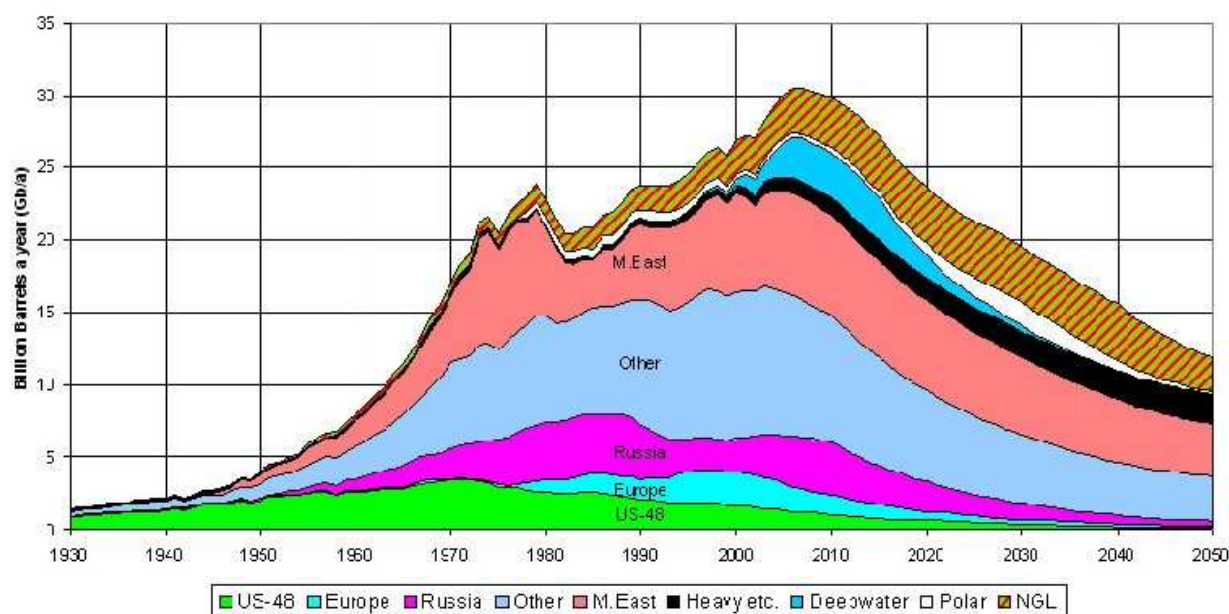


Figure 1. Scenario of Oil and gas liquids production in 2004 (Bentley 2002).

NGL = natural gas and liquids.

2. Review of literature

Chapter 2 provides a brief description of *Miscanthus*. The genus is depicted in Section 2.1. The cell wall structure, composition and biosynthesis are briefly presented in Section 2.2. Section 2.3 explains how cell wall components are applied in bio-ethanol production. Finally, the industrial conversion of lignocellulosic biomass into bio-ethanol is elaborated on in Section 2.4.

2.1 The genus *Miscanthus*

Section 2.1 reports basic facts of *Miscanthus sp.* Section 2.1.1 provides information on the genus and its natural distribution. Next, the taxonomy is presented in Section 2.1.2 and finally Section 2.1.3 elaborates on the morphology of the crop.

2.1.1 General description

Miscanthus sp. are woody rhizomatous C-4 grass species, originating from South East Asia (Jones and Walsh 2001). Its natural boundary stretches from South East Asia into Polynesia, with a few species present in Africa too (Figure 2). Currently, *Miscanthus* is found in large parts of Europe. As a non-food crop it was introduced mainly for its ornamental value. Globally, the crop is mainly distributed in tropical to sub-tropical areas, and performs well from sea level to altitudes up to more than 3000 m. It is a perennial crop with a life span of at least 10 – 15 years. Both the leaves and stems may be harvested on a yearly basis, which provide high quality lignocellulosic biomass. *Miscanthus* is well known for its high yields (10 – 25 t ha⁻¹ year expected in Northern Europe), high dry matter content at harvest, efficient use of water and nitrogen and resistance against pests and diseases. Therefore the crop is considered to be a suitable biomass crop (Jones and Walsh 2001; Heaton, Voigt et al. 2004; Hastings, Clifton-Brown et al. 2008; Yuan, Tiller et al. 2008).

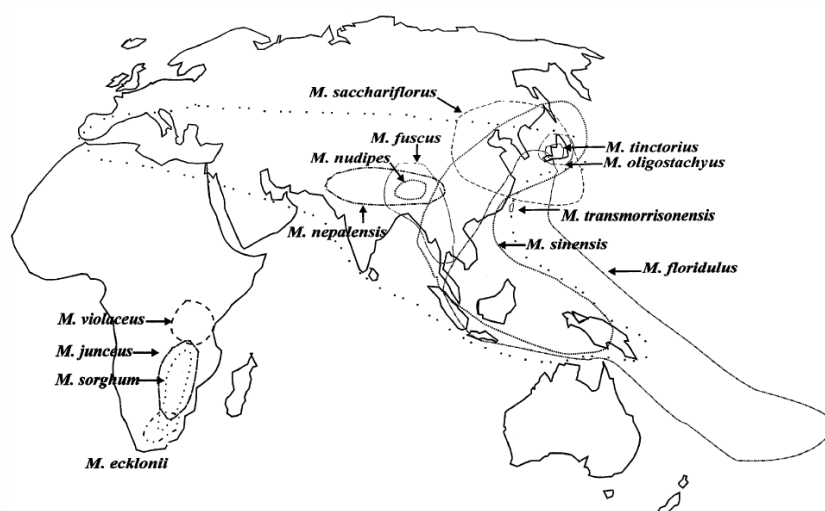


Figure 2. Major distribution areas of *Miscanthus*. (Hodkinson, Chase et al. 2002).

2.1.2 Taxonomy

Andersson was the first to describe *Miscanthus* in 1885 (Jones and Walsh 2001). The genus *Miscanthus* is part of the family Poaceae and consists of approximately 14 species (Hodkinson, Renvoize et al. 1997). Through interspecific hybridization, many hybrids have come about, such as the interesting biofuel species *Miscanthus* x *giganteus*. The hybrid *M. x giganteus* was initially collected in Japan, cultivated in Denmark and named *Miscanthus sinensis* 'Giganteus' hort. (Greef and Deuter 1993). Subsequently, the hybrid spread throughout Europe, and some authors suggest it originated from a cross between *M. sinensis* and *M. sacchariflorus* (Linde-Laursen 1993; Hodkinson, Renvoize et al. 1997). However, the evidence of this claim remains debateable and the taxonomy of this hybrid and its genus remains unsettled (Jones and Walsh 2001).

2.1.3 Morphology and diversity

Much morphological variation is present in the genus *Miscanthus*, but far less at species level (except in *M. sinensis*) (Jones and Walsh 2001). Based on multiple morphological characters (e.g. length of main axis of inflorescence) *M. sinensis* and *M. sacchariflorus* clearly separated into different groups, whilst other species (e.g. *M. fuscus*, *M. floridulus* and *M. transmorisonensis*) did not show such discrete differences at this level (Jones and Walsh 2001). Within the *Miscanthus* taxa, *M. sinensis*, *M. floridulus*, *M. oligostachyus* and *M. sacchariflorus* from South East Asia form a discrete monophyletic group which differs from African *Miscanthus* and Assum-Thailand *M. fuscus* species (Hodkinson, Renvoize et al. 1997). Table 1 illustrates the chromosome number and ploidy level of several

Miscanthus species. The basic chromosome number of *Miscanthus* is 19. Due to different ploidy levels the number of chromosomes differs between several species.

Table 1. Genetics of *Miscanthus* (Lafferty and Lelley 1994).

Species	No. chromosomes	Ploidy level
<i>M. sinensis</i>	38	di
<i>M. x giganteus</i>	57	tri
<i>M. sacchariflorus</i>	76	tetra

Figure 3 illustrates plantations of *M. x giganteus*. The plants may grow to a length of 2.5 – 3.5 meter and may produce an annual biomass of 20 – 25 tonnes dry matter per hectare (Jones and Walsh 2001). *Miscanthus* is a perennial crop with upright cane-like stems growing from a multi-tillering rhizomatous base (Hodkinson, Renvoize et al. 1997).

M. sinensis is the most commonly cultivated species throughout gardens in Europe. Several cultivars have been developed which differ in terms of height, leaf width, leaf colour and inflorescence colour. Depending on the cultivar, its colour varies from pale silver to deep purple red. Stems are retained well in the winter and continue growth in spring. *M. sacchariflorus* and *M. x giganteus* are the largest cultivated *Miscanthus* species. The triploid *M. x giganteus* differs from the tetraploid *M. sacchariflorus* and diploid *M. sinensis* in having longer rhizomes in established form and having shorter inflorescence axes. In non-flowering state, it is difficult to distinguish between the species using morphological characteristics, but can be distinguished using cytogenetic methods provide (Jones 2004).



Figure 3. *M. x giganteus* plantation with height of 3.4 meter (University_of_Illinois 2010).

2.2 Plant cell walls

Section 2.2 provides a brief overview of the composition and structure of the plant cell wall (Section 2.2.1), and a general description of the biosynthesis process of cellulose (Section 2.2.2) and lignin (Section 2.2.3). Section 2.2.4 illustrates the processing of lignocellulosic biomass into ethanol.

2.2.1 Plant cell wall structure and composition

Plant cell walls can be divided into three sections, the middle lamella, primary cell wall and secondary cell wall (Figure 4 and Figure 6). The primary cell wall develops between the plasma membrane and the middle lamella during cell growth and elongation. After growth has halted, the secondary cell wall is deposited. Lignocellulosic biomass originates from the plant cell wall and plays an important role in defining the structure of a plant and defending it against pathogens and insects. The structure, configuration and composition of cell walls depend upon plant taxa, tissue, age and cell type (Bothast and Schlicher 2005; Ding and Himmel 2006). The primary cell wall is composed of cellulose fibrils deposited in anti-parallel planes, cross linked by hemicelluloses and positioned in a pectin matrix (Figure 4). Extensin (structural glycoprotein) strengthens and adds flexibility to primary cell walls through cross-linking with pectins (Brady, Sadler et al. 1996; MacDougall, Brett et al. 2001). Monocots (i.e. grasses such as *Miscanthus*), have type II cell walls containing glucuronoarabinoxylans which make up their cross-linking glucans, and omit pectin and structural proteins (Sticklen 2008). It contains ample polysaccharides (e.g. cellulose and hemicellulose), which can be converted into bio-ethanol.

Cellulose, hemicellulose and lignin are the main components of the secondary cell wall. The secondary cell wall is typically composed of cellulose, hemicellulose and lignin (Awano, Takabe et al. 2002). Presently no detailed model has been proposed for the secondary cell wall (Obembe, Jacobsen et al. 2006). The cellulose microfibrils are cross-linked with lignin. Multiple layers (i.e. S1, S2 and S3 lamellae) of the secondary cell walls may arise during development of the cell (

Figure 9). These layers come about through the shifted arrangement of cellulose microfibrils deposition in time. The dry body mass of primary plant cell walls consists of up to 90 % polysaccharides (Rose 2003).

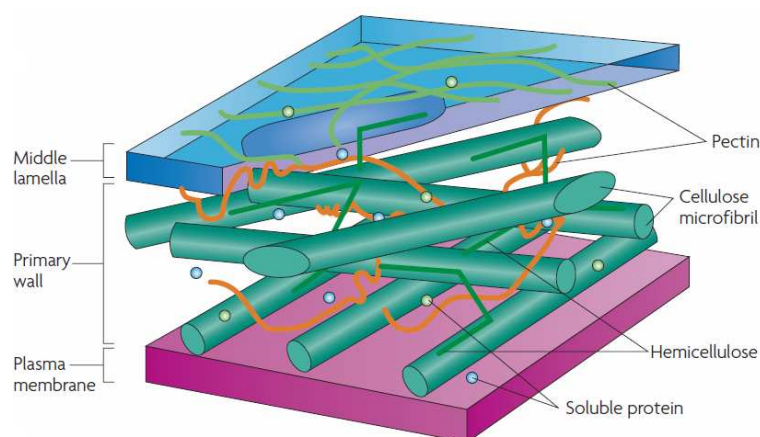


Figure 4. Schematic illustration of a plant cell wall (Sticklen 2008).

2.2.2 Cellulose structure and function

In maize coleoptiles, cellulose accommodates roughly 25 % of the dry weight of the cell wall (Carpita 1996). Cellulose is made up of unbranched chains of β -1,4-linked glucose (Figure 5). These sugar chains are coated with hemicellulose. Rosette structures (Figure 6; Figure 8) are thought to produce cellulose microfibrils (Tsekos and Reiss 1992). These structures, consisting of six catalytic subunits, are part of the biosynthesis machinery (Arioli, Peng et al. 1998). However, the detailed working of the cellulose biosynthesis by the protein complexes is not well understood (Obembe, Jacobsen et al. 2006).

Figure 7 illustrates the metabolic pathway of cellulose biosynthesis. Multiple modifications of glucose take place to produce the glucose glucan chain.

Presently cellulose is the only cell wall polysaccharide used in commercial production of bio-ethanol. This is because the currently used yeast strains are only able to convert hexose into ethanol.

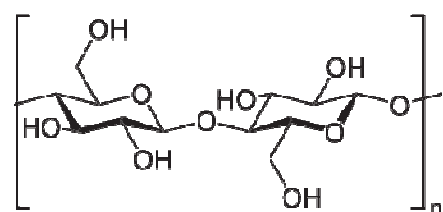


Figure 5. Cellulose molecule.

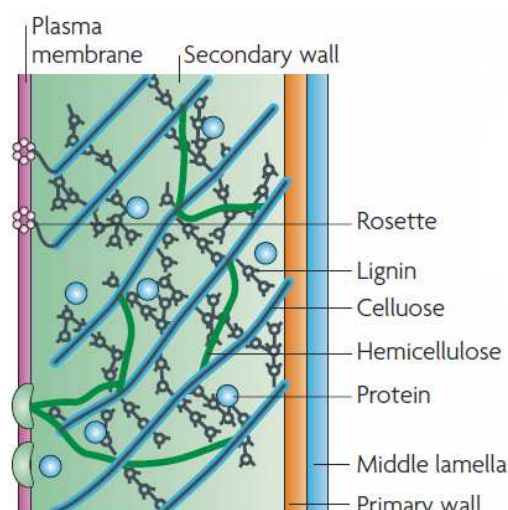


Figure 6. Cellulose synthase enzymes in rosettes in the plasma membrane (Sticklen 2008).

Cellulose bio-synthesis

Swift progress has been made in the area of cell wall biosynthesis after the first cellulose synthase (CESA) genes were isolated (Saxena, Lin et al. 1990; Pear, Kawagoe et al. 1996; Dhugga 2007). Based on mutational genetic and gene expression studies, many genes of the CESA family appear to be active in the primary cell wall formation in maize and other species (Appenzeller, Doblin et al. 2004; Djerbi, Lindskog et al. 2005; Somerville 2006). CESA1, CESA3, CESA6 genes are involved in cellulose synthesis of the primary cell wall of *Arabidopsis* (Arioli, Peng et al. 1998; Fagard, Desnos et al. 2000), whilst CESA4, CESA7 and CESA8 are involved in synthesizing cellulose for the secondary cell wall, also in *Arabidopsis* (Taylor, Howells et al. 2003). CESA proteins form hexameric arrays named rosettes that are embedded in the plasma membrane (Kimura, Laosinchai et al. 1999). The six subunits of the rosettes are encoded by three genes, but the assembly process of CESA subunits into the hexameric array is not yet understood (Cosgrove 2005). Together six CESA protein rosettes make cellulose microfibrils by means of bundling thirty-six (1,4)-linked β -D-glucan chains (Somerville, Bauer et al. 2004). It is thought that the rosette complexes are assembled in the golgi apparatus and subsequently exported to the plasma membrane (Somerville 2006).

Other proteins that influence cellulose synthesis are Kobito, Korrigan and Cobra. All these proteins have a trans-membrane domain and are embedded in the plasma membrane (Somerville 2006). The Cobra gene family has been identified to be involved in the *Arabidopsis* secondary cell wall formation and is thought to be involved in the orientation of cell wall expansion (Schindelman, Morikami et al. 2001). Recent studies have shown that the CobL4 gene from *Arabidopsis* (and orthologs in other species) is directly involved in the build up of the secondary cell wall (Li, Qian et al. 2003; Brown, Zeef et al. 2005; Ching, Dhugga et al. 2006). Other enzymes (or classes of enzymes) involved in the process are GDP-mannose pyrophosphorylase – involved in the anchor formation of

the Cobra-like proteins and glycosidases – glycosylation processing of proteins (Somerville 2006). The exact function of Korrigan and Kobito genes remains unclear (Pagant, Bichet et al. 2002; Cosgrove 2005). It is believed Korrigan could be involved in cleavage of glucosylated sitosterol primers for cellulose synthesis, or in editing the growing microfibrils to arrange packing of individual glucan chains, or in termination of the microfibril elongation (Peng, Kawagoe et al. 2002; Robert, Mouille et al. 2004). Mutations in Korrigan result in less cellulose in the primary and secondary cell wall (Szyjanowicz, McKinnon et al. 2004).

At early stages of development, secondary cell wall cellulose synthases (CESA) are expressed at a significantly higher levels than for the primary cell wall (Appenzeller, Doblin et al. 2004). The regulators of cellulose synthesis are yet to be identified (Dhugga 2007). The transcription factors that play a role in changing from primary to secondary wall synthesis have been isolated (Zhong, Demura et al. 2006; Demura and Fukuda 2007). Dhugga (2007) mention regulatory elements (in response to mechanical stress) that may provide methods for modulating cellulose formation.

Figure 7 shows the metabolic pathway of the transformation from α -D-glucose to the cellulose glucan chain. The last steps in this process entail the CESA and Korrigan products. Figure 8 illustrates the cellulose microfibril formation by rosette structures made up of CESA proteins.

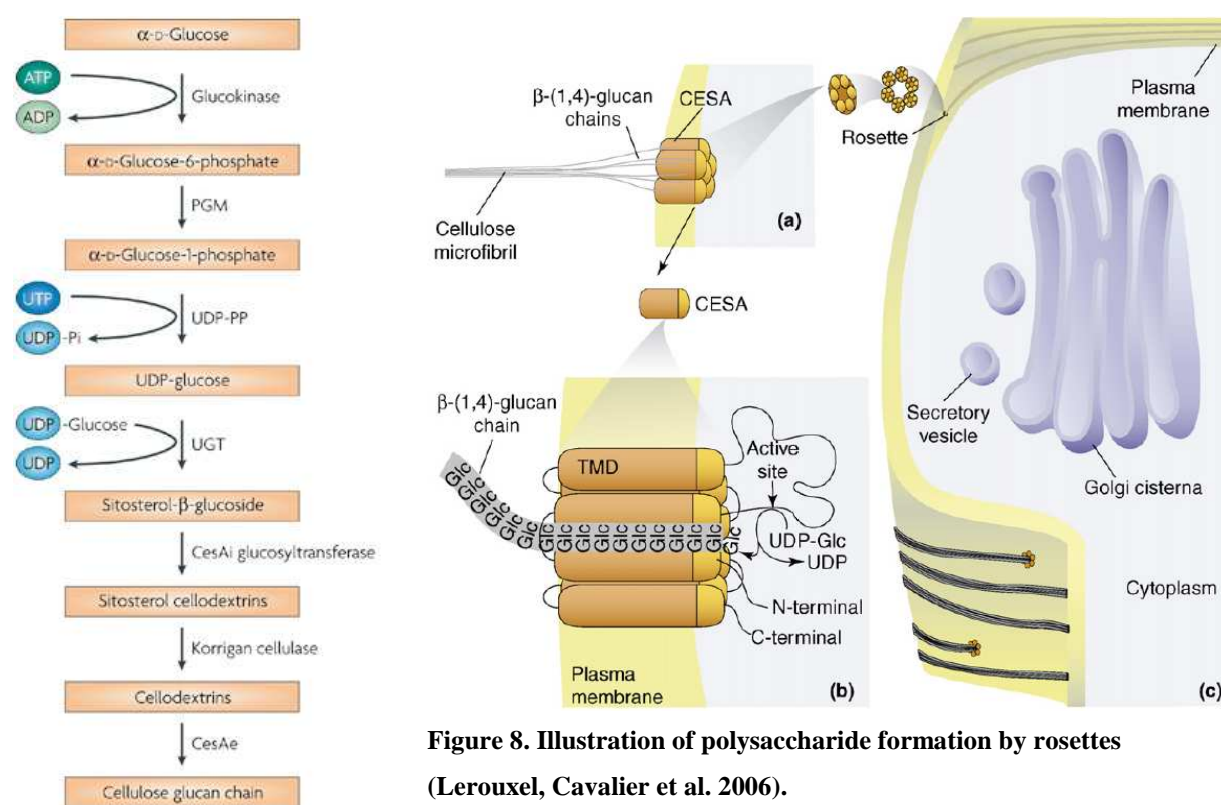


Figure 7. Steps in cellulose biosynthesis (Sticklen 2008).

Figure 8. Illustration of polysaccharide formation by rosettes (Lerouxel, Cavalier et al. 2006).

(a) Rosettes which are mobile on the membrane which produce cellulose microfibrils. (b) CESA protein complex. (c) Matrix polysaccharides are produced in the golgi apparatus before transported to the plasma membrane by vesicles.

2.2.3 Lignin structure and function

Lignin is a characteristic component of the secondary cell wall (Figure 6; Figure 9; Figure 10) (Rose 2003). It is widely accepted that lignin may be defined as an amorphous, polyphenolic material made up of an enzyme-mediated dehydrogenative polymerization of three phenylpropanoid monomers: coniferyl, sinapyl and p-coumaryl alcohols (Figure 9; Figure 11). Between 10 – 25 % of total plant dry matter is made up of this component (Sticklen 2008). At present, it is not exactly clear how the biosynthesis of lignin and cross-linking with cell wall polysaccharides takes place (Sticklen 2007). The cross linking of lignin with other wall polymers (e.g. cellulose and hemicellulose) strengthens the cell wall but increases recalcitrance of vegetative tissue to hydrolytic enzymes (Dhugga 2007). Lignin plays an important role in protecting the cell from invasive pathogens and insects and prevents water from entering the cell wall (Elkind, Edwards et al. 1990; Sewalt, Ni et al. 1997; Boerjan, Ralph et al. 2003). Lignin is thought to contribute to resistance to compression rather than tensile strength (Ching, Dhugga et al. 2006). When cells of different plants were subjected to compression, lignin synthesis was up-regulated whilst tension caused down-regulation (Donaldson, Singh et al. 1999; Joseleau, Imai et al. 2004; Andersson-Gunneras, Mellerowicz et al. 2006; Schmitt, Singh et al. 2006). Plant growth and development are affected when lignin concentration levels become too low (Shadle, Chen et al. 2007).

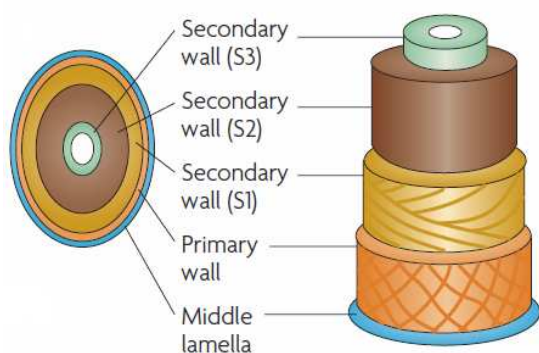


Figure 9. Layers of lignification in the cell wall (Sticklen 2008).

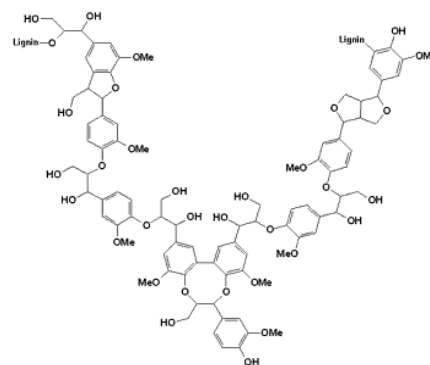


Figure 10. A random fragment of lignin.

Lignin bio-synthesis

Many enzymes are involved in the lignin biosynthetic pathway (Figure 11; Figure 36 in Appendix 9.1). The pathway has been changed many times and still remains under debate (Dixon, Chen et al. 2001; Humphreys and Chapple 2002). Lignin monomers are synthesized in the cell and subsequently transported to the cell wall where they undergo oxidation by peroxidases and/or laccases, necessary for polymerization (Vanholme, Morreel et al. 2008). A number of regulatory genes in lignin biosynthesis have been identified in multiple species, mostly *Arabidopsis* (Raes, Rohde et al. 2003;

Rogers and Campbell 2004; Rogers, Dubos et al. 2005). Most of the lignin biosynthesis genes are part of small multi-gene families and there is limited knowledge on the role of each specific gene member in the gene families (Barrière, Méchin et al. 2009). Furthermore, the developmental stage and tissue type are factors thought to play a role in this process.

Lignification of the cell wall is dependent on tissue type and plant maturity (Barrière, Riboulet et al. 2007). Lignin content increases in maize stems as the plant matures. Lignins come about from the three main pre-cursors – paracoumaryl, coniferyl and sinapyl alcohols – which are synthesized separately but in an interconnected pathway (Sticklen 2007). Lignin is composed of the three polymers: *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monomeric units which come about from the previous mentioned pre-cursors. In mature maize stalks, the S, G and H units come about in ratios of 61, 35 and 4% (Riboulet, Guillaumie et al. 2009). During plant development, the S/G ratio increases within the composition of lignified xylary and sclerenchyma cells, i.e. S-rich tissues (Buxton and Russell 1988; Chen, Auh et al. 2002). It has not been clearly established whether the proportion of lignin units H, G and S units, affect the degradability of normal, mutant and transgenic plant cell walls of by hydrolytic enzymes (Grabber, Ralph et al. 1997). Grabber, Ralph et al. (1997) found that the degradability of maize cell walls was not affected by the lignin components composition.

Many pre-cursors are involved in the biosynthesis process of lignin (Vanholme, Morreel et al. 2008). Presently little is known about the genes involved in the regulation of monolignol (source material of lignin) biosynthesis in maize and grasses (Barrière, Méchin et al. 2009). Within the monolignol pathway, the mutation of COMT in the in maize influences the transcription level of other genes (up regulation of OMT and cytochrome P450) or other pathways (i.e. hydroxylation and methylation) (Guillaumie, Pichon et al. 2007).

Many studies have been conducted whereby lignin genes have been down-regulated. This is presented in the following paragraph.

Riboulet, Guillaumie et al. (2009) discovered that a majority of lignin biosynthetic specific genes of PAL, 4CL, COMT, CAD and peroxidases were highly expressed at the first stages of development in maize stems. In a number of alfalfa studies, specific enzymes have been down regulated. Down regulation of C3H (4-coumarate 3-hydroxylase) led to a shift in lignin profile and structure, resulting in improved digestibility by ruminants (Ralph, Akiyama et al. 2006). In a separate study in alfalfa, down regulating CAD (cinnamyl alcohol dehydrogenase) did not result in decreasing the amount of lignin, but did increase in *in situ* digestibility (Baucher, Bernard-vailhé et al. 1999). A down regulation of COMT (caffeic acid O-methyltransferase) in alfalfa and maize reduced lignin content by 30 % in both species (Boerjan, Ralph et al. 2003). In this case the most apparent effect was the reduction of the syringyl phenylpropanoid units and incorporation of 5-hydroxyconiferyl alcohol into the lignin molecules (Boerjan, Ralph et al. 2003).

In dicotyledonous tobacco, enzyme OMT (O-methyl transferase) led to a higher biomass yield without decreasing the overall lignin content (Blaschke, Legrand et al. 2004). When 4CL (4-coumarate CoA ligase) was down regulated in aspen, this resulted in 45 % decrease lignin and 15 % increase in cellulose content (Hu, Harding et al. 1999). When CCR (cinnamoyl CoA reductase) was downregulated in tobacco, it resulted in a decreased lignin content and increased xylose and glucose levels of the cell wall (Chabannes, Barakate et al. 2001). Down regulation of PAL (phenylalanine ammonia-lyase) and C4H (cinnamate 4-hydroxylase) largely decreased lignin content in tobacco (Elkind, Edwards et al. 1990; Sewalt, Ni et al. 1997). PAL reduced mainly guaiacyl units, whilst C4H decreased syringyl units in lignin, which is apparent when studying Figure 11. Boerjan et al. (2003) mention three possible reasons why this takes place, such as that C4H may not be part of the metabolic route of syringyl lignin. In an expression study of lignin biosynthesis in Norway spruce, PAL and COMT did not show differential expression levels in different tissue types (Koutaniemi, Warinowski et al. 2007). Furthermore, Koutaniemi et al. (2007), indicate that certain peroxidases and laccases show differential expression levels in 1 and 40 year old trees; hence suggesting that expression of this gene affects maturation of this tree.

Down regulating CCoAOMT (caffeoyl CoA 3-O-methyltransferase) and COMT (caffeic acid 3-O-methyltransferase) in alfalfa led to higher efficiencies of acid pre-treatment and enzymatic hydrolysis of dry, milled, extractive free ligno-cellulosic residue (Chen and Dixon 2007). Suppressing genes at the beginning of the pathway is most effective in reducing lignin content (Chapple, Ladisch et al. 2007).

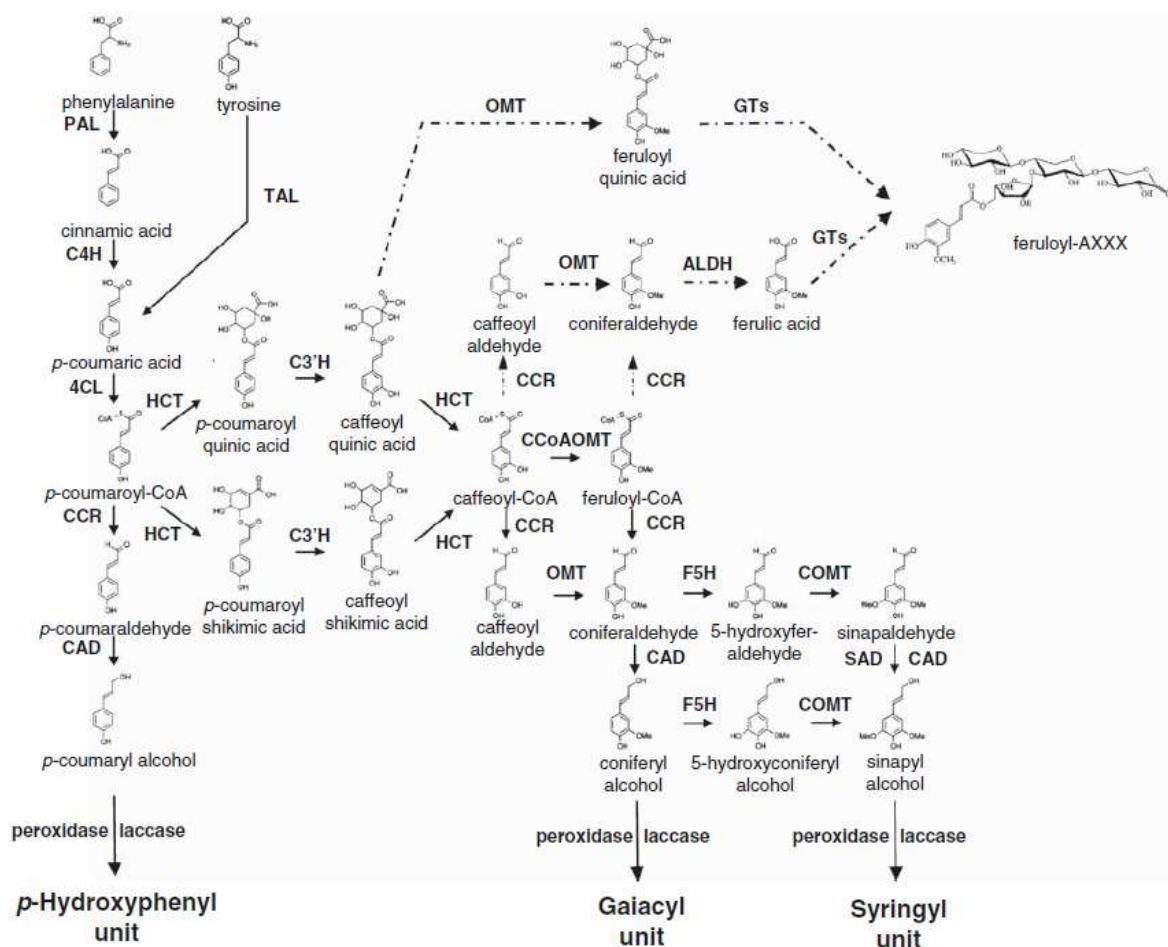


Figure 11. A putative biosynthetic pathway of monolignols, lignins and ferulic acid in maize and grasses (Barrière, Méchin et al. 2009).

2.3 Plant cell wall components and bio-ethanol production

The main monosaccharides in plant tissues are glucose and xylose and can be used to produce biofuels. Through a fermentation process (Figure 12), these monosaccharides can be converted into bio-ethanol similarly to the conversion process of starch in bio-ethanol.

Cellulosic ethanol is produced by pre-treating pulverized biomass with extreme heat and/or chemicals (e.g. acid), thus disrupting the association of lignin (Figure 10) with polysaccharides and partly hydrolyses hemicellulose and other polysaccharides (Sticklen 2008). After separating the hydrolysate from insolubles, it is fermented by yeast or bacteria into ethanol. The insoluble part is treated with cellulase and glycosidases to free glucose which is also fermented to make ethanol. The pre-treatment costs and cellulases increase the total costs of cellulosic ethanol by factor two to three, when compared to corn grain ethanol (Sticklen 2008). What is left over (mostly lignin) is burned to produce heat for use in the process. A current drawback in hydrolyzing cellulose is the vast amount of cellulase required in the process. Discovering novel enzymes (e.g. originating from termite guts and

rumen) with a higher turnover of current cellulases is certainly interesting for innovation. Making various steps in the bio-ethanol production more efficient, will lead to cost effective cellulosic bio-fuels which are less expensive than liquid fossil fuels (Somerville 2007).

2.4 Processing lignocellulosic biomass into bio-ethanol

Although lignocellulosic biomass is the most promising feedstock for bio-ethanol production, large-scale commercial production plants are not in use yet in the US or EU because of the high production costs (Somerville 2007; Balat, Balat et al. 2008; Sticklen 2008). However, large-scale implementations are underway in the US and will be operating in the near future (Lin and Tanaka 2006; Sticklen 2008). A lignocellulosic feedstock biorefinery transforms biomass (e.g. straw or stover), consisting mainly of polysaccharides and lignin, into a variety of energy and chemical products (Clark and Deswarte 2008). Figure 12 represents a schematic overview of steps in converting lignocellulosic into bio-ethanol.

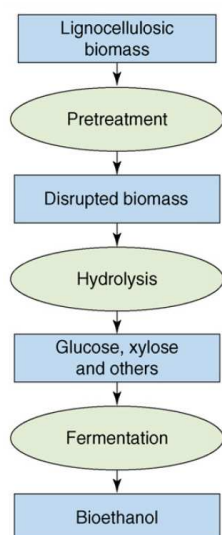


Figure 12. Processing platform of lignocellulosic biomass (Yuan, Tiller et al. 2008).

Processing lignocellulose into bio-ethanol occurs in four main steps: pre-treatment, hydrolysis, fermentation and product separation/distillation. Pretreatment is the first step in order to reduce the size of the lignocellulosic material, hence increasing the potential rate of hydrolysis in cellulose and hemicellulose (Mosier, Wyman et al. 2005). In this stage the lignocellulosic matrix is physically disrupted, which can be accomplished by different means (e.g. chemical (acid), biological and mechanical). The second step is hydrolysis: $(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6$. The third step is fermentation of sugars into ethanol. The lignocellulosic sugars come in different forms, such as glucose, xylose, mannose, galactose, arabinose and oligosaccharides. Xylose and glucose are transformed into ethanol and carbon dioxide in the following way respectively: $3C_5H_{10}O_5 \rightarrow$

$5\text{C}_2\text{H}_5\text{OH} + 5\text{CO}_2, \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{C}_2\text{H}_5\text{OH} + 2\text{CO}_2$. The fourth and final step involves a distillation step (separate ethanol from water) and removing solids in the mixture. The lignin residue is removed during the hydrolysis in the process and may be used for heat production or serve other purposes (Arshadi and Sellstedt 2008).

A major limitation in converting lignocellulosic biomass into ethanol is its recalcitrance to saccharification. By decreasing lignin contents in cell walls, biomass components can more easily be assessed by hydrolytic enzymes (Chen and Dixon 2007). Lignin modification could therefore bypass or improve the efficiency of the pre-treatment step in the process of converting lignocellulosic biomass into ethanol (see Figure 12). The production of affordable ethanol from plants is based on the knowledge of the cell wall composition and assembly (Barrière, Méchin et al. 2009).

3. Research rationale and scope

Chapter 3 describes the rationale and scope of this study. First the research goal (Section 3.1) and objective (Section 3.2) are addressed. Next, the main research question (Section 3.3) and sub-research questions (Section 3.4) are presented.

3.1 Research goal

This research project intends to contribute to the following research goal: *to develop cost effective, renewable and sustainable bio-energy crops in order to replace significant quantities of fossil based fuels necessary in society.*

3.2 Research objective

In order to contribute to the research goal, the research objective is defined as follows: *to improve the yield and fermentability of Miscanthus biomass, by investigating the morphological variation and molecular aspects of cell wall biosynthesis in stem internodes.*

3.3 Main research question

The main research question is stated as follows: *how does the stem and internode structure, biochemical composition and gene expression relate to cell wall biosynthesis changes across different developmental stages in Miscanthus?*

3.4 Sub-research questions

- 1 Do the genotypes show variation in terms of morphological characteristics of the stems and internodes?
- 2 Does a relationship (i.e. correlation) exist between morphological characteristics of the stem and internodes?
- 3 How does the biochemical composition of the stem internodes of the genotypes (i.e. cell walls) change over time?
- 4 Does the expression of cell wall biosynthesis genes change over time?

- 5 Do the genotypes show differences in terms of expression of cell wall biosynthesis genes across time?
- 6 Does the expression of cell wall biosynthesis genes differ between sections (top, middle & basal) of the stem internodes?
- 7 Does a relationship (i.e. correlation) exist between gene expression and biochemical composition of cell walls?

4. Methodology

Chapter 4 describes the materials and methods applied in this study. First, Section 4.1 describes the plant material and Section 4.1.1 the greenhouse conditions. Section 4.2 presents the methodology used to describe the morphology of the field grown genotypes. Section 4.3 and Section 4.4 depict the approach of the gene expression and biochemical analyses respectively. Section 4.5 elucidates the statistical methods. Finally Section 4.6 illustrates an overview of conceptual framework.

4.1 Plant material

A collection of *M. sinensis* (N=17), *M. sacchariflorus* (N=1), *M. x giganteus* (N=1) and inter-specific hybrid (N=1) genotypes were used in the study. This diverse set of genotypes was obtained from a breeding programme at Wageningen University. This particular set of genotypes was chosen due to their variation in length, stem thickness and density of stems. Several biochemical characteristics had already been assessed from field grown genotypes (except No. 20) (Table 2). Appendix 9.2 provides further details on the biochemical content and breeding codes of the genotypes. Within the breeding programme the genotypes had spent their existence in trial fields and/or greenhouses for a number of years. In terms of genetics the genotypes are highly heterozygous.

Table 2. *Miscanthus* genotypes with corresponding biochemical composition.

*Serial No.	Species	NDF (%)	ADF (%)	pLIG (%)	Hemicellulose (%)	Cellulose (%)	^W Ash (mg/g)
1	<i>Interspec hybrid</i>	86	54	15	32	39	5.3
2	<i>M. sinensis</i>	87	56	17	31	39	8.97
3	<i>M. sinensis</i>	86	57	16	29	42	8.68
4	<i>M. sinensis</i>	85	57	15	28	42	9.67
5	<i>M. sinensis</i>	85	56	16	29	40	6.18
6	<i>M. sinensis</i>	83	57	16	26	41	6.19
7	<i>M. sinensis</i>	82	53	20	29	33	7.03
8	<i>M. sinensis</i>	83	54	18	29	36	4.86
9	<i>M. sinensis</i>	81	53	17	27	37	5.24
10	<i>M. sinensis</i>	82	54	16	28	38	6.37
11	<i>M. x giganteus</i>	87	67	12	20	55	4.44
12	<i>M. sinensis</i>	86	59	13	27	47	5.76
13	<i>M. sinensis</i>	84	55	15	29	40	5.05
14	<i>M. sinensis</i>	83	55	15	29	40	6.57
15	<i>M. sinensis</i>	84	56	18	28	38	6.4
16	<i>M. sinensis</i>	85	60	15	25	45	6.88
17	<i>M. sinensis</i>	82	53	14	29	39	5.37
18	<i>M. sinensis</i>	85	55	15	29	40	8.16
19	<i>M. sinensis</i>	83	56	18	27	38	5.42
20	<i>M. sacchariflorus</i>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Key: NDF=Neutral Detergent Fibre, ADF=Acid Detergent Fibre, pLIG=Permanganate Lignin, Hem=Hemicellulose, Cel=Cellulose.
DM=Dry Matter, n.d.=no data.

Bold: 6x genotypes selected for gene expression and biochemical analyses in this particular study.

* Genotypes 1 to 10 had spent part of their existence in a greenhouse; whilst others were field grown (nr's 11 to 20).

[‡] Ash is sum of K, Na, Cl, Mg, Ca PO₄ & SO₄.

Pre-selection of the genotypes

Within this study, two separate sets of genotypes from Table 2 were utilized. The first set was used for the morphological study and consisted of all 20 genotypes cultivated on field plots. The second set (applied in the gene expression and biochemical analyses) comprised of the six greenhouse grown genotypes 4, 7, 9, 14, 15 and 16.

The 20 genotypes of the morphology study were grown on field plots at Wageningen University from 2009 to 2010. The stems were harvested on 19 January 2010 by cutting the stems off the rhizomes at the ground level. Subsequently, these were dried by a warm air blower for six days (25 – 27°C, 24hrs day⁻¹). Finally, the mature stems/internodes were subjected to morphological analysis (Section 4.2).

The six genotypes 4, 7, 9, 14, 15 and 16 were used for both the gene expression and biochemical analyses. This set was chosen due to their contrasting levels of cellulose and pLIG contents. Replicated rhizomes (N=2) were collected from field plots on 19 January 2010. These were subsequently placed in 7 litre pots and grown in a greenhouse in a double randomized block design (Appendix 9.3) from 19 January until 16 June 2010. The samples for gene expression and biochemical analyses consisted of tissue from second stem internode (counting from the rhizome). This particular material was used because it grew relatively fast at early stages of development, thus providing more biological material than other internodes of the same stem. The internodes were harvested periodically from the moment that the internodes reached a sufficient length to work with (i.e. enough stem material for RNA extraction). This was done due to the fact that expression of multiple genes involved in lignin bio-synthesis are expressed relatively high at early stages of development (stage of silking) in maize (Riboulet, Guillaumie et al. 2009). The replicated sets of internodes were harvested at four moments in time (3 - 5 March, 6 - 9 April and 3 - 7 May and 16 June 2010) and directly frozen in liquid nitrogen. In an attempt to synchronize the developmental stages of the harvested internodes, the number of visible leaves on the stems was used as a developmental marker (Appendix 9.4). Due to a limiting number of internodes growing from the rhizomes, only a single stem was collect at each time of harvest. The second internode was cut longitudinally in half and transversely into three separate sections in the second to fourth stage of development (Figure 14). The harvested internodes of the first stage of development of genotypes 4, 7, 9 and 16 were too small to be split up into separate parts. Therefore the tissue of the complete second internode was used for the gene expression analyses.

4.1.1 Greenhouse conditions

In the greenhouse the average temperatures and relative humidity (RH) ranged between 16 - 22 °C and 60 - 70 % (Figure 13); fluctuations in average RH were considerable. The plants received two water and fertilizer treatments per week. During January to February the plants received extra lighting. In May, an infection (i.e. etiolated leaves) within the genotypes was observed (assumeably due to *Pythium sp.*). There after, the plants were treated with fungicide Aliette® (Bayer CropScience). Slowly the leaves turned back to their normal green colour.

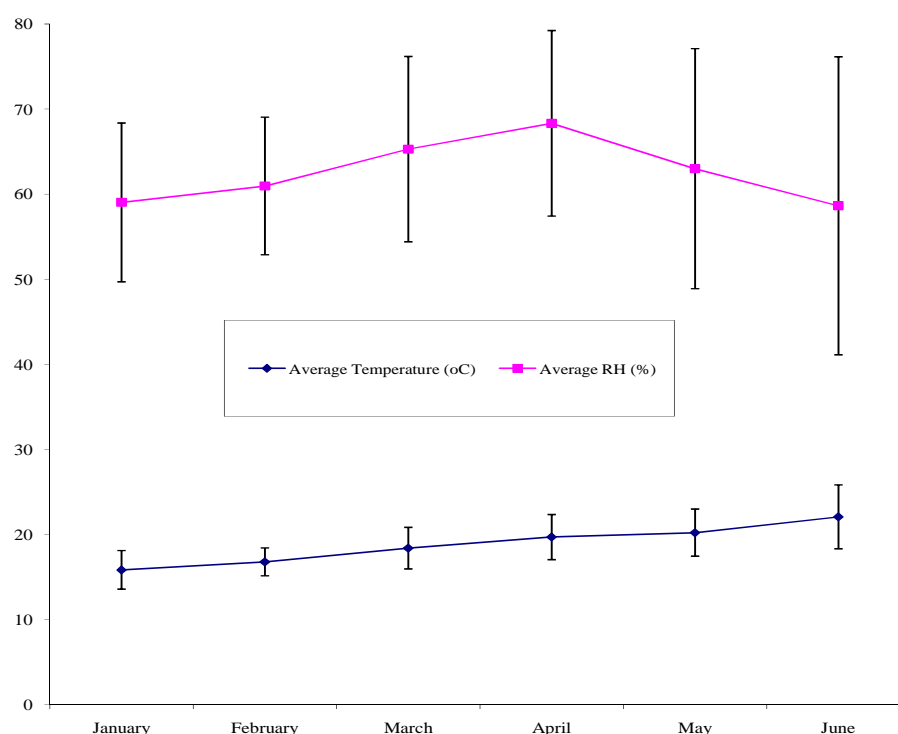


Figure 13. Average temperature and relative humidity (RH) in the greenhouse with S.D. bars.

4.2 Characterization of stem and internode morphology

The morphology of the stems and the internodes were characterized in terms of mass, length and diameter. The diameter of the stems was measured at the centre of the internodes. Because the stems were slightly oval, the stem diameter was recorded at the narrowest distance. The samples (per genotype) consisted of a batch of approx. 20 stems. From this batch, three stems were randomly picked out for subsequent quantification. Prior to taking the measurements, the leaves were completely stripped off the stems. Measurement data was collected from a gravimetric scale and measurement tape.

4.3 Gene expression analysis

Section 4.3 illustrates the molecular methods applied needed to conduct the qPCR. Section 4.3.1 presents the choice of the candidate genes. Section 4.3.2 illustrates the materials used for conducting the gene expression analysis.

4.3.1 Choice of candidate genes

The expression PAL, 4CL, LAC, CESA, β -tub and GADPH were monitored by means of qPCR. These genes were selected due to their putative pivotal location (i.e. di-/convergent sections) in the lignin biosynthetic pathway (except for CESA). CESA was included due to its role in cellulose synthesis of the cell walls. The primers of CESA were readily available from previous research at Wageningen University. The primers of PAL were designed from an available *Miscanthus* sequence fragment at Wageningen University. The qPCR primers of 4CL and LAC were developed by designing initial primers from aligned sequences of these genes in related species (e.g. maize and rice), isolating the DNA fragment, sequencing the fragment and finally designing the qPCR primers in exons regions. Primers of the reference genes β -tubulin and GADPH were available from literature (Iskandar, Simpson et al. 2004). Moreover, earlier *Miscanthus* research at Wageningen University confirmed that these primers of β -tubulin and GADPH were functional within the genotypes.

Within the lignin biosynthetic pathway, a failed attempt was made to develop qPCR primers for TAL, HCT, C3'H, COMT and CAD. Functional primers could not be developed for the isolation of PCR fragments using Genbank sequence data (www.ncbi.nlm.nih.gov).

4.3.2 Materials used for gene expression

Section 4.3.2 describes the methods of DNA extraction, fragment isolation and cloning, sequencing, development of primers, RNA extraction, cDNA synthesis and the qPCR approach.

DNA extraction

DNA was extracted for the purpose of testing primers and necessary for the isolation of the putative fragments of 4CL and LAC. In order to carry out DNA isolation, young leaf material was collected from 5 week old plants (pooled across the replicated genotypes: N=2). The harvested leaves were directly frozen in liquid nitrogen and stored at -80°C. A modified version Tanksley's method (Fulton,

Chunwongse et al. 1995) was carried out to isolate the DNA from the leaf material (Appendix 9.5). After isolation, the DNA quality was inspected on electrophoresis agarose gel. Moreover, a spectrophotometer (Nanodrop ND-1000, ISOFEN Life-Science) was used to quantify the DNA concentrations. Absorbance ratios of ~1.8 and 1.8 – 2.2 for 260/280nm and 260/230nm respectively were used to determine the purity and quality of the DNA samples. The DNA samples were diluted with Milli-Q water to generate working solutions with concentrations of 25ng/μl.

Fragment isolation and cloning

Putative fragments of 4CL and LAC were needed for sequencing and development of qPCR primers. The fragments were synthesized in a PCR using Phusion® High-Fidelity DNA Polymerase, (cat. No. F-530S, Finnzymes Oy.). Phusion® polymerase was used due to its high accuracy (i.e. low error rate). After the addition of gelred, the DNA fragments lighted up in agarose gel by UV light and were subsequently removed from the gel by means of a razor blade. In the case of 4CL, two fragments bands were observed under the UV light and the upper band was selected; LAC showed multiple bands and the brightest band was chosen. The fragments were extracted from the gel by using MinElute® gel extraction kit (QIAGEN). Subsequently, poly-A tails were added to the fragments (Appendix 9.6) and were purified with DNA Clean & Concentrator™-5 (Zymo Research, cat. No. D4004).

Cloning was performed by inserting the fragments into ultra-competent *E. coli* cells (XL10-Gold Ultracompetent Cells, Stratagene) using the 3kB pGEM-T Easy Vector (by Progema) (see Appendix 9.6 for details). Due to the size of the fragments (~700bp), a ligation mix with 1:3 ratio (vector:insert) was used. The competent cells were cultivated (overnight at 37°C) in LB-ampicillin agar plates. Next, the positively transformed (white stained) cells were multiplied through incubation (overnight at 37°C) in 120 μl LB medium to be used in a PCR which is needed for the sequence reactions.

Sequencing

Sequencing was performed in order to design qPCR primers based on *Miscanthus* DNA. A forward and reverse PCR sequence reaction (see Appendix 9.7 for details) was carried out on the cloned fragments (Section 4.3.2) of 4CL and LAC. Before carrying out the sequence reaction, the fragments of the PCR were cleaned using Sephadex (Appendix 9.8). The forward and reverse fragments were sent to Greenomics (Wageningen UR) for sequencing. The sequences of 4CL and LAC were aligned using software (DNASar Lasergene v.8 – Seqman) and compared (BlastX procedure) to NCBI Genbank (www.ncbi.nlm.nih.gov) data. This was done in order to confirm the (putative) identity of the gene (

Table 7 in Section 5.2.1). The exons of consensus sequence of 4CL and LAC were subsequently used for developing qPCR primers.

Development of primers

Two sets of primers were developed in this study: (1) initial primers – for the isolation of the putative gene fragments needed for sequencing and (2) qPCR primers.

The initial primers (Table 3) were designed from aligned sequences of 4CL and LAC of related *Miscanthus* species (e.g. rice, maize, sorghum and other grasses) utilizing NCBI Genbank data (www.ncbi.nlm.nih.gov). Consensus contigs were made using software (Seqman, Lasergene package v.8, DNASTar Inc.). Primers were designed using software (Gene Runner v3.05, Hastings Software Inc.).

qPCR primers were designed from exons of the consensus fragments of the sequences. Table 5 (in this section) provides an overview of the primers used in the qPCR experiments.

Table 3. Overview of initial primers used for fragment amplification.

Gene	Abbr.	Function	Orientation	Primer sequence	Melting T (°C)	Size (bp)
Laccase *	LAC	Lignin biosynthesis	F	5-TCAACKCGCACCTCTTCTTC-3	60	~650
			R	3-CTGSAGCACGAYCTCCACC-5	64	
4-coumarate:	4CL	Lignin biosynthesis	F	5-GGACAGGGBTAYGGGATG-3	61	~800
CoA ligase §			R	3-GWAGAYCTTGTGCAGCCTC-5	58	

* LAC based on alignment of NCBI accessions: AY897209, AY897208, AF465470 and BQ739797. § 4CL based on alignment of NCBI accessions: AX204867, AX204867, BT034270, NM_001068470 and AF052221.

RNA extraction

mRNA was extracted for the purpose of synthesizing cDNA. Total RNA was extracted from three separate sections (i.e. top, middle and basal) of the second stem internode (counting from the rhizome upwards). Replicated internode sections (N=2) were pooled. The bast and stem pith tissue were not separated from each other because it is rather difficult to separate these. At the first stage of development the internodes of genotypes 4, 7, 9 and 16 were too small to be split up. Therefore, the complete internodes of these genotypes were used for RNA extraction. The internodes were cut and divided as shown Figure 14 (except for genotypes 4, 7, 9 and 16 at the first stage of development). In an attempt to increase discrimination between the three internode sections, a small part (2-5mm) was removed in between the sections (for RNA extraction). An adapted CTAB-based method was applied to isolate RNA from the stem material (Appendix 9.9). Validation of successful RNA isolation was done by inspecting the presence of typical RNA bands on electrophoresis agar gel. Furthermore,

spectrophotometer (Nanodrop ND-1000 of ISOFEN Life-Science) quantification was carried out to determine the final RNA concentrations at an OD 260/280 ratio.

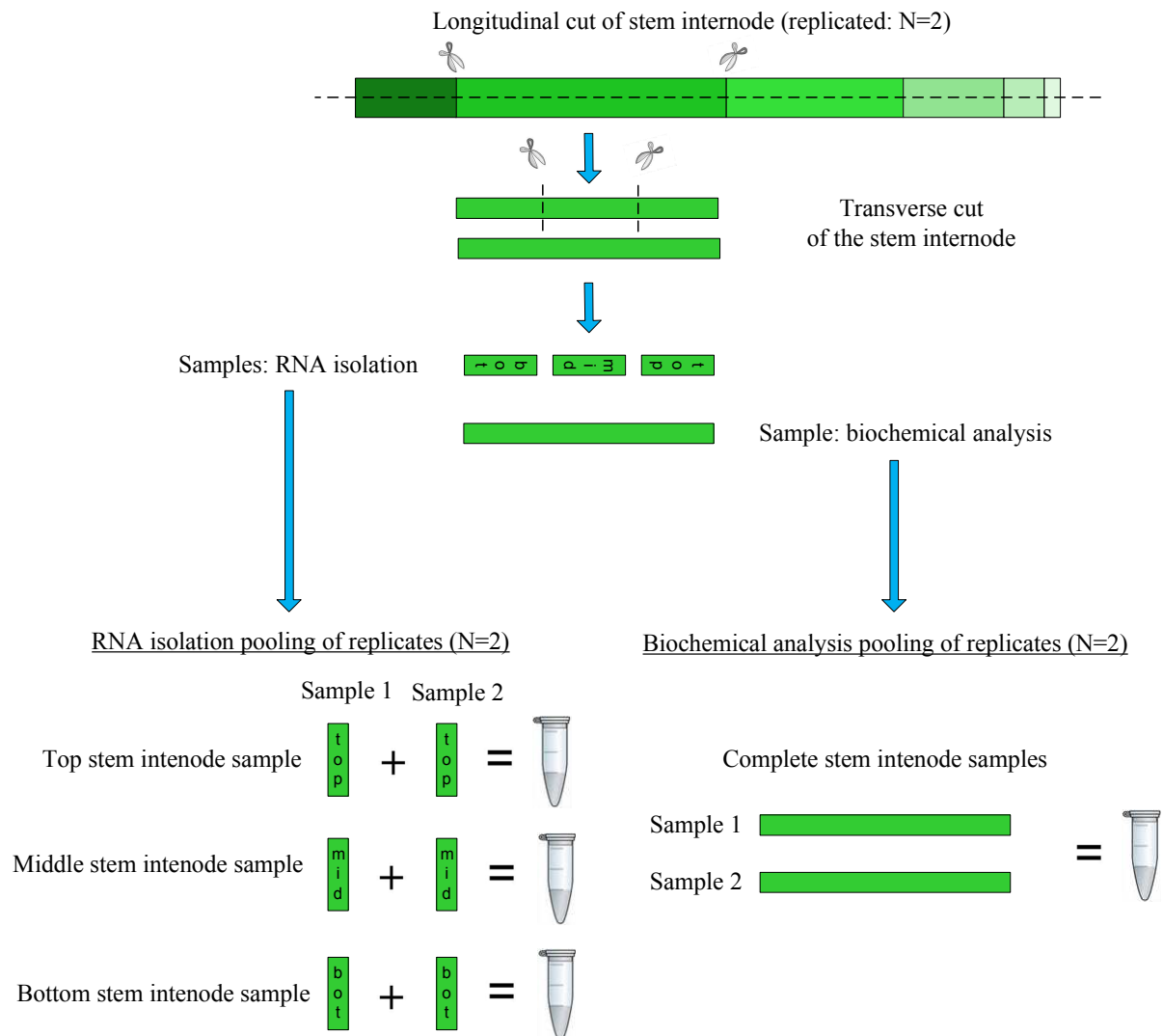


Figure 14. Separation and pooling of stem internode tissue.

Note: the internodes of genotypes 4, 7, 9 and 16 at the first stage of development were not split into three sections due to limiting biological sample material.

cDNA synthesis

First, the RNA samples were treated with DNase I Amplification Grade kit (cat. no. 18068-015 by Invitrogen) to remove all DNA. This was confirmed by a PCR using the RNA DNase treated samples as template. Next, cDNA was constructed by applying the iScript cDNA Synthesis Kit (cat. no. 170-8890 by Bio-Rad Laboratories, Inc).

Quantitative PCR (qPCR)

Gene expression was measured by means of the qPCR. Expression levels of the genes PAL, 4CL, LAC and CESA were compared to the candidate reference genes: GADPH and β -tubulin. This comparison was made by means of the Δ Ct-calculation method (i.e. Δ Ct-value = reference Ct-value – sample Ct-value). To minimize the effect caused by the amount of cDNA in the samples, the average transcript level of each sample were normalized to the level of the reference genes. Expression levels were measured by using the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc). Table 4 presents the iQ-SYBR® Green supermix qPCR mix (Bio-Rad). The qPCR programme was as follows: 95°C 3', (95 °C 15'', 60 °C 1') x 40, 95 °C 10'', 65 °C 5'', 95 °C 5''.

Table 4. qPCR mix per well

cDNA (60 ng/ μ l)	2 μ l
Buffer (iQ-SYBR® Green supermix)	5 μ l
Forward primer (10 pmol/ μ l)	0.3 μ l
Reverse primer (10 pmol/ μ l)	0.3 μ l
MQ	2.4 μ l
Total volume	10 μ l

The candidate reference genes were analyzed together with the samples in duplo (N=2) on the same qPCR plate. The samples were derived from a single mastermix tube (buffer and MQ). The mastermix was divided into six new tubes (for each gene) and the primers were added. Next, the content of each tube (buffer, MQ and primers) was divided into several new tubes and the cDNA samples were added. Finally, the duplo samples were pipetted from this tube into the qPCR plate.

qPCR primers

Table 5 presents the genes with subsequent primers applied in the qPCR. Four cell wall biosynthesis genes were investigated (i.e. lignin: PAL, 4CL and LAC; cellulose: CESA), along with two endogenous reference genes (β -tubulin and GADPH).

Table 5. Overview of primers applied in the qPCR.

Gene	Abbr.	Function	Orientation	Primer sequence	Melting T (°C)	Size (bp)
Phenylalanine- ammonia-lyase #	PAL	Lignin biosynthesis	F	5-GCCATGGCGTCCTACTGCT-3	55	110
			R	3-ACGTCCTGGTTGTGCTGCTC-5	55	
4-coumarate: CoA ligase †	4CL	Lignin biosynthesis	F	5-ACTTACGGGACGACGGCG-3	56	120
			R	3-CATCGTCGACCGGCTCAAG-5	56	
Laccase †	LAC	Lignin biosynthesis	F	5-CAGCACCGCCATGTAGTAG-3	49	90
			R	3-GGACACGCTCGTTCTCGC-5	54	
Cellulose synthase •	CESA	Cellulose biosynthesis	F	5-GAACCGCACACCAACAATC-3	51	200
			R	3-AGCCTCTTCAAATAATAGTCACG-5	50	
β-tubulin Δ	β-tub	Reference gene	F	5-CCAAGTTCTGGGAGGTGATCTG-3	55	130
			R	3-TTGTAGTAGACGTTGATGCGCTC-5	54	
Glyceraldehyde- 3-phosphate- dehydrogenase Δ	GADPH	Reference gene	F	5-CACGGCCACTGGAAGCA-3	53	130
			R	3-TCCTCAGGGTTCCTGATGCC-5	56	

Key: # Primers developed from available gene sequence contig at Wageningen University. † Primers developed through bioinformatics and sequencing in this particular project • Primers available from Wageningen University. Δ Primers from study (Iskandar, Simpson et al. 2004).

4.4 Biochemical analysis

The biochemical analyses were carried out in order to quantify the cellulose, lignin and ash contents of the second stem internodes of genotypes 4, 7, 9, 14, 15 and 16. This information was needed for the analogy between the gene expression and biochemical components of these genes. The sequential method (Goering and Van Soest 1970) using the ANKOM 2000 Fiber Analyzer (ANKOM Corporation, USA) with the filter bag system was applied. The acid detergent fibre (ADF), permanganate lignin (pLIG) and total ash contents were determined by carrying out the modified protocol of ANKOM (Torres Salvador 2010). ADF is the sum of cellulose, lignin and ash contents in the samples.

Due to a limiting amount of biomass of the samples, most samples could not be replicated (except genotype 16 at the 3rd and 4th stage of development: N=2). Only genotypes 14 and 15 were analyzed at the first stage of development. Many samples weighed less than the minimal mass (0.45g) specified in the protocol (Appendix 9.10).

4.5 Analogy of gene expression with biochemical content

A comparison between gene expression and biochemical contents was made in an attempt to discover a relationship between expression PAL, 4CL, LAC and CESA with their biochemical counterpart. This was done by comparing the ΔC_t -values with pLIG and cellulose content in the internodes. The comparison was not investigated statistically because only two stem internode samples could be replicated in the biochemical analyses (due to lacking amount of biological material).

4.6 Data analysis

Data analysis was carried out using the statistical software packages Genstat version 12.1.0 and PASW Statistics version 17.0. Analysis of variance (ANOVA) was carried out to investigate differences between the treatments. The significance (p-values) of the post-hoc tests are based on Bonferroni, Sidak and Tukey methods due to their differing levels of stringency. The relationship between data attributes was assessed by means of Pearson's correlation analysis. Threshold values were based on the cut off value of $\alpha=0.05$. Normality of the data was inspected by viewing the Q-Q plots and equality of variance was tested by means of Levene's test.

4.7 Conceptual framework

The conceptual framework of this study is presented in Figure 15. The process inside the large box represents structure of this study, whereas the small boxes (with green arrows pointing towards the large box) illustrate the potential factors of influence. The genotypes from the greenhouse were subjected to gene expression and biochemical analyses. The field grown genotypes were subjected to stem internode analysis.

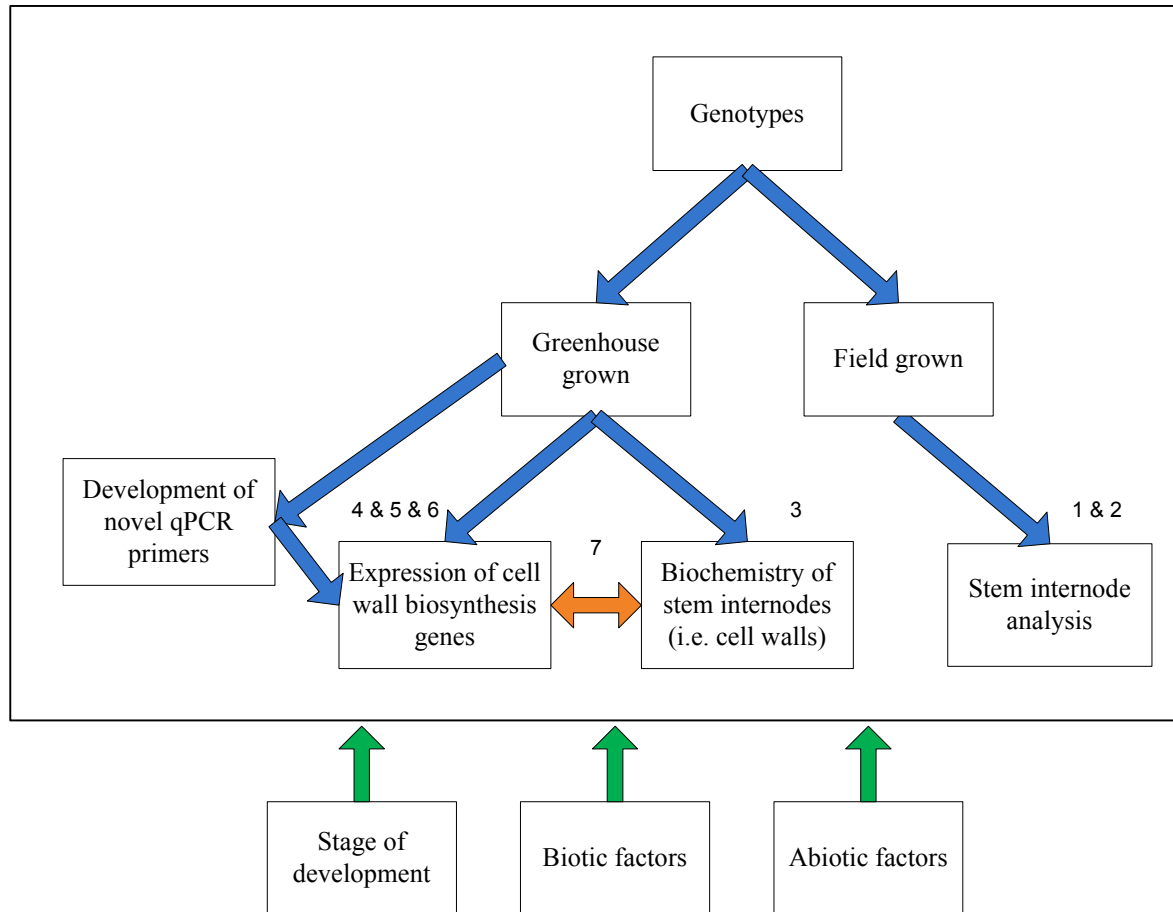


Figure 15. Conceptual framework of the research.

Blue arrows indicate a flow. The orange arrow indicates the correlation analysis. Green arrows indicate influential factors on cell wall biosynthesis. Numbers 1 – 7 indicate sub-research questions posed in Section 3.4.

5. Results and discussion

Chapter 5 presents the results and discussion of the experiments. Section 5.1 presents the results of the morphological investigation of the stems and internodes grown on field plots. Next, Section 5.2 and Section 5.3 depict the results and discussion on gene expression and biochemical composition of the greenhouse grown genotypes respectively. Finally, Section 5.4 presents the analogy between gene expression patterns and biochemical composition of the stem internodes.

5.1 Morphology of stems and stem internodes

Section 5.1 presents the results of the stems collected from the field plots. The stems were collected at the end of the growing season (see Section 4.1 for details). The results reflect upon the means and confidence interval bars (95%) of the stem mass, stem length, inflorescence length, number of internodes, internode length, internode mass and internode diameter. The results are presented in a descriptive manner and should not be used for drawing hard conclusions upon. Section 5.3.4 presents the discussion.

5.1.1 Stems

Section 5.1.1 presents the morphological characteristics of the stems of the twenty *Miscanthus* genotypes.

Figure 16 illustrates the mean mass of the stems (including the inflorescence which was not present on all genotypes). Genotype 11 (*M. x giganteus*) has the highest stem mass (43g). Stems of genotypes 9, 10, 16, 17 and 20 have a relatively high stem mass (>20g), whilst those of genotype 1, 6, 7, 12 and 13 have a relatively low mass (<10g). *M. sinensis* genotypes (2 – 10 & 12 – 19) show much variation in terms of this attribute; many of these genotypes differ significantly from each other (see 95% confidence interval bars).

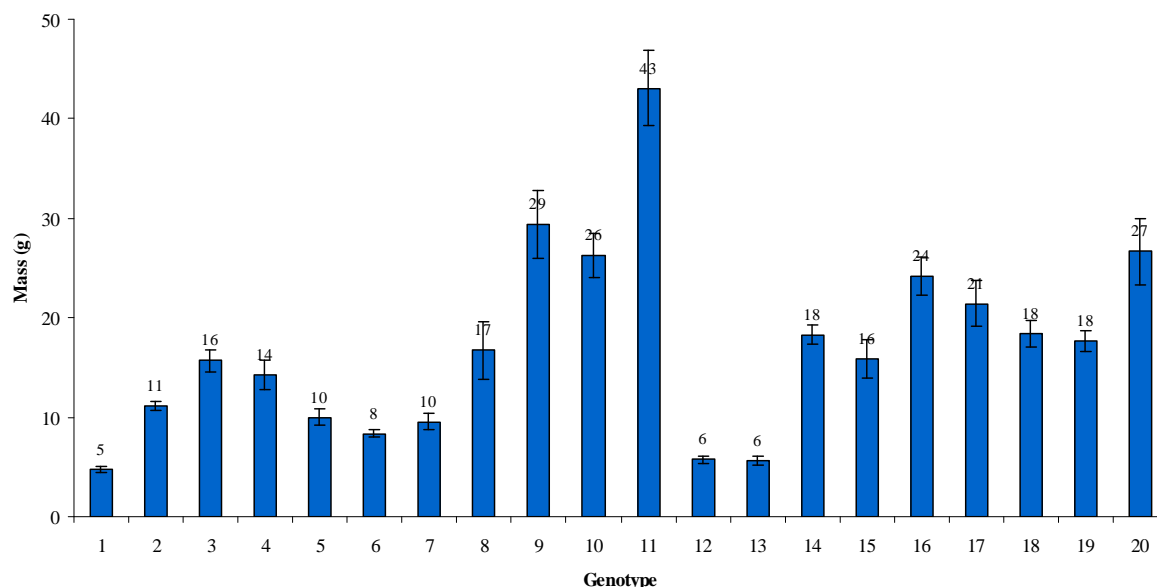


Figure 16. Mean stem dry mass including inflorescence with 95% C.I. bars of field lines.

Figure 17 illustrates the mean stem length (excluding the inflorescence). The mean stem length of approximately half of the genotype is less than 200 cm. Genotypes 9, 10, 17 and 18 have the longest stems, whilst genotypes 1, 6, 7, 12, 13, 16 and 20 have relatively shorter stems. *M. sinensis* genotypes (2 – 10 & 12 – 19) show much variation in terms of mean stem length; many of these genotypes differ significantly from each other (see 95% confidence interval bars).

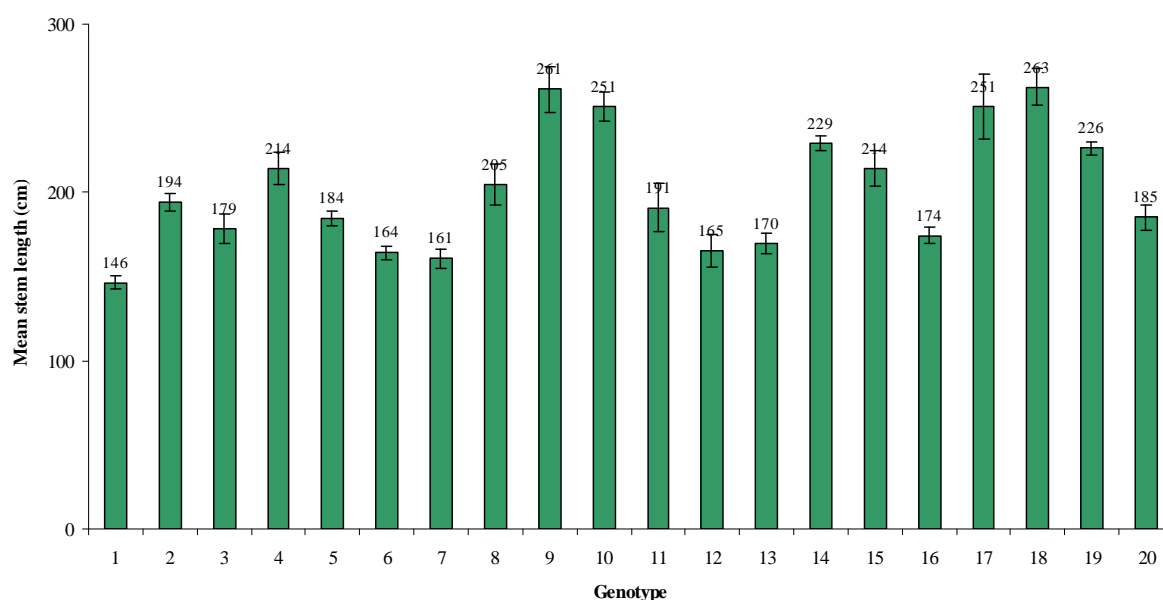


Figure 17. Mean stem length excluding inflorescence with 95% C.I. bars of field lines.

Figure 18 illustrates the mean inflorescence length of the genotypes. In genotypes 11, 16 and 20 the inflorescences were missing, presumably because they had broken off the main stem. The mean inflorescence length ranged between approximately 19 – 38 cm. Genotypes 9, 10 and 14 have the longest flowers, whilst genotypes 1, 12, 13 and 17 have relatively the shortest. *M. sinensis* genotypes (2 – 10, 12 – 15 and 17 – 19) show much variation in terms of mean inflorescence length; a number of these genotypes differ significantly from each other (see 95% confidence interval bars).

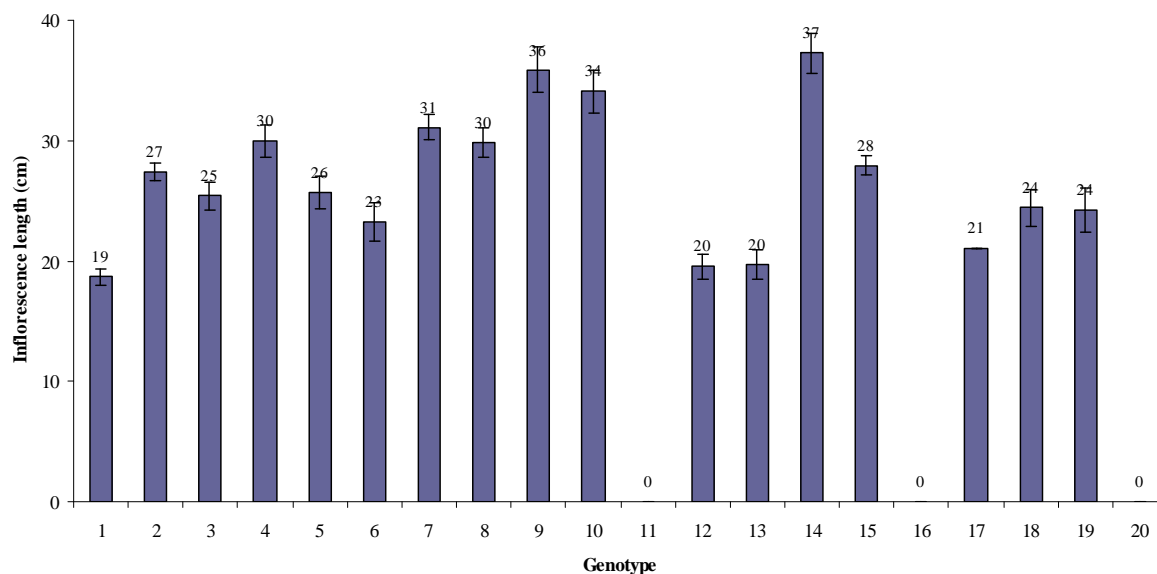


Figure 18. Mean length of inflorescences with 95% C.I. bars of field lines.

Note: genotype 11, 16 and 20 did not have inflorescences, presumably broken off the stems.

Figure 19 illustrates the mean number of internodes of the stems. Genotypes 17 and 20 have a high number of internodes, whilst genotypes 2, 7, 8, 12 and 13 have the least. *M. sinensis* genotypes (2 – 10 & 12 – 19) show much variation in terms of mean number of internodes; many of these genotypes differ significantly from each other (see 95% confidence interval bars).

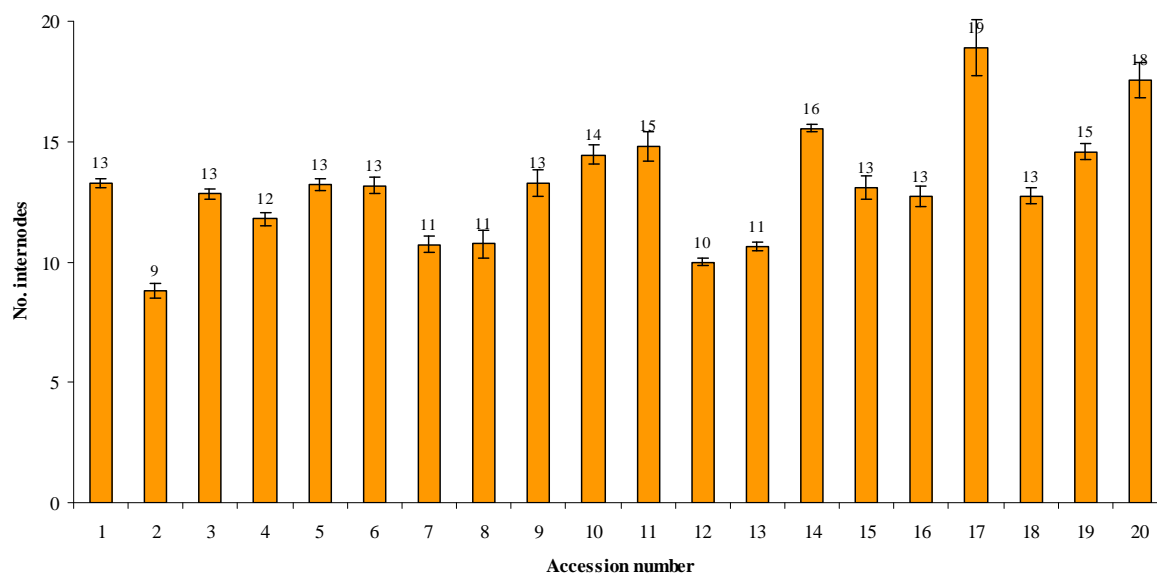


Figure 19. Mean number of internodes with 95% C.I. bars of field lines.

5.1.2 Internodes

Section 5.1.2 presents the results of the stem internodes (length, mass and diameter). The results per genotype are based on the data of three randomly sampled stems (N=3). Therefore, the results are presented in a descriptive manner.

Figure 20 presents the mean length of the internodes. Genotypes 2, 8, 9, 10, 15 and 18 have relatively long internodes, whilst genotypes 1 and 20 score low on this characteristic. *M. sinensis* genotypes (2 – 10 & 12 – 19) show much variation in terms of mean number of internodes; a number of these genotypes differ significantly from each other (see 95% confidence interval bars).

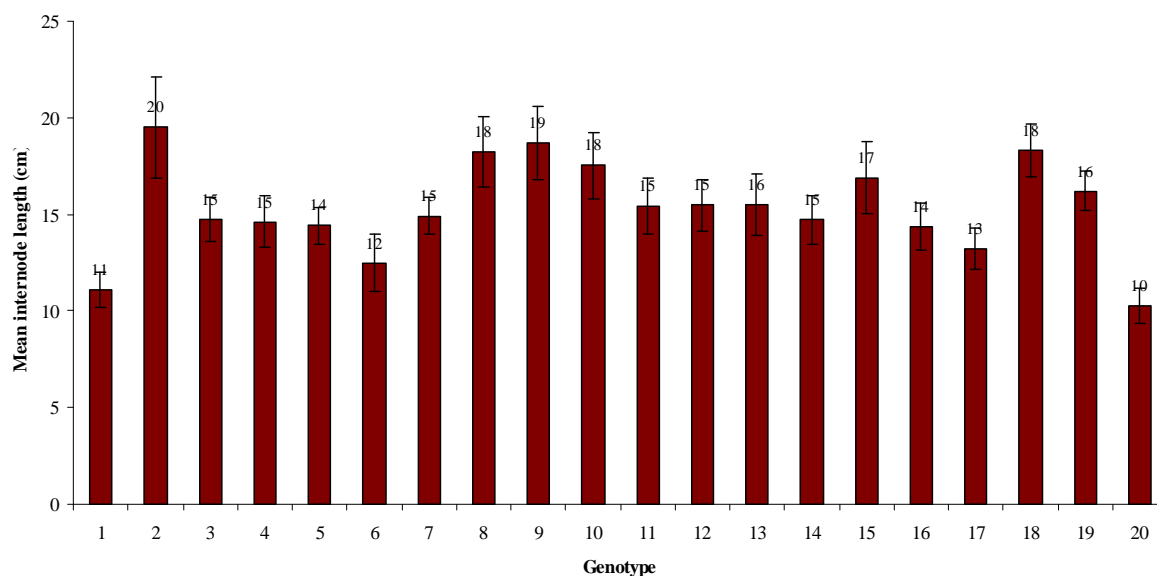


Figure 20. Mean internode length with 95% C.I. bars of field lines.

Figure 21 presents the mean mass of the genotype internodes. It is evident there are ample differences between the genotypes. The internodes of genotype 1 (interspecific hybrid) weights the least whilst genotype 11 (*M. x giganteus*) the highest.

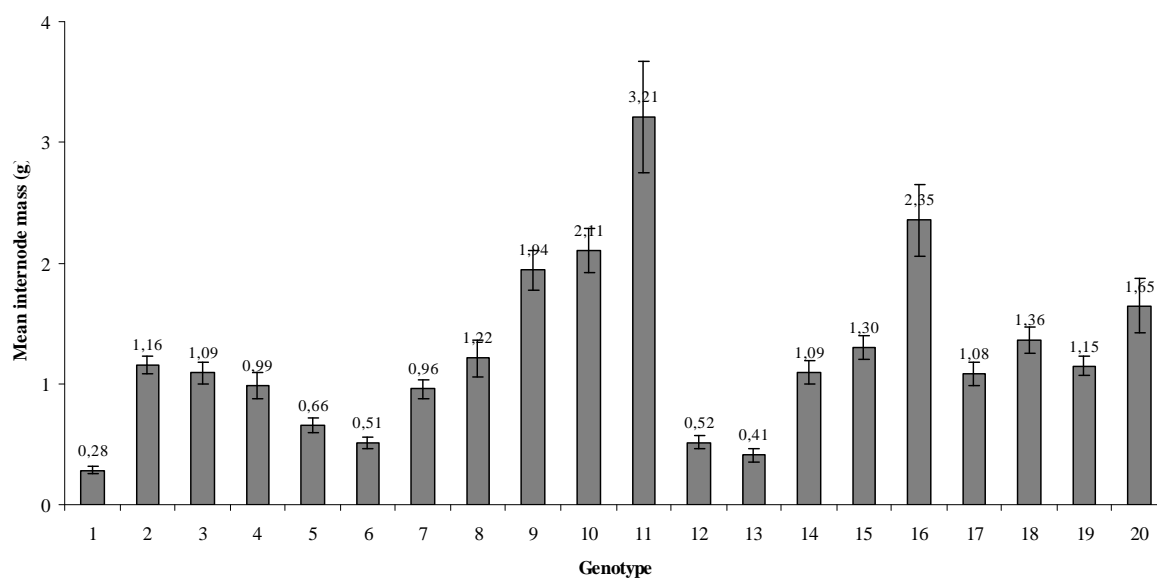


Figure 21. Mean mass of dry internodes with 95% C.I. bars.

Figure 22 illustrates the mean diameter of the internodes. Genotypes 9, 10, 11 and 16 have a relatively large diameter, whilst genotypes 1, 12 and 13 score lower on this characteristic.

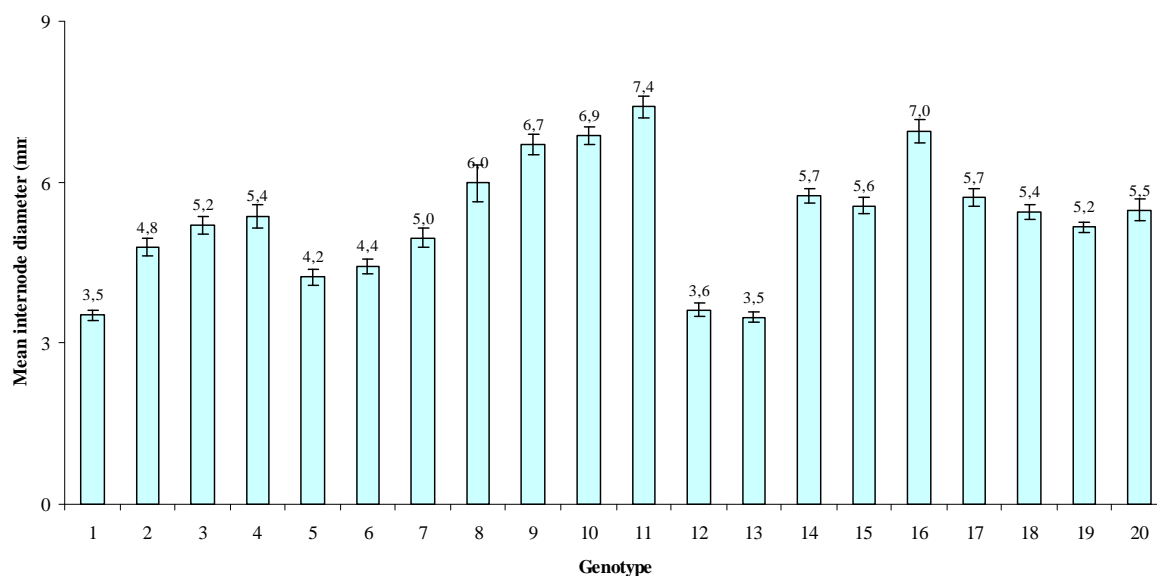


Figure 22. Mean diameter of internodes with 95% C.I. bars.

5.1.3 Correlations between the morphological characteristics

Table 6 presents the inter-item correlations between the stem and internode characteristics. Nine significant correlations are found between the attributes and ranged between -.5 and .94. Relatively high correlations (>.9) are found between: stem mass - internode mass, stem mass - internode diameter and internode mass - internode diameter. A negative correlation (-.5) was found between no. internodes & internode length.

Table 6. Correlations between stem and internode characteristics for all genotypes (N=20).

	Stem mass	Stem length	Flower length	No. internodes	Internode mass	Internode length
Stem length	.48					
Sig.	.030					
Flower length	-.36	.41				
Sig.	.121	.068				
No internodes	.53	.39	-.27			
Sig.	.015	.087	.259			
Internode mass	.94	.36	-.40	.30		
Sig.	<.001	.117	.082	.194		
Internode length	.14	.52	.50	-.50	.25	
Sig.	.559	.018	.026	.026	.281	
Internode diameter	.90	.55	-.13	.38	.92	.30

Sig.	<.001	.011	.573	.096	<.001	.198
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Key: **Bold** = significant result (p<.05).

5.1.4 Discussion

The morphological results of the stems and internodes are based on a random collection of stems from the field plots at Wageningen University. Three stem internodes (N=3) were randomly selected per genotype and subsequently measured. Because the data is based on three stem samples of a single growth season of a single growth location, the data may not be accurately representative for the genotypes over time.

A solution to overcome these potential drawbacks would be to analyze a higher number of stem samples from different growth seasons and locations in order to gain a more precise insight into the morphological characteristics of these genotypes.

5.2 Gene expression

Section 5.2 presents the results regarding the qPCR. Section 5.2.1 exhibits information on the annotation of the sequences used for designing the qPCR primers. Subsequently, Section 5.2.2 depicts information about the reference genes needed to define their suitability for application in the qPCR analysis. Finally, Section 5.2.3 details on the gene expression patterns of the genes.

5.2.1 Overview of genes and primers

The DNA sequences used to design the qPCR primers show significant resemblance with other sequences of related *Miscanthus* species (

Table 7) in Genbank (www.ncbi.nlm.nih.gov). PAL, 4CL, LAC and CESA sequences correspond with those of rice, sugarcane, maize and sorghum. Moreover, the putative identity of the genes corresponding with the fragments is confirmed due to the fact that the related species of *Miscanthus* show a high similarity with the sequences. PAL and 4CL show the same E-values between the Genbank accessions of the different species. Both LAC and CESA show the highest resemblance with maize (LAC: laccase 1; CESA: cellulose synthase 1).

Table 7. Gene sequence analysis and annotation to closely related *Miscanthus* species.

Gene	Genbank accession no.*	Species	Putative function	E-value
PAL	NP_001055608.1	Rice	phenylalanine ammonia-lyase	$1 * 10^{-45}$
	ABM63378.1	Sugarcane	phenylalanine ammonia-lyase	$1 * 10^{-45}$
	ACG44219.1	Maize	phenylalanine ammonia-lyase	$1 * 10^{-45}$
	BAJ09365.1	Sorghum	phenylalanine ammonia-lyase	$1 * 10^{-45}$
4CL	ABR25503.1	Rice	4-coumarate: CoA ligase 2	$1 * 10^{-18}$
	AAF37733.1	Ryegrass	4-coumarate: CoA ligase 2	$1 * 10^{-18}$
	BAD05189.1	Rice	4-coumarate: CoA ligase 1	$1 * 10^{-18}$
LAC	NP_001105789.1	Maize	laccase 1	$7 * 10^{-73}$
	AAL73970.1	Ryegrass	laccase LAC5-4	$7 * 10^{-20}$
	BAD82649.1	Rice	putative laccase LAC6-8	$6 * 10^{-13}$
CESA	NP_001105574.1	Maize	cellulose synthase 1	$1 * 10^{-67}$
	NP_001105574.1	Maize	cellulose synthase 2	$1 * 10^{-67}$
	AAF89963.1	Maize	cellulose synthase 3	$1 * 10^{-63}$

* Results are based on Genbank BlastX procedure (www.ncbi.nlm.nih.gov).

5.2.2 Choice of reference genes

Table 8 shows Ct-value descriptives of the two qPCR reference genes: β -tubulin and GADPH. The mean Ct-value of β -tubulin is 1.5 higher in comparison to GADPH. Both genes have a stable relative mean Ct-value and standard error of mean. Inter-item correlation are high ($\geq .982$) (Table 9). Therefore the average Δ Ct-value of the two endogenous genes is used as a suitable reference for the qPCR analyses.

Table 8. Ct-value descriptives of the reference genes.

Gene	Mean Ct-value	Standard error of mean
β -tubulin	25.56	0.174
GADPH	24.04	0.156

Table 9. Correlations between Δ Ct-values of the reference genes.

	Δ Ct- β -tubulin	Δ Ct-GADPH	Δ Ct-average
Δ Ct- β -tubulin	1		
Δ Ct-GADPH	.930 ^A	1	
Δ Ct-average*	.982 ^A	.983 ^A	1

* Δ Ct-average = average value of Δ Ct- β -tubulin and Δ Ct-GADPH

^A Correlation significance $p < .001$

5.2.3 Gene expression patterns

Section 5.2.3 presents the results of the gene expression analyses. Due to unbalanced data in the first stage of development, analysis of variance (ANOVA) is conducted on the data from the second to fourth stage of development.

Phenylalanine ammonia-lyase (PAL)

Main effects and interactions between second and fourth stage of development

All main effects (Stage of development; Genotype; Section) and interactions (Stage of development * Genotype) are significant for PAL (Table 10). The model fits the data relatively well ($R^2 = .878$), hence 12.2% of the variance remains is unexplained (i.e. residual). The interaction Stage of development * Genotype * Section could not be conducted in the ANOVA due to insufficient degrees of freedom.

Table 10. PAL ANOVA: 2nd to 4th stage of development.

Source of variation	d.f.	s.s.	m.s.	F-stat.	sig.
Stage of development	2	105.64	52.82	52.42	<.001
Genotype	5	22.34	4.47	4.43	0.006
Section	2	6.94	3.47	3.44	0.049
Stage of development * Genotype	10	26.57	2.66	2.64	0.026
Genotype * Section	10	6.92	0.69	0.69	0.726
Residual	23	23.18	1.01	17.72	
Sample * Units * stratum	53	3.01	0.06		
Total	105	189.03			

$R^2 = .878$

PAL expression patterns across stages of development

As indicated in

Table 10, gene expression of PAL differs across the second to fourth stage of development

($F(10,105)=2.64$; $p=.026$). Figure 23 illustrates the ΔC_t -values of PAL of the complete internodes of the genotypes across the stages of development.

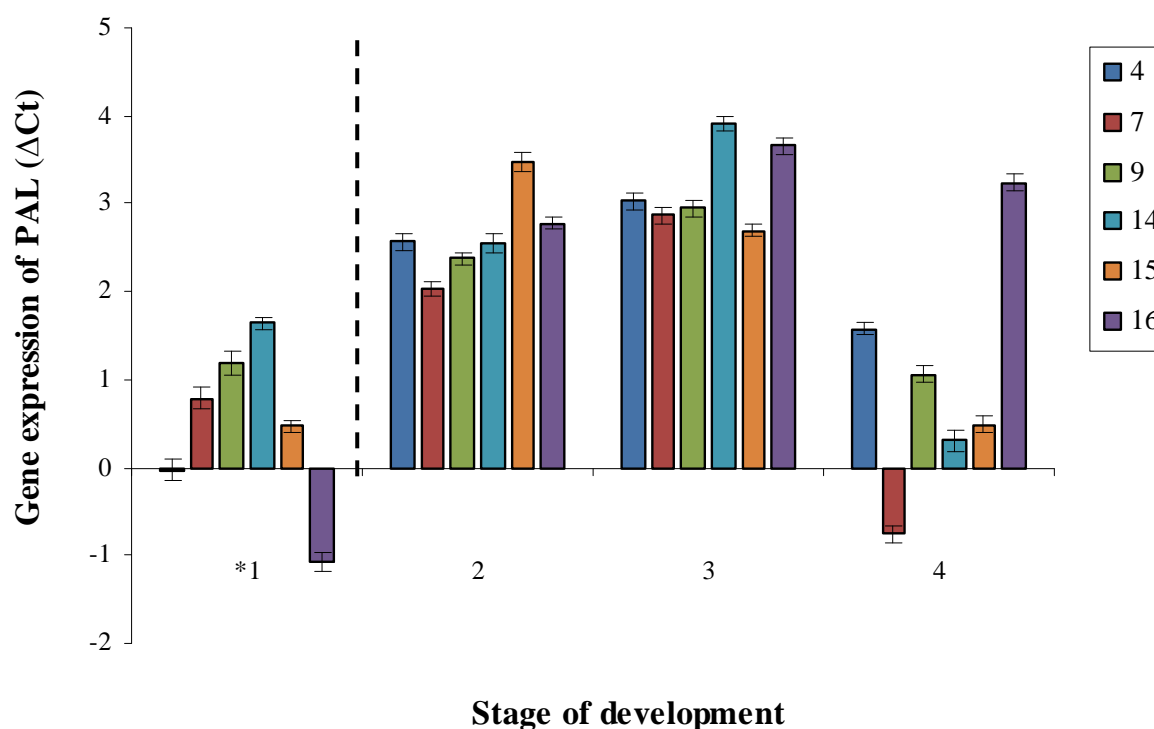


Figure 23. PAL expression (ΔCt) across stages of development with standard error bars.

* Data of the first stage of development originates from a different data set than second to fourth (i.e. unbalanced comparison). Hence a statistical analysis is not possible between the first and other stages of development due to limited degrees of freedom.

Within the complete internodes, expression of PAL is highest during the second or third stage of development in the genotypes (Table 11). From this peak, expression subsequently declines substantially.

Table 11. Relative expression of PAL.

Genotype	PAL fold change relative to first stage		
	Stage 2	Stage 3	Stage 4
4	3.6	4.1	2.6
7	1.5	1.8	-2.5
9	2	2.5	-0.1
14	1.6	2.4	-5.4
15	7.4	5.8	1
16	4.8	5.7	5.3

PAL expression patterns across stem internode sections

Expression of PAL does not differ significantly between the internode sections of the stems across the genotypes ($F(10,105)=.69$; $p=.726$). Although this result is non-significant, all genotypes express PAL higher in the basal internode section than in the top (Table 12). Furthermore, genotypes 4, 9, 14 and 16 show an increasing trend of expression from top to middle to basal stem section.

Table 12. PAL Δ Ct-value of internode sections.

Genotype	Δ Ct-value of internode section		
	Top	Middle	Basal
4	2.10	2.31	2.84
7	1.03	0.92	2.16
9	1.87	2.24	2.32
14	1.90	2.49	2.54
15	1.60	2.71	2.37
16	2.83	3.12	.769

Note: positive Δ Ct-value is high expression.

L.S.D. (5%) = 1.24

4-coumarate:CoA ligase (4CL)

Main effects and interactions between second and fourth stage of development

The main effect 'Stage of development' and interaction 'Stage of development * Genotype' are significant for 4CL (Table 13). The model fit with the data (R^2) is .765, hence 23.5% of the variance remains is unexplained (i.e. residual). The interaction Stage of development * Genotype * Section could not be conducted in the ANOVA due to insufficient degrees of freedom.

Table 13. 4CL ANOVA: 2nd to 4th stage of development.

Source of variation	d.f.	s.s.	m.s.	F-stat.	sig.
Stage of development	2	17.37	8.69	6.30	.007
Genotype	5	6.26	1.25	0.91	.493
Section	2	1.29	0.64	0.47	.633
Stage of development * Genotype	10	67.77	6.78	4.91	<.001
Genotype * Section	10	5.05	0.51	0.37	.949
Residual	23	31.72	1.38	12.57	
Sample * Units * stratum	53	5.81	.11		
Total	105	135.11			

$R^2 = .765$

4CL expression across the stages of development

As indicated in Table 13, expression of 4CL differs across the second to fourth stage of development ($F(10,105)=4.91$; $p<.001$). Figure 24 illustrates the ΔCt -values of 4CL of the complete internodes for the genotypes across the stages of development.

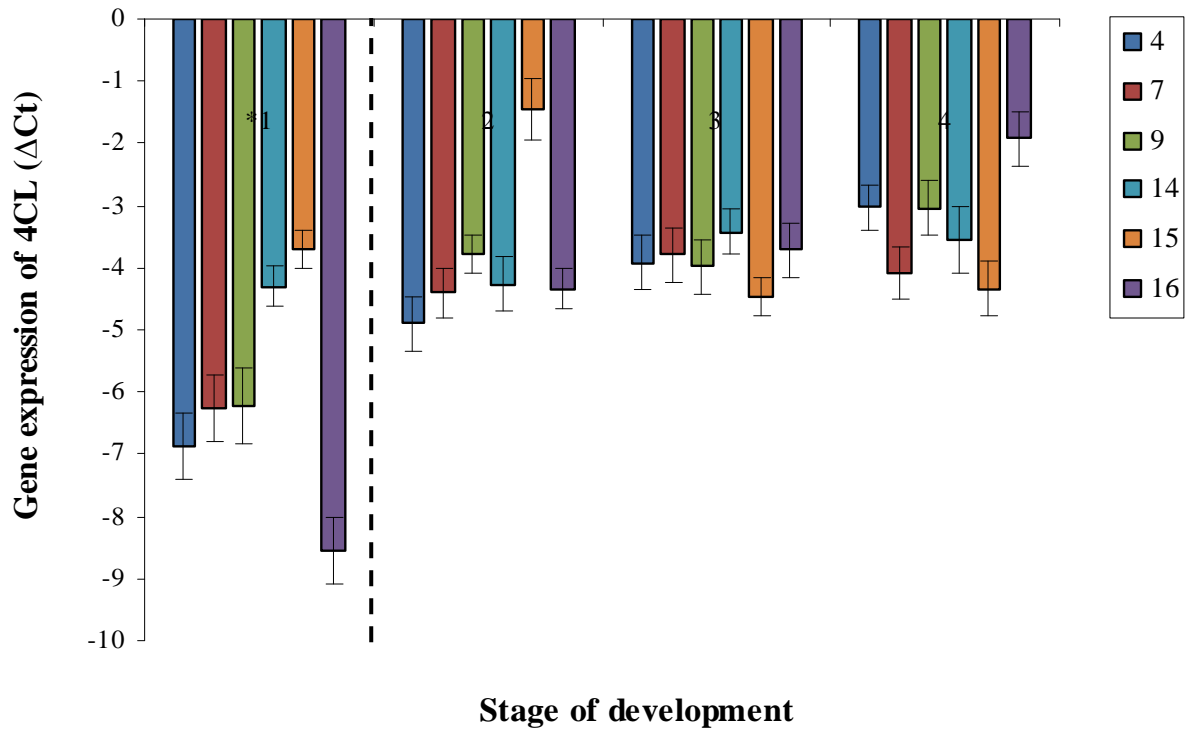


Figure 24. Gene expression of 4CL (ΔCt) across stages of development with standard error bars.

* Data of the first stage of development originates from a different data set than second to fourth (i.e. unbalanced comparison). Hence a statistical analysis is not possible between the first and other stages of development due to limited degrees of freedom.

Across all genotypes, relative expression of 4CL differs significantly over the second to fourth stage of development (Table 13). Genotypes 4 and 16 increase their expression of 4CL trough all four stages of development. Genotypes 7 and 14 show a maximum expression of PAL at the third stage. It is apparent that genotype 15 shows a large decrease in expression of 4CL at the third and fourth stage of development.

Table 14. Relative expression of 4CL in comparison to the first stage of development.

Genotype	4CL fold change relative to first stage		
	Stage 2	Stage 3	Stage 4
4	3	4	4.9
7	2.9	3.5	3.2
9	3.4	3.2	4.2
14	1	1.9	1.8
15	3.2	-4.1	-2.7
16	5.2	5.9	7.6

4CL expression patterns across stem internode sections

The 4CL ΔC_t -values of the stem internode sections do not differ significantly ($F(10,105)=.37$; $p=.949$). No apparent trend in expression of 4CL is observed within the internode sections of the genotypes (Table 15). In genotypes 4, 7 and 9 expression is higher in the top part than in the basal part; for genotypes 14, 15 and 16 the opposite is true.

Table 15. 4CL ΔC_t -value of internode sections.

Genotype	ΔC_t -value of internode section		
	Top	Middle	Basal
4	-3.82	-4.07	-3.97
7	-3.98	-4.35	-4.27
9	-4.11	-4.14	-4.28
14	-3.87	-3.83	-3.56
15	-4.12	-3.43	-3.04
16	-4.42	-4.19	-3.68

Note: positive ΔC_t is high expression.

L.S.D. (5%) = 1.40

Laccase (LAC)

Main effects and interactions between second and fourth stage of development

The main effects of 'Stage of development' and 'Genotype' and the interaction 'Stage of development * Genotype' differ significantly across the stages of development for 4CL (Table 16). The model fit with the data (R^2) is .805, hence 19.5% of the variance remains is unexplained (i.e. residual). The interaction Stage of development * Genotype * Section could not be conducted in the ANOVA due to insufficient degrees of freedom.

Table 16. LAC ANOVA: 2nd to 4th stage of development.

Source of variation	d.f.	s.s.	m.s.	F-stat.	sig.
Stage of development	2	51.05	25.52	4.72	.019
Genotype	5	158.66	31.73	5.86	.001
Section	2	14.52	7.26	1.34	.281
Stage of development * Genotype	10	191.32	19.13	3.53	.006
Genotype * Section	10	90.75	9.08	1.68	.147
Residual	23	124.50	5.41	32.64	
Sample * Units * stratum	53	8.79	.17		
Total	105	639.32			

$R^2 = .805$

LAC expression across stages of development

Across all genotypes the relative gene expression of LAC differs significantly across the 2nd to 4th stage of development ($F(10,105)=3.53$; $p=.006$) (Table 16). Figure 25 illustrates the ΔCt -values of LAC for the genotypes across the stages of development.

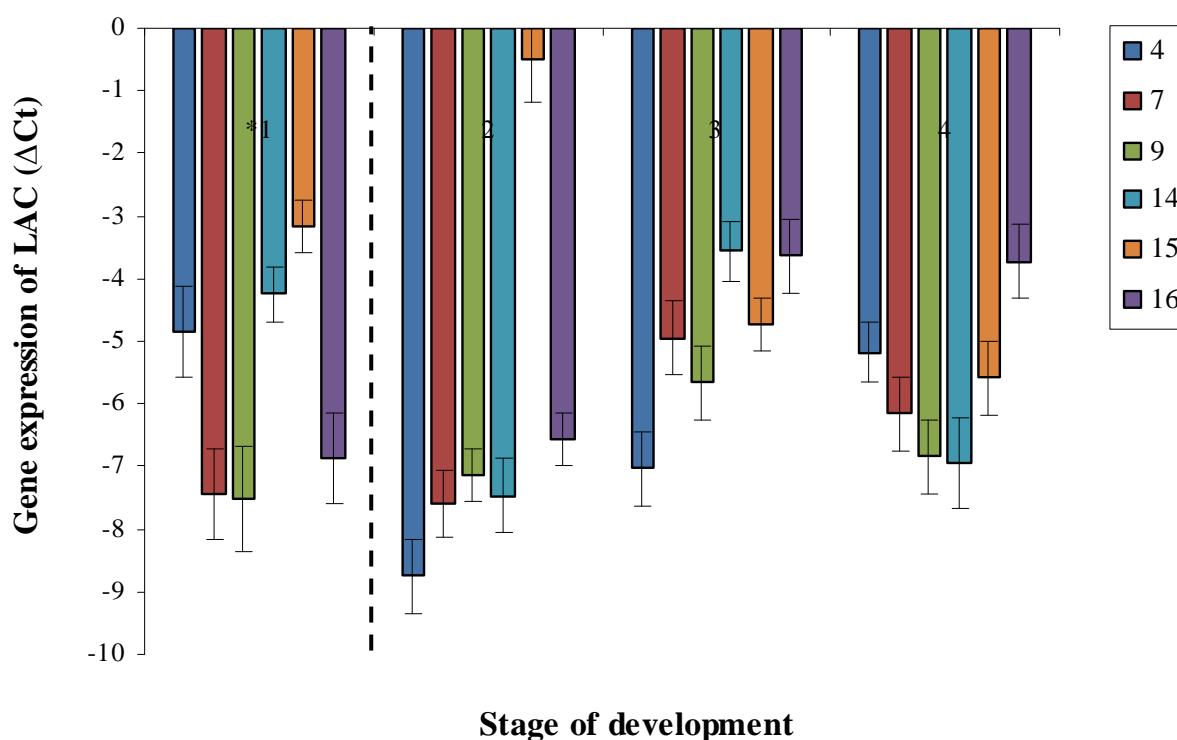


Figure 25. Gene expression of LAC (ΔCt) across stages of development with standard error bars.

* Data of the first stage of development originates from a different data set than second to fourth (i.e. unbalanced comparison). Hence a statistical analysis is not possible between the first and other stages of development due to limited degrees of freedom.

No apparently clear trend in the expression of LAC is observed within the genotypes across the stages of development (Table 17). Genotypes 9 and 16 express LAC relatively higher at stages 2, 3 and 4 (compared to stage 1), whilst genotypes 7 and 14, first show a decrease and subsequently an increase in expression.

Table 17. Relative expression of LAC in comparison to the first stage of development.

Genotype	LAC fold change relative to first stage		
	Stage 2	Stage 3	Stage 4
4	-4.9	-0.4	-0.1
7	-0.2	3.5	2.3
9	1.4	2.9	1.7
14	-4.2	1.2	-2.8
15	1.8	-1.8	-3.4
16	1.3	4.2	4.2

LAC expression patterns across stem internode sections

The LAC Δ Ct-values of the stem internode sections do not differ significantly ($F(10,105)=1.68$; $p=.147$). No apparent trend in expression of LAC is observed within the internode sections of the genotypes (Table 18). The only clear exception is genotype 15, major differences (up by factor 3.56 gene fold expression) are observed between the top, middle and basal section.

Table 18. LAC Δ Ct-value of internode sections

Genotype	Δ Ct-value of internode section		
	Top	Middle	Basal
4	-6.93	-7.24	-7.81
7	-6.89	-6.07	-6.07
9	-6.69	-7.11	-7.40
14	-6.34	-5.71	-6.30
15	-6.80	-3.24	-1.18
16	-5.19	-5.11	-5.20

Note: positive is high expression.

L.S.D. (5%) = 2.78

Cellulose synthase (CESA)

Main effects and interactions between second and fourth stage of development

The main effects ‘Stage of development’ and ‘Genotype’ and interaction ‘Stage of development * Genotype’ are significant for CESA (Table 19). The model fit with the data (R^2) is .767, hence 23.3% of the variance remains is unexplained (i.e. residual). The interaction Stage of development * Genotype * Section could not be conducted in the ANOVA due to limiting degrees of freedom.

Table 19. CESA ANOVA: 2nd to 4th stage of development.

Source of variation	d.f.	s.s.	m.s.	F-stat.	sig.
Stage of development	2	20.98	10.49	4.27	.026
Genotype	5	77.99	15.60	6.35	<.001
Section	2	3.51	1.76	0.72	.500
Stage of development * Genotype	10	56.93	5.69	2.32	.046
Genotype * Section	10	24.53	2.45	1.00	.472
Residual	23	56.47	2.46	24.14	
Sample * Units * stratum	51	5.19	0.10		
Total	103	242.11			

$R^2 = .767$

CESA expression across stages of development

Across all genotypes the expression of CESA differs across the 2nd to 4th stage of development ($F(10,103)=2.32$; $p=.046$) (Table 19). Figure 26 illustrates the ΔC_t -values of CESA for the genotypes across the stages of development.

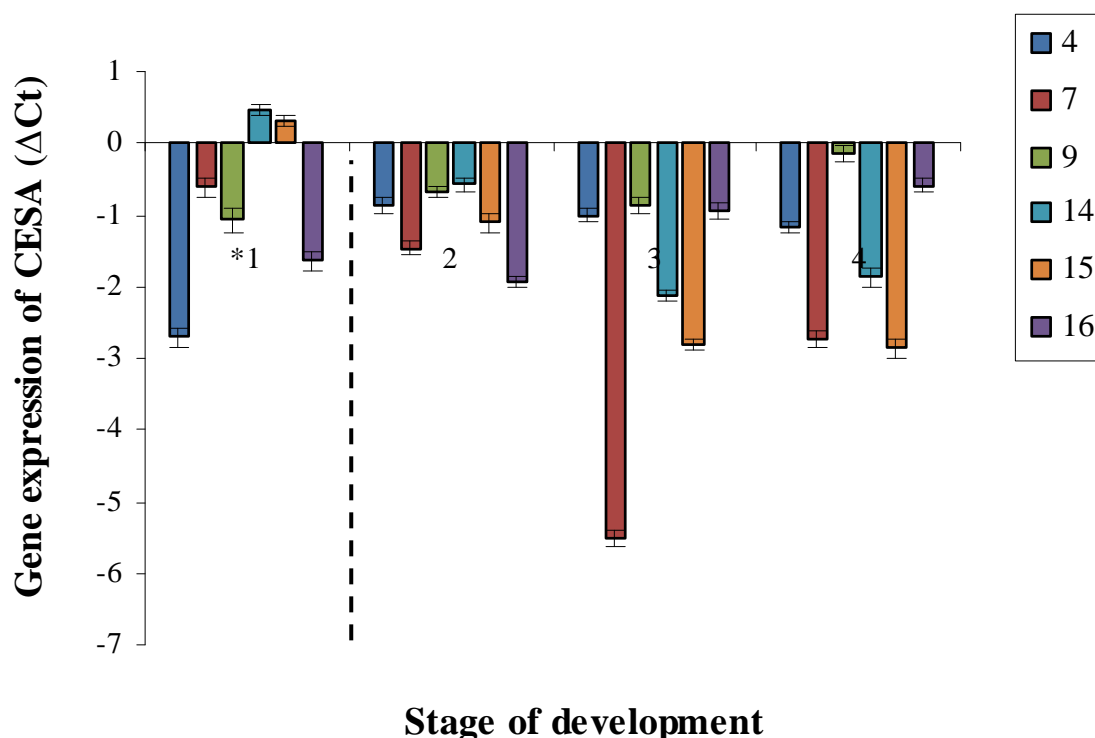


Figure 26. Gene expression of CESA (ΔCt) across stages of development with standard error bars.

* Data of the first stage of development originates from a different data set than second to fourth (i.e. unbalanced comparison). Hence a statistical analysis is not possible between the first and other stages of development due to limited degrees of freedom.

No apparently clear trend in the expression of CESA is observed within the genotypes across the stages of development (Table 17). Genotypes 4 and 9 express LAC relatively higher at stages 2, 3 and 4 (compared to stage 1), whilst all other genotypes show a relatively lower expression at these stages.

Table 20. Relative expression of CESA in comparison to the first stage of development.

Genotype	CESA fold change relative to first stage		
	Stage 2	Stage 3	Stage 4
4	2.8	2.7	2.5
7	-0.1	-6	-1.5
9	1.4	1.2	1.9
14	-1.4	-3.6	-2.9
15	-1.5	-4.2	-4.2
16	-0.8	-1.5	-1.8

CESA expression patterns across stem internode sections

The CESA ΔCt-values of the stem internode sections do not differ significantly ($F(10,105)=1.00$; $p=.472$). No apparent trend in expression of LAC is observed within the internode sections of the

genotypes (Table 21). Genotypes 4, 7, 15 and 16 show a relatively higher expression in the basal internode than the top.

Table 21. CESA Δ Ct-value of internode sections

Genotype	Δ Ct-value of internode section		
	Top	Middle	Basal
4	-1.25	-1.20	-.63
7	-2.60	-4.72	-2.44
9	-.74	-.39	-.80
14	-2.11	-2.53	-2.14
15	-1.89	-1.79	-1.01
16	-1.76	-0.99	-1.95

Note: positive is high expression.

L.S.D. (5%) = 1.87

5.2.4 Discussion

Experimental setup

The interaction between the expression levels of the genes and stage of development differed significantly across the genotypes for PAL, 4CL, LAC and CESA. *A priori*, one would expect that expression levels of lignin and cellulose biosynthesis genes are higher in genotypes with a relatively higher lignin and cellulose content. Based on the results in Section 5.2.3, this is not the case. This may be due to the following factors of influence: the assumed putative gene function, SNPs and alleles of the genes, type of tissue used as a sample, pooling of the RNA samples and the developmental stage of the harvested material.

The exact putative functions of PAL, 4CL, LAC and CESA remain unknown. Furthermore, the genes are part of multi-gene families. For instance, LAC has been associated with multiple physiological functions (e.g. formation of phenoxyl radicals and phenolic cross-linking in the cell wall) (Liang, Haroldsen et al. 2006), but in particular with lignin biosynthesis (Cai, Davis et al. 2006). However, studies show that over expression (Dean, LaFayette et al. 1998) and down regulation (Ranocha, Chabannes et al. 2002) of LAC did not alter lignin content. The genetic evidence of LAC involvement with in lignification remains under debate (O'Malley, Whetten et al. 1993; Sato, Wuli et al. 2001). In aspen, 4CL two isomers of the gene family have been proposed to be specifically involved in lignin biosynthesis and participation in flavonoid formation (Hu, Kawaoka et al. 1998). It cannot be concluded that PAL is strictly related to lignin biosynthesis because it is involved in other pathways too (van den Broeck, Maliepaard et al. 2008).

The highly heterozygous genotypes used in this study may contain multiple alleles (and SNPs). Therefore the exact type of PAL, 4CL, LAC and CESA genes in the genotypes remains unknown and may bring forth a bias effect of expression (

Table 7 in Section 5.2.1). Riboulet et al. (2009) illustrate that different contigs of PAL, 4CL and LAC in maize are differentially expressed across plant development.

In this study, the tissue (i.e. bast and pith core) within the internode sections (used for RNA isolation) was not separated. Van den Broeck et al. (2008) show that the expression PAL, 4CL and LAC show differential expression between the bast and core pith tissues in fibre hemp. Although fibre hemp and *Miscanthus* are different species, the “pooling” of the bast and core pith may bring about a certain unknown bias.

Each RNA sample used for cDNA synthesis, consisted of two pooled stem internode sections (see Figure 14). It was attempted to pool two internodes of the same stage of plant development (by means of the biological marker: no. of visible leaves). However, the preciseness of this marker is unknown and may bring forth error (when RNA samples of differing stages of development are pooled). Therefore, this potential error of pooling may have influenced the gene expression levels by averaging out the high and/or low values.

Biotic factors

It is known that biotic factors influence the biosynthesis of lignin (cf. Vanholme, Demedts et al. 2010). A fungal infection (assumed to be *Pythium sp.*) was observed in the plants in the greenhouse during the 3rd to 4th stage of development. This phenomenon may have induced an alteration in expression levels at the time of infection and there after.

Potential improvements

A possible solution to overcome (a part of) the potential biases, would be to perform this study in a model species (e.g. *Arabidopsis* or maize). In such a case, homozygous (inbred) lines may be used with more research and bioinformatics data resources available (e.g. DNA sequences and SNPs). Hence, primers of specific genes (with known function and family membership) may be monitored. The developmental stages may be synchronized more efficiently by growing the plants from seeds instead of rhizomes.

5.3 Biochemical composition of the stem internodes

Section 5.3 presents the results of the biochemical analyses of the complete second stem internode at the four stages of plant development. The biochemical content entails the dry matter content (DM), acid detergent fiber (ADF), permanganate lignin (pLIG), cellulose and ashes (Section 5.3.1). Due to a lack of biomass material, only genotypes 14 and 15 were analyzed at the first stage of development. In most cases the measurements are based on a single data point (N=1). Only genotype 16 was replicated (N=2) at the 3rd and 4th stage of development. Therefore no statistically concrete conclusions can be drawn from differences between the genotypes at a single developmental stage. Statistical analyses are possible between the stages of developmental and are addressed in Section 5.3.2.

5.3.1 Biochemical composition within each stage of development

First stage of development

Genotypes 14 and 15 show relatively large differences in terms of ADF, cellulose and ash, and a small discrepancy between pLIG (Figure 27). The DM content is similar between the two genotypes.

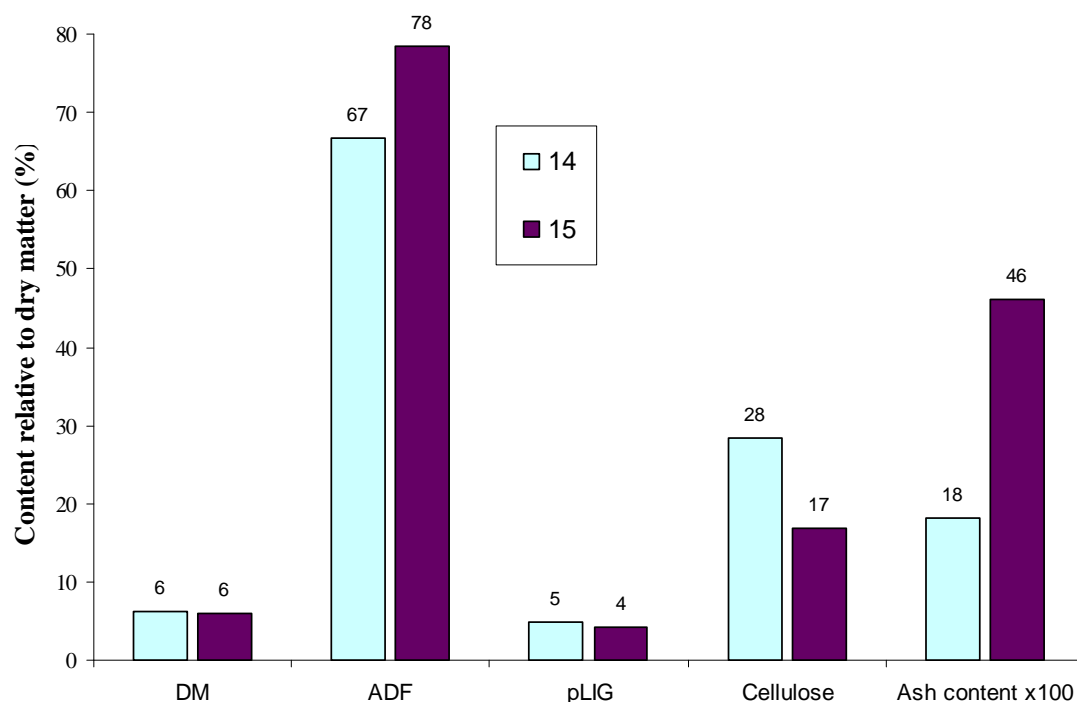


Figure 27*. Biochemical composition of the complete second stem internodes at developmental stage 1.

* Data is based on a single measurement (N=1). Genotypes 4, 7, 9 and 16 could not be analyzed due to insufficient biomass material at this stage of development. Figures are rounded off to whole numbers.

Second stage of development

In terms of DM content, genotypes 4, 15, 16, 9, 14 and 7 are ranked highest to lowest respectively (Figure 28). The genotypes show approximately the same DM values, and the maximum DM difference between the genotypes (4 and 7) is 5.4%. ADF ranks highest to lowest for genotypes 7, 15, 4, 14, 9 and 16. The maximum ADF difference is 8.2% (between 7 and 16). pLIG values do not show very much difference between the genotypes. The largest difference (between sample 16 and 15) is 3.2%. Cellulose composition ranked from high to low in genotypes 16, 9, 14, 15, 4 and 7. The largest cellulose difference (5.3%) is between sample 16 and 7. Ash content differs considerably differences between the genotypes. Genotype 16 scores the highest (0.73 mg/g DM) and 4 (28 mg/g DM) the lowest on ash content.

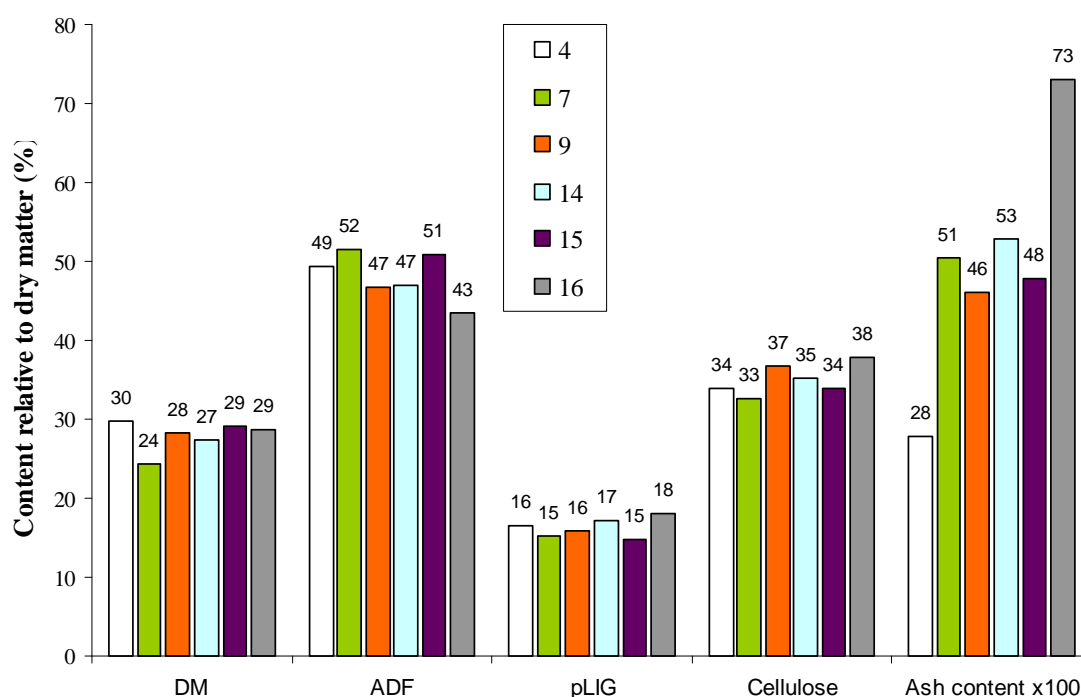


Figure 28*. Biochemical composition of the complete second stem internodes at developmental stage 2.

* Data is based on a single measurement (N=1). Figures are rounded off to whole numbers.

Third stage of development

DM content ranges between 29.1 – 46.6 % for the genotypes. Genotype 16 has a standard deviation (SD) of 4.5 (10% of mean value). In terms of ADF all genotypes show relative approximate values

(43.6 – 46.1 %DM), the SD of genotype 16 is 0.6. pLIG values are highest for genotypes 15, 9 and 16 and lower for 14, 4 and 7. The SD of genotype 16 is 1.3. Cellulose content ranges between 32.3 – 36.9 % between the genotypes; the SD of genotype 16 is 1.9. Ash content showed ample difference between the genotypes (0.376 – 0.767 mg/g DM). Genotypes 9 and 4 have the highest ash content, whilst 14, 7, 15 and 16 contain much less. The SD of genotype 16 in ash is 0.2.

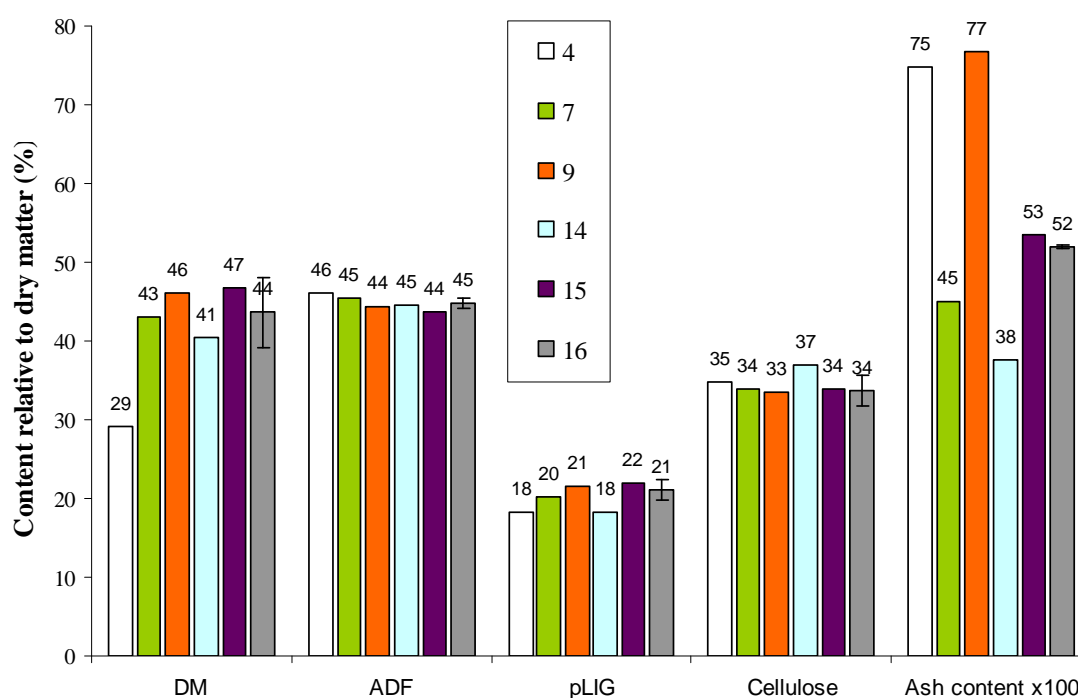


Figure 29*. Biochemical composition of the complete second stem internodes at developmental stage 3 with S.D. bars.

* Data is based on a single measurement (N=1), except for genotype 16 (N=2). Figures are rounded off to whole numbers.

Fourth stage of development

The DM content ranged between 36.9 – 53.8 % (Figure 30). The standard deviation (SD) of DM in genotype 16 is 5.0 (10% of mean). ADF content vary between 44.8 – 52.5 % DM, with genotype 14 and 9 showing the highest and lowest values respectively. The SD of ADF in genotype 16 is 0.4. pLIG values ranged between 18.1 – 21.4 % DM, the SD in genotype 16 is 0.4. Cellulose levels ranged between 28 – 33.2 % DM. The SD of cellulose in genotype 16 is 0.8. Ash content showed relatively more variation (between 0.4 – 0.65 mg/g DM). The SD of ash in genotype 16 is 9.6.

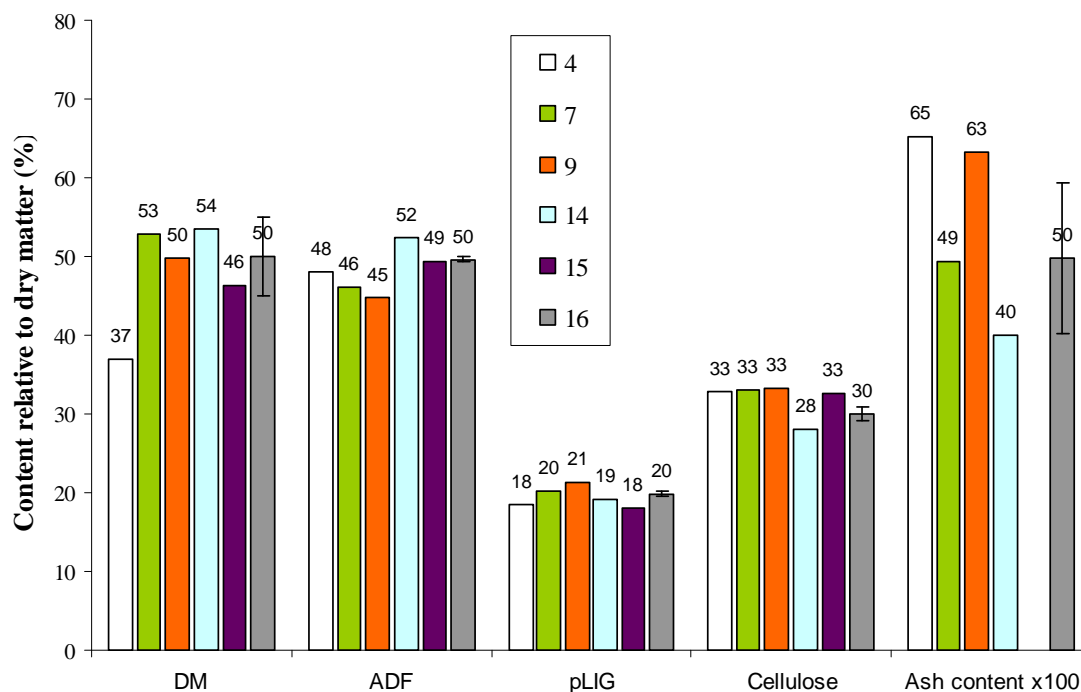


Figure 30*. Biochemical composition of the complete second stem internodes at developmental stage 4 with S.D. bars.

* Data is based on a single measurement (N=1), except for genotype 16 (N=2). Ash has missing value for genotype 15. Figures are rounded off to whole number.

5.3.2 Biochemical composition between the stages of development

Investigating the statistical assumptions

Normality of all the data was validated after having inspected the Q-Q plots. Levene's statistic indicated that DM ($p=.204$), pLIG ($p=.354$) and Ash ($p=.820$) have equal variances, whilst ADF ($p<.001$) and cellulose ($p<.001$) do not.

Dry matter content

As shown in Figure 31 DM content is not equal over the four stages of development ($F(3,18)=47.3$; $p<.001$). A clear positive trend in DM content is observed across plant development. The pair-wise comparisons between all stages of development are significant for Tukey's method ($p<.049$).

Bonferroni and Sidak methods consider all comparisons significant, except between development 3 and 4 ($p=.065$ and $p=.063$ respectively).

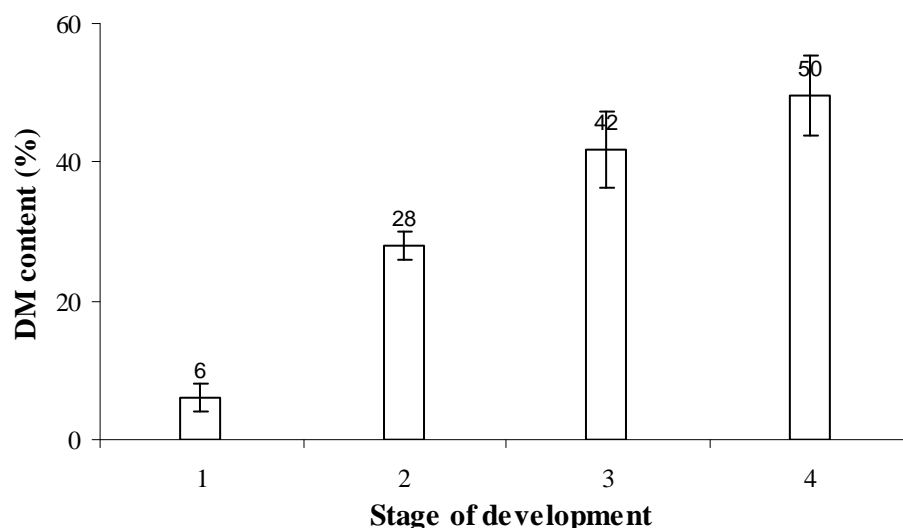


Figure 31. DM content across the stages of development with values and 95% C.I. bars.

Acid detergent fiber content

Figure 32 shows that ADF content is not equal over the four stages of development ($F(3,18)=47.0$; $p<.001$). ADF content is relatively high at the 1st stage of development, then declines and remains approximately stable through the rest of the stages. Pair-wise comparisons (Tukey, Bonferroni and Sidak) show a non-significant difference between stages of development 2 & 3, 2 & 4 and 3 & 4 (Tukey p-values: .208, .994 and .114 respectively). All comparisons with the first stage of development are highly significant ($p<.001$).

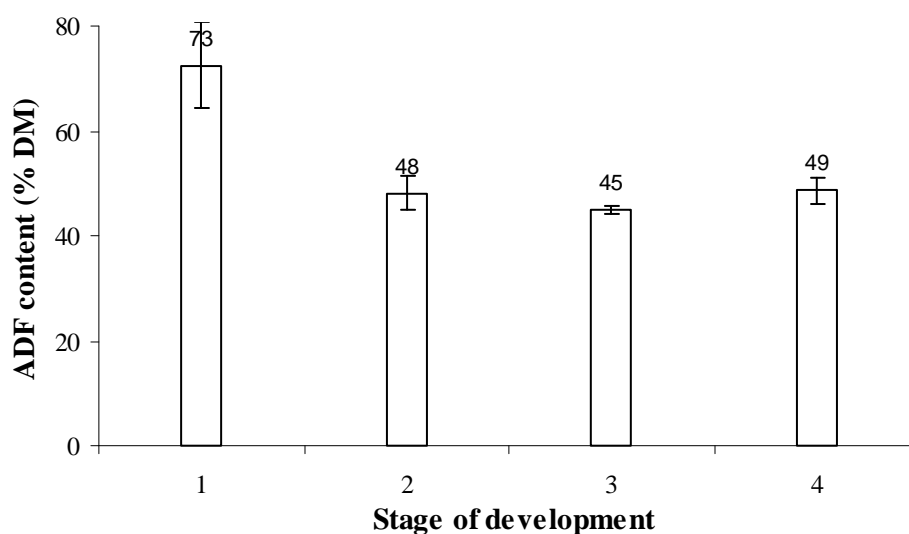


Figure 32. ADF content across the stages of development with 95% C.I. bars.

Permanganate lignin content

As presented in Figure 33, pLIG content is unequal across all stages of development ($F(3,18)=86.5$; $p<.001$). pLIG content clearly increases over stages 1 to 3. From stage 3 to 4 no significant change is observed (Tukey $p=.680$; Bonferroni $p=1.0$; Sidak $p=.856$). All other comparisons are significantly different from each other ($p\leq.001$).

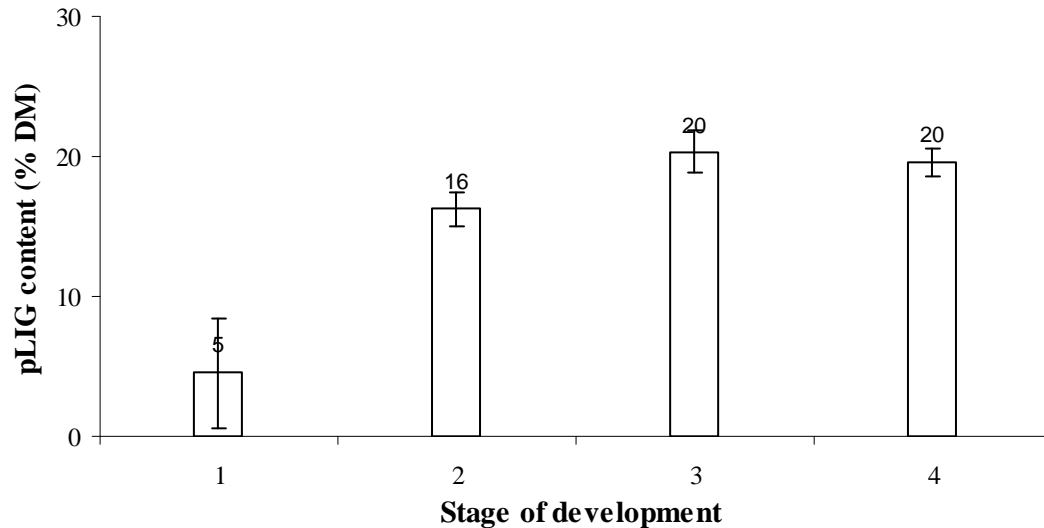


Figure 33. pLIG content across the stages of development with 95% C.I. bars.

Cellulose content

As shown in Figure 34, the cellulose content is unequal over all stages of development ($F(3,18)=12.9$; $p<.001$). ADF content increases from stage 1 – 2, and then gradually decreases from 2 – 4. The pair-wise comparisons between stages 1 & 2, 1 & 3 and 1 & 4 are unequal to each other ($p\leq.004$). All other comparisons are non-significant ($p\geq.089$).

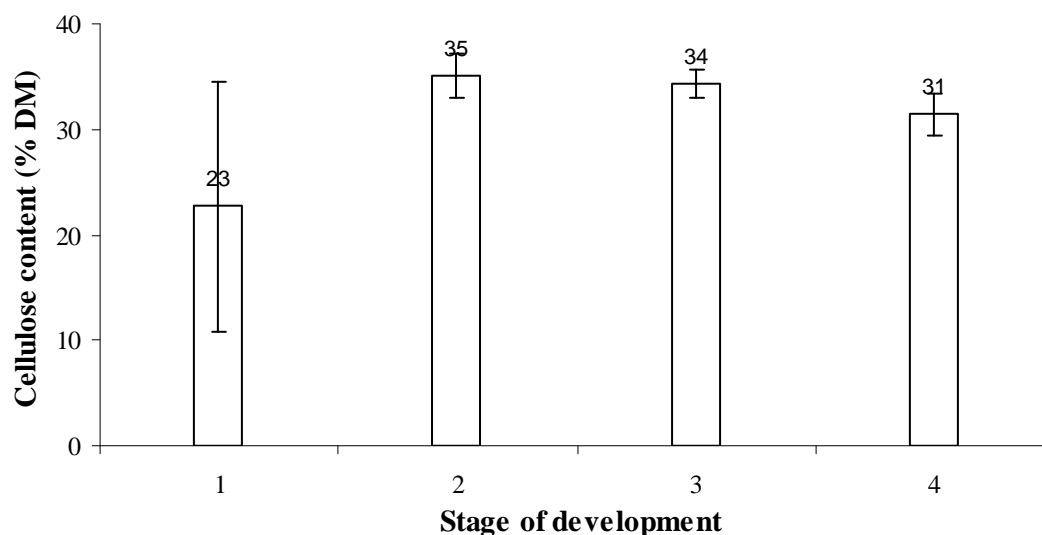


Figure 34. Cellulose content across the stages of development with 95% C.I. bars.

Ash content

As presented in Figure 35, ash content does not differ significantly across the stages of development ($F(3,18)=1.58$; $p=.231$). Furthermore, the pair-wise comparisons between all the stages of development are highly non-significant on Tukey ($p\geq.181$), Bonferroni ($p\geq.253$) and Sidak ($p\geq.284$) methods.

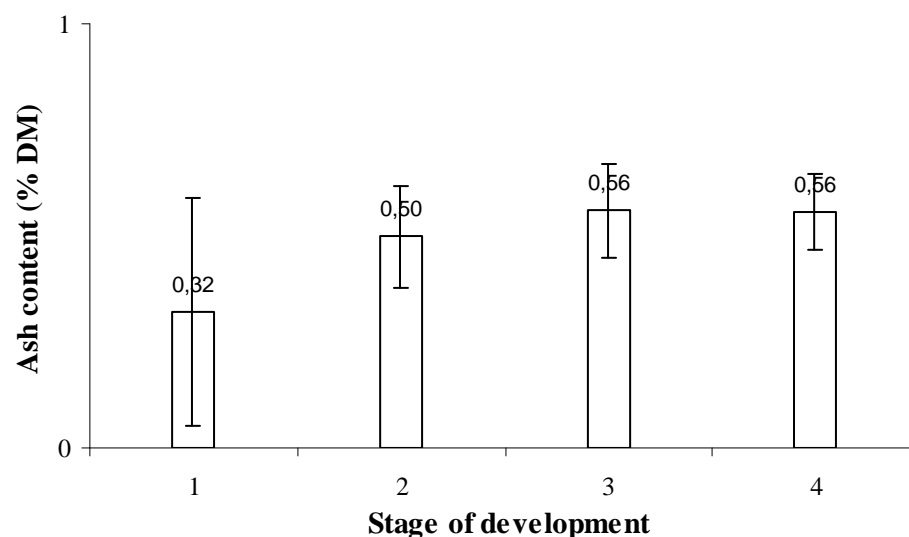


Figure 35. Ash content across the stages of development with 95% C.I. bars.

5.3.4 Discussion

In this study, a number of potential influential factors may have caused a bias in the biochemical analysis of the stem internodes. These factors may entail the sampling of the biological material in the analysis and the method of analysis.

The rhizomes were not all of identical size when placed in the greenhouse pots. In a number of rhizomes small shoots from the previous season had already developed. These were the first internodes to grow tall during plant growth. In general, these stems were the first to be harvested because they were taller than the others (i.e. more biological material for analysis). Due to this effect there may be a bias in the results.

The ANKOM method requires biomass samples between 0.45-0.55g. However due to limiting biological material this was not the case in 12 of the 22 samples (Appendix 9.10). Furthermore, most samples were based on a single measurement ($N=1$); merely two samples were replicated ($N=2$). Thus the measurement error in most samples is unknown.

Potential improvements

A solution to these potential biases would be to make use of plants that are grown in field plots from rhizomes of identical size and developmental stage. In this way, sufficient biological material would be available for the biomass samples and replicates.

5.4 Relationship between gene expression and biochemical composition

Section 5.4 aims to illustrate the relationship between expression of PAL, 4CL, LAC and CESA with lignin and cellulose in the second stem internode.

Table 22 presents the ΔC_t -values ($=C_t\text{-endogenous} - C_t\text{-sample}$) with corresponding pLIG and cellulose contents across the four stages of development. The relative expression data in

Table 22 should be considered per gene within each stage of development.

First stage of development

No apparently clear relationship is observed between the gene expression levels and biochemical contents. Cellulose content differs considerably between two genotypes (11.5 % DM), whilst the differential expression between the samples of CESA is minimal ($\Delta Ct = 0.1$). PAL, 4CL and LAC display ΔCt differences between 0.5 - 1.1, yet pLIG content varies by only 0.6 % DM.

Second stage of development

No strikingly coherent relationship is observed between the gene expression levels and biochemical contents. Cellulose and pLIG content varies between 32.6 – 37.9 and 14.8 – 17.9 % DM respectively. Genotypes 9, 14, 15 & 16 show a moderate relationship between CESA expression and cellulose content, whilst genotypes 9 and 16 clearly do not. PAL expression of genotypes 4, 7, 14, 15 & 16 shows association with pLIG values, but genotypes 4 and 15 do not. In general, 4CL and LAC expression matches the association with pLIG values, except for genotype 15.

Third and fourth stage of development

Seemingly there is no clear relationship between the gene expression levels and biochemical contents at the third and fourth stage of development. Expression of CESA, PAL, 4CL and LAC do not show a plausible relationship with cellulose and pLIG content. In a number of cases expression is high whilst the cell wall contents of the stems are low and vice versa (e.g. PAL expression genotype 14 and 15 at stage 3 respectively).

Table 22. Gene expression levels and biochemical composition of the stem internodes.

Stage of development	Genotype	Relative gene expression * (Δ Ct-value)				Biochemical composition (% DM)	
		PAL	4CL	LAC	CESA	pLIG	Cellulose
1	14	++ (1.6)	+ (-4.2)	+ (-4.2)	+ (0.2)	4.8	28.4
	15	+ (0.5)	++ (-3.7)	++ (-3.2)	+ (0.1)	4.2	16.9
2	4	+ (2.6)	+ (-4.9)	-- (-8.8)	+ (-0.3)	16.5	34.0
	7	- (2.0)	+ (-4.4)	- (-7.6)	- (-0.5)	15.3	32.6
	9	+ (2.4)	+ (-3.8)	- (-7.1)	+ (-0.2)	15.9	36.8
	14	+ (2.5)	+ (-4.3)	- (-7.5)	+ (-0.2)	17.2	35.3
	15	++ (3.5)	+++ (-1.5)	+++ (-0.5)	- (-0.4)	14.8	33.8
	16	+ (2.8)	+ (-4.3)	- (-6.6)	- (-0.6)	17.9	37.9
3	4	+ (3.0)	- (-3.9)	-- (-7.0)	+ (-0.3)	18.3	34.8
	7	+ (2.9)	- (-3.8)	- (-5.0)	-- (-1.8)	20.3	33.9
	9	+ (2.9)	- (-4.0)	- (-5.7)	+ (-0.3)	21.4	33.4
	14	++ (3.9)	+ (-3.4)	+ (-3.6)	- (-0.7)	18.2	36.9
	15	+ (2.7)	-- (-4.5)	- (-4.7)	- (-0.9)	22.0	33.8
	16	++ (3.7)	- (-3.7)	+ (-3.6)	+ (-0.3)	21.1	33.7
4	4	++ (1.6)	+ (-3.0)	- (-5.2)	- (-0.4)	18.4	32.9
	7	- (-0.8)	- (-4.1)	- (-6.2)	- (-0.9)	20.2	33.1
	9	+ (1.1)	+ (-3.0)	-- (-6.8)	++ (0.0)	21.4	33.2
	14	- (0.3)	+ (-3.5)	-- (-7.0)	- (-0.6)	19.1	28.0
	15	- (0.5)	- (-4.3)	- (-5.6)	-- (-0.8)	18.1	32.5
	16	+++ (3.2)	++ (-1.9)	+ (-3.7)	+ (-0.2)	19.8	30.0

* Relative expression across complete stem internode indicated at 4 levels: --, -, +, ++. Relative expression should be considered per gene within each stage of development. Positive Δ Ct-values represent relatively high expression in comparison with the endogenous genes (and vice versa).

5.4.1 Discussion

In Section 5.4 the attempt is made to identify a relationship between the expression of the genes with corresponding cell wall component. This analogy may be biased by a number of factors namely: the putative function of the genes, the accuracy of the biochemical analyses, the time lag between gene expression and biosynthesis of cell wall components.

Firstly, the genes show a relatively high correspondence with their putative candidate but it remains debatable if the genes are truly PAL, 4CL, LAC and CESA. Secondly, the biochemical analyses may bring forth a certain error because the samples did not all have the minimum mass as specified in the protocol. These two factors of influence are integrated together in the analogy and may bring forth a bias.

Furthermore, in cells a time lag exists between transcription (expression) and translation (synthesis). Therefore, comparing the expression levels with biochemical contents both at the same stage of

development (i.e. same moment in time) may not be an inaccurate procedure of analogy. For example, if expression of CESA is relatively high at time point 1 the effect may not be noticeable at time point 1 but at time point 2 or 3.

Potential improvement

In order to correct for the time lag between transcription and translation, the biological samples for gene expression may be harvested earlier than those for the biochemical analyses. However, in such as case it remains unclear as to how much time should between these steps. In an ideal situation, real time information would be gathered on both expression and biochemical content but this is not possible in practice. Therefore, a potential improve could be to collect many more samples to analyze the changes across the stages of development. This could be possible if this study is performed on genotypes grown in the field from large rhizomes with abundant stem material.

6. Conclusions

Chapter 6 provides statements that provide answer to the sub-research questions posed in Section 3.4. The conclusions are presented per topic in Section 6.1 (morphology of the stems and internodes), Section 6.2 (gene expression patterns), Section 6.3 (biochemistry of the stem internodes) and finally Section 6.4 (the relationship between gene expression and biochemistry of stem internodes).

6.1 Morphology of stems and internodes

Considerable variation in terms in the number of internodes per stem, length, mass and diameter. *M. x giganteus* has the highest internode mass and diameter. The interspecific hybrid has a relatively low internode length, mass and diameter. Considerable morphological variation is present in the stem and internodes characteristics of the *M. sinensis* genotypes.

Within all 20 genotypes, eight positive and a single negative correlation are found between the stem and internodes characteristics. Strong correlations (>0.9) were present between stem mass - internode mass, stem mass - internode diameter and internode mass - internode diameter.

6.2 Gene expression patterns

Expression of PAL, 4CL, LAC and CESA alter significantly over the stages of development across the genotypes. In general, expression of PAL and LAC reach their maximum expression at the third stage of development. Genotypes 14, 15 and 16 express PAL and LAC relatively high across a number of stages of development.

6.3 Biochemistry of stem internodes

Across the stages of development the levels of DM, ADF, pLIG and cellulose are not constant for the genotypes 4, 7, 9, 14, 15 and 16. DM and pLIG content increases through all four stages of development. Cellulose levels are the highest at the second stage of development and subsequently decline in the third and fourth stage. ADF content reaches the highest point in the third stage of development.

6.4 Relationship between gene expression and biochemistry of stem internodes

No clear relationship is observed between the expression of PAL, 4CL and LAC with pLIG content; and CESA with cellulose content. In the case of lignin biosynthesis, rather a mild negative relationship is observed. Genotypes with lower amounts of pLIG show higher relative expression for PAL, 4CL and LAC. Therefore, no hard evidence is found to associate PAL, 4CL, LAC and CESA (of this study) with cell wall biosynthesis function.

7. Recommendations

Chapter 7 provides recommendations for research on cell wall biosynthesis in *Miscanthus* and other species.

Future *Miscanthus* research should be carried out using genotypes grown on field plots. In doing so the environmental conditions of the experiments will be identical to how *Miscanthus* is cultivated in society for bio-based products. Hence, the research results will not be biased by differential environmental conditions in a greenhouse. Growing the genotypes in the field will yield more stems than in the greenhouse and more pooling and replication will be possible in the analyses. Also the genotypes should be grown from rhizomes of identical size. In doing so, plant developmental stages can be synchronized accurately. The application of inbred lines would make genetic analysis far less complex.

In order to gain insight into fundamental concepts of cell wall biosynthesis, it is recommended to perform the research in model species such as *Arabidopsis* or maize. In such a case, homozygous (inbred) lines may be used with ample bioinformatics resources available (e.g. on primers, sequences, QTLs and SNPs). Primers of specific genes (with known function, isomeric form and family membership) may be monitored. Moreover, the developmental stages may be synchronized more efficiently by growing the plants from seeds instead of rhizomes.

In comparison to this study, an alternative methodological approach is to analyze the expression of cell wall biosynthesis genes between bast and pith tissue of the stem internodes (see study in Hemp (van den Broeck, Maliepaard et al. 2008)). In this way, the expression of the putative genes may discriminate in the different tissue types. This may be used as a tool to validate that these genes are likely to be involved in their putative function.

In future research, more bioinformatics data will be available through databases. These should be exploited to design qPCR primers for cell wall biosynthesis genes that were omitted in this study. Moreover, it is also interesting to investigate the expression of genes in pathways prior to lignin such as the shikimate and aromatic amino acid biosynthesis pathways.

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9. Appendix

9.1 Phenylpropanoid (lignin) biosynthesis pathway

Figure 36 illustrates the phenylpropanoid biosynthesis pathway in Sorghum (KEGG 2010).

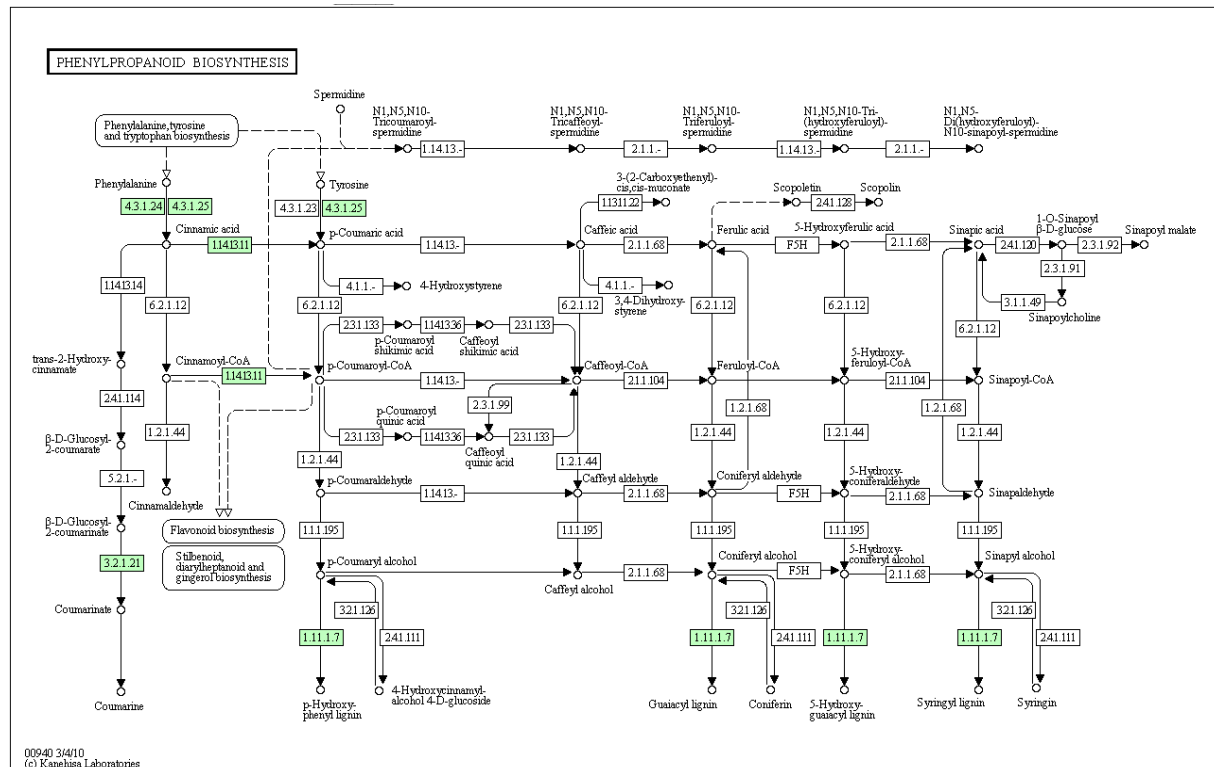


Figure 36. Phenylpropanoid biosynthesis pathway in Sorghum (KEGG 2010).

Codes in the boxes illustrate the EC enzyme codes.

9.2 Further specifications of genotypes

Table 23 presents the details of the genotypes (breeding programmes codes and trace elements) used in this study. Two studies describe genotype MS-88-110 in further detail (Jørgensen 1997; Clifton-Brown, Lewandowski et al. 2001).

Table 23. Specifications of the field and greenhouse genotypes.

Serial						Trace elements (mg/g)						
No.	Clone	Genotype	Trial	Plot	Entry_Name	Cl	PO4	SO4	Na	K	Mg	Ca
1	G0106	H0144	OD0608	168	EMI 8	.00	2.17	.58	.05	.67	.70	1.15
2	G0107	H0146	OD0608	170	EMI 13	.00	3.47	.93	.06	1.52	1.28	1.73
3	G0112	H0160	OD0608	184	MB 23	.13	2.73	.98	.48	2.63	.83	.92
4	G0114	H0180	OD0608	204	MB 54	.03	3.29	.90	.30	3.62	.41	1.13
5	G0117	H0210	OD0608	233	MB 148	.00	2.25	.68	.13	1.62	.67	.83

6	G0118	H0223	OD0608	245	Sirene	.03	2.40	.54	.15	1.23	.52	1.33
7	G0119	H0227	OD0608	249	Prof.Richard Hansen	.00	1.96	.47	.15	2.26	.55	1.65
8	G0120	H0233	OD0608	256	MB 162	.00	1.70	.64	.08	.98	.73	.74
9	G0121	H0242	OD0608	265	MB 171	.00	1.97	.38	.16	1.42	.52	.81
10	G0123	H0244	OD0608	267	MB 173	.00	2.44	.55	.11	1.37	.82	1.09
11		H0118	OD0307	118	Giganteus	.02	.95	.81	.41	1.35	.49	.42
12		H0119	OD0307	190	MS-90-2	.06	1.52	.77	.07	1.52	.78	1.06
13		H0120	OD0307	120	MS-88-110	.00	1.46	.68	.22	1.38	.56	.76
14		H0151	OD0608	175	MB 11	.01	2.25	.53	.20	2.45	.38	.76
15		H0165	OD0608	189	MB 31	.00	1.86	.62	.13	2.36	.56	.88
16		H0211	OD0608	234	MB 150	.00	2.45	.52	.29	2.07	.66	.92
17		H0238	OD0608	261	MB 167	.00	1.84	.42	.14	1.31	.69	.98
18		H0241	OD0608	264	MB 170	.31	2.27	.57	.54	3.46	.38	.66
19		H0248	OD0608	271	MB 190	.01	1.68	.67	.12	1.74	.43	.78
20		H0139	OD0608	(162)	EMI 5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

9.3 Double randomized block design

Figure 37 illustrates the randomized block design of the genotypes grown in the greenhouse. The first two left columns make up the first block; the next two the second block.

168	249	16	261
267	184	118	271
189	234	245	170
233	204	267	256
162	118	120	265
265	175	175	204
120	264	233	189
170	16	168	184
245	271	264	249
256	261	162	234

Figure 37. Double randomized block design of the greenhouse grown genotypes.

9.4 Morphological characteristics of the harvested stems

Table 24 - Table 27 provide basic information of the harvested plant material (i.e. stem internodes for gene expression and biochemical analyses) at the four stages of development.

Table 24. Harvested stems at first stage of development

Harvesting date	Acession Nr. / Genotype	No. leaves	Plant height (cm)	Base stem diameter (mm)	Max. broadness leaf (mm)	Leaves from previous season
3-3-2010	204.1 / 16.1	8	38	5	10	yes 1
3-3-2010	204.2 / 16.2	8	51	4	10	yes 2

3-3-2010	249.1 / 7.1	8	59	6	10	yes 3
3-3-2010	249.2 / 7.2	8	57	6	10	yes 3
3-3-2010	265.1 / 9.1	8	60	7	12	no
5-3-2010	265.2 / 9.2	7	54	4	10	no
5-3-2010	234.1 / 16.1	7	73	8	19	no
3-3-2010	234.2 / 16.2	8	44	5	12	yes 3
3-3-2010	175.1 / 14.1	12	120	13	22	yes
5-3-2010	175.2 / 14.2	9	130	10	23	yes
5-3-2010	189.1 / 15.1	12	108	12	26	yes
3-3-2010	189.2 / 15.2	11	97	11	23	yes

Table 25. Harvested stems at second stage of development

Harvesting date	Acession Nr. / Genotype	No. leaves	Plant height (cm)	Base stem diameter (mm)	Max. broadness leaf (mm)	Leaves from previous season
3-3-2010	204.1 / 16.1	15	161	9	24	yes 2
3-3-2010	204.2 / 16.2	13	140	6	22	yes 2
3-3-2010	249.1 / 7.1	12	130	6	14	yes 2
3-3-2010	249.2 / 7.2	14	146	7	13	yes 3
3-3-2010	265.1 / 9.1	14	156	7	23	yes 1
5-3-2010	265.2 / 9.2	13	160	7	18	yes 1
5-3-2010	234.1 / 16.1	12	198	8	16	yes 3
3-3-2010	234.2 / 16.2	12	169	10	18	yes, 3
3-3-2010	175.1 / 14.1	17	174	11	25	yes 6
5-3-2010	175.2 / 14.2	14	203	9	23	yes 3
5-3-2010	189.1 / 15.1	16	135	8	22	yes 3
3-3-2010	189.2 / 15.2	14	137	9	20	yes 3

Table 26. Harvested stems at third stage of development

Harvesting date	Acession Nr. / Genotype	No. leaves	Plant height (cm)	Base stem diameter (mm)	Max. broadness leaf (mm)	Leaves from previous season
3-3-2010	204.1 / 16.1	13	112	8	22	yes 1
3-3-2010	204.2 / 16.2	14	177	9	23	yes 3
3-3-2010	249.1 / 7.1	15	111	7	13	yes 4
3-3-2010	249.2 / 7.2	16	135	5	14	yes 4
3-3-2010	265.1 / 9.1	15	137	6	20	no
5-3-2010	265.2 / 9.2	16	194	6	21	yes 3
5-3-2010	234.1 / 16.1	16	256	12	26	yes 3
3-3-2010	234.2 / 16.2	16	252	9	25	yes 4
3-3-2010	175.1 / 14.1	18	206	14	24	yes 4
5-3-2010	175.2 / 14.2	17	214	9	23	yes 3
5-3-2010	189.1 / 15.1	16	150	5	20	yes 3
3-3-2010	189.2 / 15.2	16	150	5	20	yes 5

Table 27. Harvested stems at fourth stage of development

Harvesting date	Acession Nr.	No. leaves	Plant height	Base stem diameter	Max. broadness	Leaves from	Remarks
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						leaf	previous
/ Genotype			(cm)	(mm)	(mm)		season
3-3-2010	204.1 / 16.1	11	67	4	13	yes 1	slightly etiolated leaves
3-3-2010	204.2 / 16.2	14	147	6	21	yes 1	starting to flower slightly
3-3-2010	249.1 / 7.1	13	216	5	9	yes 1	slightly etiolated leaves
3-3-2010	249.2 / 7.2	12	197	5	14	yes 3	fully flowering
3-3-2010	265.1 / 9.1	14	97	4	22	yes 2	etiolated leaves
5-3-2010	265.2 / 9.2	14	161	5	16	yes 2	almost flowering
5-3-2010	234.1 / 16.1	15	214	7	18	yes 2	
3-3-2010	234.2 / 16.2	16	189	6	22	yes 2	
3-3-2010	175.1 / 14.1	21	244	6	23	yes 6	
5-3-2010	175.2 / 14.2	18	178	6	20	yes 6	
5-3-2010	189.1 / 15.1	15	177	6	20	yes 3	starting to flower
3-3-2010	189.2 / 15.2	21	115	6	18	yes 7	

9.5 DNA isolation protocol (Tanksley's modified method)

DNA was isolated from the leaves using the following protocol:

- 1 Place the leaves in a 2 ml tube with liquid nitrogen and mill it completely fine
- 2 Add 800 µl fresh buffer* (extraction buffer + lysis buffer + sarcosyl solution (5 % w/v) + sodium bisulfate) and vortex well
- 3 Place tubes in 65 °C bath for 1 hour (vortex 4x in between the hour)
- 4 Add 800 µl chloroform and shake the tubes carefully (40x)
- 5 Spin down for 5 minutes
- 6 Add 700 µl supernatant into new 1.5 ml tube
- 7 Add 560 µl isopropanol and shake the tubes carefully once again (40x)
- 8 Spin down for 5 minutes
- 9 Pour off the supernatant
- 10 Wash the pellet with 1000 µl 70 % ethanol
- 11 Dry the pellet and dissolve it in 50 µl TE buffer*
- 12 Measure the DNA concentration.

* Details of buffers are found in the books of the molecular lab of the Plant Breeding department.

9.6 DNA fragment isolation and cloning

Section 9.6 shows the technical details of how the poly-A tails were added to the purified PCR fragments (Section 9.6.1) and the molecular cloning took place in the competent cells (Section 9.6.2).

9.6.1 Addition of poly-A tails to the purified PCR fragments

<i>Master mix (for 15 samples):</i>	7.8 µl	dream taq. pol.
	19.5 µl	10x taq. buffer
	2.7 µl	0.25mM dATP
	2.7 µl	MQ

Total volume: 2.18µl per well + 10µl purified PCR fragments.

Incubation programme: 25' 72 °C, ∞ 10 °C.

9.6.2 Cloning procedure

<i>Ligation mixture:</i>	0.25 µl	vector (50ng/µl)
	1 µl	ligase
	5 µl	2x buffer
	3.75 µl	MQ

Total volume: 10 µl per sample and left over night at 16 °C.

9.7 Sequence PCR

The sequence PCR was carried out to synthesize forward and reverse fragments (of the isolated PCR fragments) needed for sequencing.

Forward and reverse PCR reaction mix

4 µl purified PCR fragments

4 µl DETT

0.5 µl M13F primer (forward reaction)/ M13R primer (reverse reaction) (10 pmol/µl)

1 µl MQ

Total volume = 10 µl per sample.

Sequence PCR programme: 30'' 94°C, (20'' 94°C, 15'' 50°C, 1' 60°C) 25x, ∞ 10°C.

9.8 Sephadex cleaning method

The sephadex cleaning method (see list below) was applied to purify the DNA fragment of the sequence PCR.

- 1 Add sephadex (G50-fine, Amersham) to the 96 well multiscreen filter plates (Millipore cat. No. MAHVN4550) using column loader (Millipore cat. No. MACL09645)
- 2 Add 330 µl MQ to the number of wells to be used
- 3 Let it stand for 4 hours at room temperature or overnight at 4°C
- 4 Place the waste plate under the filter plate and centrifuge for 5 minutes at 729g
- 5 Replace waste plate with collection plate and add the forward and reverse sequence PCR samples to the wells
- 6 Centrifuge for 5 minutes at 729g
- 7 Continue with sequence reaction or store at -20°C.

9.9 Adapted CTAB-MINI RNA extraction protocol

RNA was isolated from the stem internodes using the following protocol:

- 1 Crush and grind the internode completely in a cleaned mortar with liquid nitrogen
- 2 Add 400 µl pre-warmed CTAB buffer and incubate for 10 minutes at 65°C
- 3 Vortex thoroughly. Emulsify tissue/buffer mixture with 800 µl chloroform and vortex. Spin down in microfuge for 2 minutes at room temperature
- 4 Transfer top phase to fresh microfuge (700 µl), add the same amount of chloroform and vortex thoroughly. Spin in microfuge for 5 minutes at room temperature and carefully pour off supernatant
- 5 Add 500 µl wash buffer (80 % ethanol) and leave for 2 minutes. Spin down 3 minutes and discard supernatant with pipette
- 6 Dry pellet on ice and dissolve it in 100 µl MQ
- 7 Add 33 µl 8M LiCl. Place at -20°C for 30 minutes. Spin down (cold centrifuge) for 30 minutes at 14.000 RPM
- 8 Rinse with cold 80 % ethanol. Spin down for 2 minutes at 14.000 RPM (cold centrifuge)
- 9 Dry pellet and dissolve in 25 µl MQ.

9.10 Mass of samples in biochemical analysis

Table 28. Mass of samples in biochemical analysis.

Sample	Stage of development	Sample mass (g)
14	1	.19
15	1	.08
4	2	.45
7	2	.29
9	2	.34
16	2	.49
14	2	.50
15	2	.39
4	3	.27
7	3	.26
9	3	.35
16 (rep.1)	3	.53
16 (rep.2)	3	.51
14	3	.49
15	3	.33
4	4	.21
7	4	.48
9	4	.28
16 (rep.1)	4	.50
16 (rep.2)	4	.52
14	4	.51
15	4	.28

9.11 Conducted activities during thesis study

During the period of research a wide range of activities were conducted. Table 29 provides the chart illustrating which activities were carried out during the 36 week period.

Table 29. Conducted thesis project activities during the study.

Project activity	January				February				March				April				May				June				July				August				September			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Review literature																																				
Write proposal																																				
Make a planning																																				
Stem internode analysis																																				
Molecular lab activities																																				
Collecting stem material																																				
Gene expression																																				
Biochemical analysis																																				
Holidays/break																																				
Write thesis report																																				
Examination of draft thesis report																																				
Examination of final thesis report																																				
Colloquium and thesis defence																																				

