LIGNIN BIOSYNTHESIS IN WHEAT BIOMASS AND ITS RESPONSE TO GENETIC AND ENVIRONMENTAL VARIATIONS

By

Tran Nguyen Nguyen

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ABSTRACT

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Production of bioethanol from lignocellulosic biomass, particularly from cereal crop residues such as wheat straw, has been considered as a viable approach to meet the ever increasing demands for energy without affecting the quality of our environment. However, lignin, one of the major constituent of lignocellulose that contributes mechanical strength to plants, hinders the success of efficient production of bioethanol as it confers recalcitrance of lignocellulosic biomass to hydrolysis. Genetic reduction of plant biomass lignin content or alteration of its composition without affecting its agronomic functions, can reduce biomass recalcitrance, however, application of this strategy requires a detailed understanding of the molecular mechanisms underlying lignin synthesis in plant tissues. To this end, this thesis performed comprehensive analysis of the expression of wheat lignin biosynthesis candidate genes and identified genes that are predominant across different tissues. Using three commercial wheat cultivars that exhibit variation in stem resistance to lodging, it investigated the association of expression of these predominant genes with tissue lignin content. Furthermore, this identified transcriptional changes mediating the response of lignin biosynthesis in wheat to changes in soil moisture conditions.

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ABBREVIATIONS

AC (AC-rich elements)	cytosine and adenosine
ADF	acid detergent fiber
ARF	auxin response factor
ATAF	*Arabidopsis thaliana* activating factor
CA	coniferyl alcohol
CAD-CA	catalytic activity of CAD to covert coniferaldehyde to coniferyl alcohol
CAD-SA	catalytic activity of CAD to covert sinapaldehyde to sinapyl alcohol
cDNA	complimentary deoxyribonucleic acid
CO_2	carbon dioxide
CUC	cup-shaped cotyledon
DEPC	diethylpyrocarbonate
DNase	deoxyribonuclease
ER	endoplasmic reticulum
EST	expressed sequence tag
G	guaiacyl
H	hydroxyphenyl
K_2O	potassium Oxide
KNOX	knotted1-like homeobox
LSD	least significant difference
MYB	myeloblastosis
N	nitrogen
NAC	NAM, ATAF1,2, CUC2

NAD^+	oxidized form of nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenine dinucleotide
NAM	no apical meristem
NDF	neutral detergent fiber
PCR	polymerase chain reaction
qPCR	real-time quantitative PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase- polymerase chain reaction
S	syringyl
SA	sinapyl alcohol
SND	secondary wall-associated NAC domain

FORWARD

This thesis is written in manuscript style. A general introduction about the research project and a literature review precedes the manuscript. An abstract, introduction, materials and methods, results and discussion form a complete manuscript. The manuscript is followed by a general discussion and conclusions, a list of references and appendices.

1. GENERAL INTRODUCTION

At present more than 80% of world energy consumption comes from fossil fuel (United States Energy Information Administration [USEIA], 2012). The United States Central Intelligence Agency (USCIA) World Factbook (2014) predicts that if we continue consuming fossil fuel at the present rate, this energy source would run out by the end of this century. Moreover, fossil fuel use is the primary factor contributing to global warming (Intergovernmental Panel on Climate Change [IPCC], 2014). These problems have triggered increased efforts to utilize plant biomass as a feedstock for the production of biofuel and bioproducts. A major form of biofuel currently in use is bioethanol, which is primarily produced from sugar cane (Brazil), sugar beet (European Union) and other cereal crops such as corn and wheat (Canada, China and United States of America) (United States Department of Energy [USDE], 2013). The use of these crops as a source of biofuel has caused the competition between fuel and food demands leading to the 'Food versus Fuel' debate (European Biofuels Technology Platform [EBTP], 2013b). Therefore, the use of lignocellulosic plant biomass for the production of bioethanol could be a win-win solution. A potential biomass resource that has attracted significant study efforts is the residues from the cultivated food crops such as rice, wheat and corn (Demura and Ye, 2010). The plant remains, especially stems and leaves, after grain collection in these crops contain a great amount of lignocellulosic compounds that can be used to generate renewable and storable liquid transportation fuels (Simmons et. al, 2010).

Plant biomass is composed of cell wall, which in turn consists of cellulose, hemicellulose, pectin and lignin. One of the major impediments for conversion of lignocellulosic biomass to biofuels is the contents and composition of lignin as it interferes with the digestion of cell wall polysaccharides either chemically or biochemically. Therefore, optimization of biomass lignin

content of crop plants is one of the viable strategies to produce biofuels and bioproducts from crop residues in an economically efficient way (Simmons et al., 2010). However, lignin also plays a role in structural support and defensive system of plants (Vanholme et al., 2010a), and thus the ideal lignin content and composition would be the one that can reduce the delignification cost without compromising the agronomic performance of plants. Lignin is a phenolic polymer synthesized from phenylalanine through the general phenylpropanoid and monolignol-specific pathways, which involve ten enzymes that appeared to be encoded by gene families (Vanholme et al., 2010a). Manipulation of the expression of lignin biosynthetic genes in several species has been successful to produce plants with lower lignin content and altered lignin composition that could facilitate the conversion of cell walls to bioethanol (Burton and Fincher, 2014).

Previous studies have analyzed the spatio-temporal expression pattern of candidate lignin biosynthesis genes and their response to pathogen attack, and the expression of three wheat lignin genes has been investigated in relation to stem lignin content and lodging resistance (Ma, 2007; 2009; 2010; Bi et al., 2011). Despite these results, very little is known about the molecular mechanisms regulating lignin deposition and stem mechanical strength in wheat, and the response of wheat lignin biosynthesis to abiotic stress factors such as excess soil moisture is poorly understood. To gain further insights into the subject matter, this thesis performed a comprehensive and comparative transcriptomic analysis of wheat lignin biosynthetic genes in different tissues along with measurements of the activities of selected lignin biosynthetic enzymes and profiling the levels of lignin and other constituents of lignocellulose including cellulose and hemicellulose. Moreover, the study investigated molecular elements mediating the response of lignin biosynthesis to excessive soil moisture using both molecular and biochemical approaches. The findings of this study have the potential to enhance our understanding of lignin biosynthesis in wheat, which is a

requirement to develop molecular tools to facilitate wheat breeding for enhanced feedstock quality of wheat biomass/straw.

2. LITERATURE REVIEW

2.1 Background

At present the world fossil energy reserves are about 900 billion tons oil equivalent (USEIA, 2012). While the demand for energy constantly increase year by year, the USCIA World Factbook (2014) predicts that if we continue consuming fossil fuel at the present rate, this energy source, which took hundreds of millions of years for its formation, would run out by year 2088. The 2012 USEIA report indicated that 81.1% of the primary energy consumed globally in the year 2011 was derived from fossil fuel. This portion of fossil fuel consisted of oil (32.4%), coal/peat (27.3%) and gas (21.4%). Fossil fuel use contributes 57% of global CO_2 emission, making it the primary factor of global warming (IPCC, 2014). Therefore, the current energy-use patterns can lead not only to the exhaustion of fossil fuel supplies but also to the unsustainable earth environment. Biofuels are one of the solutions to mitigate fossil fuel-associated problems because biofuels are renewable and the use of biofuels releases much less greenhouse gases as compared with fossil fuels (Ryan et al., 2006)

Biofuel is the fuel produced from carbon that is fixed biologically (Giampietro et al., 1997). Biofuels, particularly bioethanol, can be produced from starch and sugar derived from crops such as corn, wheat, sugarcane and sugar beet (Ajanovic, 2011). They can also be produced from lignocellulosic biomass of energy crops that do not compete with food crops for lands; wastes and residues of agriculture, forestry and other organic materials productivity; and other novel sources such as algae (EBTP, 2013a). Although the share of biofuel to the world total primary energy supply was less than 10% until 2010 (USEIA, 2012), its use and demand has been increasing continually. However, the use of starch and sugar as sources for biofuel has affected the global

food price dynamics, and recently the Food and Agriculture Organization of the United Nations (FAO) called for a renewed debate on this issue (EBTP, 2013a). Although the efficiency of converting lignocellulosic biomass to biofuels is still lower as compared with sugar and starch (USDE, 2014), the use of lignocellulosic biomass for biofuel production has therefore been given due consideration (Simmons et al., 2010).

2.2 Biofuels

2.2.1 Introduction to biofuels

Biofuels are solid, liquid or gaseous fuels that are produced from material of biological origin, referred to as biomass (Giampietro et al., 1997; International Energy Agency, 2011). Biomass does not include the material of biological origin that is embedded in geological formations and transformed to fossil (FAO, 2009a). Biofuels can be produced from plant matter, vegetable oils, animal fats, algae, municipal solid waste, and sewage. The conventional biofuels or first generation biofuels, whose production have been established, are used on a commercial scale and are produced using crops grown mainly for food. On the other hand, advanced biofuels or second/third generation biofuels, which are produced from non-food feedstocks (biomass), are currently in the phases of research, development and demonstration (United Nations Environment Programme [UNEP], 2009). The primary conventional biofuels used on a commercial-scale today are bioethanol, biodiesel and biogas. Conventional bioethanol is made from the fermentation of sugary/starchy crops, while conventional biodiesel can be produced from oil crops such soybean and oil palm; and they can be blended with regular gasoline and diesel, respectively. Biogas, mainly methane, is made from the anaerobic fermentation of manure biomass and used for cooking, heating, and fueling up natural gas vehicles (Giampietro et al., 1997). Advanced biofuels

also include bioethanol, biodiesel and biogas; however they are produced from woody biomass, grasses, agricultural by-products, algae, seaweed, municipal solid waste and sewage. Next generation biofuels would be the longer chain alcohols such as n-butanol, iso-butanol and pentanol, and isoprenoids.

In 2002, the global biofuels production was equivalent to about 12 million tons of oil. It increased constantly and reached 60 million tons in 2012 (British Petroleum [PB], 2014). Only 38 billion liters of bioethanol was produced worldwide in 2005, and it was double in 2009 and expected to reach 120 billion liters by the year 2017. Biodiesel production also sharply increased from 4 billion liters in 2005 to 18 billion liters in 2009, however the increase has since then slowed down and the production may not exceed 20 billion liters by 2017 (Food and Agricultural Policy Research Institute, 2008).

2.2.2 Advantages of biofuels over fossil fuels

The use of biofuels can reduce greenhouse gas emission which causes climate change, increased independence from using non-renewable fossil fuels, and thereby enhance energy security. At the beginning of the industrial age (1760s), the CO_2 concentration in the air was 260 ppm; and after nearly two hundred years by the end of World War II, it was still only 320 ppm. At present CO_2 concentration has just crossed the 400 ppm mark (IPCC, 2014). Burning fossil fuels is responsible for more than half of this increase, and it is the primary reason for global warming, which can be noted by abnormal events such as the melting of mountain glaciers, Greenland ice sheet and arctic sea ice, the rise of sea level by ~ 3 mm per year and increases in the frequency and intensity of severe weather (IPCC, 2014). Despite this fact, the global rate of fossil fuel consumption is rising mainly due to the dramatic rise of energy demand in newly industrialized countries, particularly

China and India. From 1980 to 2004, the oil consumption in China and India alone increased about 3.5 times (PB, 2014). Therefore, if the fossil fuel consumption continues at such rate, we not only cause almost irreversible damage to our ecosystem but also put energy security at risk as all of oil, charcoal/peat, and gas would be depleted by the end of this century (USEIA, 2012). McGlade and Ekins (2015) reported that to maintain the average global temperature throughout 21st century at a level not exceeding 2^{o}C above the average global temperature of pre-industrial times, 80% of current coal reserves, half of the gas reserves, and a third of the oil reserves around the world should remain unused for a period of 40 years (2010-2050). Thus far, biofuel shares only about 10% of total primary energy supply, and increasing its use is one of the promising solutions for these fossil fuels associated issues. A major portion of CO_2 emitted by the production and use of biofuel will get absorbed by the biomass, which then will serve as the feedstock of the next cycle of biofuel production (Ryan et al., 2006).

2.3 Bioethanol

2.3.1 Conventional bioethanol

Conventional bioethanol is sugar-based ethanol and starch-based ethanol. The production of sugar-based ethanol, particularly which from sugar cane requires less upstream processing and has highest yields. In contrast, the production of starch-based ethanol requires extensive upstream processing to break down amorphous polymers into monomers (Gray et al. 2006).

2.3.2 Bioethanol from lignocellulosic biomass

Plant biomass is composed of cell wall, which in turn consists of cellulose, hemicellulose, pectin and lignin; the content of cellulose and hemicellulose that can be converted to ethanol are about

80% in both crop and woody plants (McKendry, 2002). There is a wide range of biomass feedstock for production of advanced bioethanol; it can be produced from the agricultural or forest residues (straw, corn stover, logging wastes), non-food crops/trees (hemp, willow) and energy plants (switchgrass, *Miscanthus*). Therefore, an apparent advantage of lignocellulose-based ethanol production is the availability of biomass feedstock. For example, in recent years, about 400 million ton of biomass per year is available in USA and this is expected to reach 600 million ton per year by 2020, which, if converted to bioethanol, can meet about 21% of the transportation fuel demand (Union of Concerned Scientists, 2012). Thus far, the production of advanced bioethanol is lower than that of conventional bioethanol. At present about 330 liters of ethanol can be obtained from one ton of dry lignocellulose-based feedstock while about 380 liters and over 700 liters of ethanol can be obtained from one ton of dry starch-based feedstock and sugar-based feedstock, respectively. However lignocellulose-based ethanol production shows a much higher energy balance (energy out/energy in ratio) as compared to the conventional ethanol. The energy balance of wheat straw or hemp ethanol is about 8.5 and it is as high as over 30 in *Miscanthus* or willow ethanol; this ratio is only 1.5 in conventional ethanol (Tan et al., 2008). In addition, more CO_2 emission can be saved by using advanced ethanol instead of conventional ethanol. While the CO_2 equivalent emission savings per 1000 L ethanol are only 1.2 ton and 0.4 ton for sugar and starch crops, respectively, they are 2.5 ton for either lignocellulose residues or lignocellulosic energy plants (Ryan et al., 2006). To solidify the advantage of lignocellulose-based ethanol, a number of energy crops, such as *Miscanthus*, switchgrass, reed canarygrass, willow, poplar and alder have been developed to meet the required features including ability to grow on marginal land; lower requirement of pesticide, water and fertilizer inputs; perennial growth habit; high biomass yield; and increased ethanol productivity and quality (Chen and Dixon, 2007; Simmons et al., 2010;

Burton and Fincher, 2014).

2.3.3 The conversion of lignocellulosic biomass to bioethanol

Lignocellulose biomass can be converted to bioethanol by a three step process (Keating et al., 2006). The first step, which is called pretreatment, reduces the interactions between lignin and cell-wall polysaccharide components. Different pretreatment methods have been used, including the use of steam, liquid hot water, dilute acid, ammonia, lime, carbon dioxide and organosolve. The second step involves the production of hydrolytic enzymes (cellulase/hemicellulase) which are then used for hydrolyzing the polysaccharide, converting cellulose and hemicellulose to simple hexose/pentose sugars (saccharification). Finally ethanol is obtained from sugar (C5 and C6 sugars) fermentation.

A new technology called Consolidated Bioprocessing has been developed based on the use of microorganisms that are able to digest cellulose and hemicellulose components of biomass. In this method, the enzymes production, hydrolysis (step 2) and fermentation (step 3) would be combined into a single step offering lower cost and higher efficiency than non-integrated processes (Lynd et al. 2005). Increased effort has also been put in the improvement of lignocellulose biomass, mainly modifying lignin content as well as its composition (Li et al., 2008; Simmons et al., 2010; Fu et al., 2011a), to reduce the cost of the pretreatment step.

2.3.4 Lignin limits the digestion of cell wall polysaccharides

The formation of lignin-carbohydrate complex in plant cell wall makes the plant biomass recalcitrant to microbial, enzymatic and chemical digestion. The linkages that connect carbohydrate with lignin include phenyl glycoside, benzyl ether, γ-ester, and hydroxycinnamates.

Lignin contains a significant amount of *p*-coumaric and ferulic acids, which are known to be involved in cross-linking lignin and cell wall polysaccharide. The *p*-coumaric and ferulic acids, for example, constitute about 6% and 9%, respectively, of wheat straw (Zeng et al., 2013), Ferulic acid is etherified with lignin and esterified with cell wall polysaccharide; on the other hand, *p*-coumaric acid is extensively esterified with lignin (Eraso and Hartley 1990; Jacquet et al., 1995; Ralph et al., 1995). Ferulate esters play a more important role as they can be oxidatively cross-linked in many ways (Scheller and Ulvskov 2010), and the ferulate or diferulate esters have been found to cross-link the chains of glucuronoarabinoxylan and/or lignin (Grabber et al., 2002; Obel et al., 2002). It has been reported that ferulate related linkages are predominant in wheat straw followed by benzyl ether and γ-ester linkages (Zeng et al., 2013). In woody lignin, however, phenyl glycoside, benzyl ether and γ-ester linkages are the predominant ones. These cross-links, together with the impermeability of lignin, limit the accessibility of cell wall polysaccharide to hydrolytic enzymes, making lignocellulosic biomass recalcitrant to enzymatic saccharification. Moreover, lignin can also adsorb hydrolytic enzymes. Thus, the efficient conversion of lignocellulose biomass into ethanol requires the reduction of these cross-links as well as the removal of lignin through costly pre-treatment (Mielenz, 2001, Keating et al., 2006, Chen and Dixon, 2007). This fact has triggered increased effort to improve biomass feedstock quality through modifying lignin content as well as its composition (Li et al., 2008; Simmons et al., 2010; Fu et al., 2011a).

2.3.5 Advantages of advanced biofuels over conventional biofuels

Conventional biofuels are produced using available technology and crops that are grown for food production. For example, conventional bioethanol is made from the fermentation of sugar or starch derived from important food crops such as sugar cane, sugar beet, corn and wheat; and biodiesel is obtained from oil palm, soybeans and canola (USDE, 2013). These crops have been developed

mainly for the purpose of food not for biofuel production, therefore the production of conventional biofuels can cause not only the Food versus Fuel debate (EBTP, 2013b), but also other problems such as deforestation (to have more land for crops) and limited greenhouse gas savings (due to the low energy out/energy in ratio). In contrast, advanced biofuels will be made mainly from algae, agricultural/forestry waste/by-product, municipal solid waste, and grasses and trees that have been developed for this purpose, thereby having advantages over conventional biofuels in terms of sustainability of feedstock (more available, efficient land-use, and maintaining biodiversity) and greenhouse gas savings (UNEP, 2009).

2.4 Bioethanol and wheat productions in Canada

2.4.1 Production and consumption of fuel ethanol

Canada consumed about 800 million liters of fuel ethanol in the year 2006 alone. The consumption has increased since then and appeared to be doubled every four years; it was 1,600 million litres in 2010 and expected to be almost 30,000 million liters in 2014. The consumption of fuel ethanol exceeds the production every single year. In 2006, Canada produced only 200 million litres, meeting only 25% of its consumption, and the production has since reached about 70% of the demand. For example, 1,200 million litres was produced in 2010 and about 2000 million litres is predicted to be produced in 2014 (Global Agricultural Information Network, 2013). To date, all of bioethanol produced in Canada come from starch-based feedstock (wheat and corn). The pilot plants for lignocellulosic ethanol are currently under construction, and the first commercial plant is expected to be deployed during 2015 to 2020 (Barnabé, 2014).

2.4.2 Wheat straw - a potential feedstock

Wheat, barley and oats are the major cereal crops produced in the Canadian Prairies. In 2013 alone,

over 34.7 million tons of wheat and approximately 13 million tons of barley and oats were produced from 11.2 million hectares (Statistics Canada, 2014). It can be deduced from this figure that a similar amount, 37.5 million tons, of wheat straw was also produced, of which about 11 million tons could be used for soil conservation protecting against wind and water erosion (Sokhansanj et al., 2006). Wheat straw is also used for livestock production as feeding and bedding materials, which consumes about 6 million tons every year (Sokhansanj et al., 2006). Therefore, a great amount of wheat straw, up to more than 20 million tons, appears to be available every year for conversion into bioethanol without any competition for its use in protecting the soil and livestock production.

2.4.3 Abiotic stress and crop agriculture in the Canadian Prairies.

The Canadian Prairies region that has been used for crop cultivation is classified as semi-arid desert steppe with a cool continental climate (Phillips, 1990). The growing seasons (frost-free period) of these regions are short, usually less than 115 days, during which plants could experience two key abiotic stresses, temperature (cold and heat) and water deficit stress (Bueckert and Clarke, 2013). The total precipitation across the cropping regions of the Canadian prairies is only 300 to 450 mm annually with infrequent rainfall events; and the total heat units is only from 1400 to 2300, but summer day time temperature sometimes can exceed 32^{o}C (Padbury et al., 2002). Therefore, the major factor for the success of crop production in this region has mainly been crop adaptation to these stress conditions, particularly cool temperature and water deficiency (Bueckert and Clarke, 2013). Winter wheat needs to have the abilities to survive the frozen soil for many winter months; spring wheat requires tolerance to cold stress that can occur in the spring when plants germinate and emerge. In addition, cold stress caused by late spring frost, early fall frost could be damaging

when plants are at the stages of flowering and crop maturity, respectively. Wheat plants can also be exposed to water-deficit stress at any time during the growing season, although it is more likely to occur after flowering (Bueckert and Clarke, 2013).

2.4.4 Excessive soil moisture as an abiotic stress factor

In addition to the above stress factors, flooding/excessive soil moisture can be a problem in some years for wheat plants grown on low lying fields of the Prairies (Environment Canada, 2010; Bueckert and Clarke, 2013). Since snowmelt runoff is a main reason for the occurrence of excessive soil moisture, global warming and other climate changes are more likely to make the problem increasingly serious. A report on the amount of precipitation in the Prairie region during the period of April 1 to September 9, 2014 indicated a great portion of Manitoba and Saskatchewan received 115 to 200% of the average precipitation (Agriculture and Agri-Food Canada, 2014). Such excess precipitation might affect the local agriculture including the production of wheat which is a major cultivated crop in the region.

Waterlogging can cause hypoxia (deficiency of oxygen) or anoxia (complete absence of oxygen) in the soil environment. The response of wheat plants to this stress condition might involve morphological and metabolic changes. Some of the changes include development of aerenchyma channel to transfer oxygen from shoot to root, enabling the root to aerobically respire under anaerobic condition. There are two types of aerenchyma in stems and roots, lysigenous and schizogenous, which are developed through two different processes. While the separation of cells from each other creates schizogenous aerenchyma (Huang et al., 1997; Colmer et al., 2004), lysigenous aerenchyma is formed by the death of cells in the primary cortex (Haque et al., 2010). Some wheat genotypes may even develop barriers to reduce radial oxygen loss, which refers to the

loss of oxygen in aerenchymatous roots to the rhizosphere via radial diffusion, by increasing the deposition of suberin or lignin on the epidermis or exodermis of root (Hossain and Uddin, 2011). In wheat, aerenchyma can be formed within five to seven days after the start of waterlogging (Thomson et al., 1990).

Metabolic changes due to excessive soil moisture are mainly those reflecting the shift of energy metabolism from aerobic to anaerobic strategy (anaerobic respiration). This includes increased glycolytic and fermentative enzyme activities, mainly for ethanolic fermentation or lactate fermentation to recycle the NADH producing NAD^+ required for glycolytic pathway (Ricard et al., 1994; Drew, 1997), elevated content of soluble sugar that acts as a substantial storage of carbohydrates for fermentation; and stimulation of oxidative stress resistance mechanism. Wheat plants suffering from excessive moisture exhibit reduced root growth, reduced number of tillers and dry matter; and their leaves senesced prematurely (Hossain and Uddin, 2011). Root growth is more affected by excessive soil moisture as compared to that of shoots. Huang et al. (1994) reported a reduction in total root dry weight in all wheat genotypes included in their study except for one genotype after 14 days of waterlogging treatment; particularly, the total length of seminal roots in the sensitive genotypes was reduced by up to 50% as compared to that of the control plants.

2.5 Lignin

2.5.1 Functions of lignin in plants

Lignin, a complex phenolic polymer, is the second most abundant organic carbon after cellulose (Battle et al., 2000). Lignin is a constructional element of vascular plant cell wall, particularly of the secondary wall, and is also found in the middle lamella region between cells (Ralph et al.,

2004). Lignin is deposited in the walls of the cells that are specialized for mechanical support and conduction, mainly sclerenchyma cells (fibres and sclereids) and tracheary elements (tracheids and vessel member [or vessel element]). It imparts increased strength, rigidity and impermeability (hydrophobicity) to the cellulose and hemicellulose matrix enabling the plant to withstand gravity, mechanical stress, and pressure caused by transpiration (Roger and Campbell, 2004; Bonawitz and Chapple, 2010). Lignin can also be found in cell wall of root endodermal tissue, anthers and seed pods (Zhong and Ye, 2009). Lignin makes the cell wall rigid by filling the space between cellulose-hemicellulose microfibrillar elements as an adhesive; and the non-condensed GS units (G and S units are connected by β- 0-4 interunit linkages) might be responsible for the cohesion among these microfibers (Chabannes et al., 2001).

Glucuronoarabinoxylan is the major hemicellulose in grass cell walls, particularly in the secondary cell wall (Scheller and Ulvskov, 2010). The hydrogen-bonds between the stretches of unsubstituted glucuronoarabinoxylan and cellulose severs as the cross-links among cellulose microfibrils (Carpita, 1996); and ferulic acids (also *p*-coumaric acids) form ferulate esters or diferulate esters not only with the glucuronoarabinoxylan chains but also with that of lignin (Grabber et al., 2002), cross-linking the chains of glucuronoarabinoxylan and/or lignin. A study by Obel et al. (2002) showed the relationship among hemicellulose deposition, lignin deposition and the contents of ferulic and *p*-courmaric acids in the leaf of wheat seedlings when the majority of cells in the tissue transition from division and expansion stage to mature stage, implying that lignin and hemicellulose at some level function similarly in strengthening cell wall rigidity.

The crosslinking of polysaccharides by lignin also makes the cell wall impervious, owing to the hydrophobicity of lignin, while other plant cell wall polysaccharides are hydrophilic (Campbell and Sederoff, 1996). As the result, the functions of supporting and conducting tissues

in plant are closely related to lignin content and structure (Vanholme et al., 2010a). However, in order to get lignin with different physiological functions, e.g. water or nutrient conduction and resistance to compression forces, specific tissues biosynthesize different forms of lignified polymeric matrices (Anterola and Levis, 2002). Lignin also plays important role in plant response to biotic stresses such as infection by pathogens and abiotic stresses such as low temperatures, water deficit, excessive light, mechanical injuries and abnormal concentration of mineral element in the soil (Moura et al., 2010; Moura-Sobczak, 2011). For example, the phenylpropanoid pathway, which takes part in lignin biosynthesis, has been reported to respond to pathogen attack (Dixon et al., 2002, Naoumkina et al., 2010). Lignin formation will reinforce the cell wall, and the complexity and insolubility of the lignin polymers makes the cell wall more resistant to degradation by microorganisms (Campbell and Sederoff, 1996). However, lignin biosynthesis is a highly energy-consuming and irreversible process (Zhao and Richard, 2011), and delignification during bioethanol production is a costly process. Given all these, the ideal lignin content and composition would be the one that contributes to the reduction of delignification cost while maintaining all lignin-related beneficial traits such as its contribution to resistance to lodging and pathogen attack.

2.5.2 Lignin and stem mechanical strength in cereal crops

Stem mechanical strength, the bending strength of the culm, in cereal crops such as wheat is closely associated with not only stem weight and diameter, but also structure and composition of the cell wall, particularly cellulose and lignin, (Kokubo et al., 1991; Li et al., 2003; Wu et al., 2011; Tripathi, 2013). In most cereal crops, stem strength is the main determinant of resistance to lodging, which is a well-known factor in affecting harvesting efficiency, yield and quality (Foulkes

et al., 2011). Cell walls of wheat stem consist mainly of cellulose, hemicellulose, pectin, protein and lignin; and lodging resistance has been reported to be closely associated stem cellulose content (Tripathi et al., 2003; Wang et al., 2012).

Through comparative analysis of stems derived from 16 wheat cultivars with different lodging resistance behavior, Wang et al. (2012) concluded that the contribution of cellulose in conferring lodging resistance is more than that of lignin, indicating that cellulose is the primary component responsible for the differences. This is well supported by the inconsistent relationship observed between lodging resistance and stem lignin content. For example, Mulder (1954) found low lignin content in the basal internodes of lodged wheat, and Tripathi et al. (2003), by studying 12 wheat genotypes, reported a negative relationship between stem lignin content and lodging score; higher lodging score inferring greater susceptibility to lodging. However, other studies, which involved the use of plant growth regulators, chlormequat chloride and ethephon, in wheat, revealed no association between stem lignin content and enhanced lodging resistance (Clark and Fedak, 1977; Knapp et al., 1987) Furthermore, a study that involved comparative analysis of lodging susceptible (Arthur 71) and lodging resistant (Auburn) cultivars reported no association between lignin content and lodging resistance (Knapp et al., 1987), and the authors suggested that the arrangement and interaction of lignin and other cell wall structural carbohydrates may play a more important role in lodging resistance than the concentration of these components.

The relationship between lignin content and lodging resistance could vary with growth stage. The second basal internode derived from two wheat cultivars with contrasting lodging resistance behavior showed no substantial difference in lignin content at elongation stage; however at the heading and milky stages, the lodging resistant cultivar exhibited 66.3 and 42.1%, respectively, higher lignin content than that found in susceptible cultivar (Ma, 2009). Although the

close association between lodging resistance and stem lignin content has not always been apparent as observed for cellulose, recent studies appear to increasingly strengthen the covariation of stem strength/lodging resistance and stem lignin content (Ma, 2009; 2010, Wu et al., 2011; Wang et al., 2012). Therefore, stem lignin content can be considered as an important trait for breeding wheat varieties with enhanced lodging resistance. In addition to structural carbohydrates, stems of crop plants also contain storage carbohydrates that can influence stem strength. For example, resistance to typhoon induced lodging in the S1 line of rice, which is a near isogenic line derived from the advanced backcross progeny of Koshihikari and Kasalath cultivars and that includes a QTL for resistance to lodging during typhoon (*lrt5*), has been attributed to the high accumulation of starch in the stem tissues (Ishimaru et al., 2008).

2.5.3 The structure of lignin

Lignin is an aromatic heteropolymer in which the monolignols, coniferyl alcohol, sinapyl alcohol, and p-coumaryl alcohol are the three main building blocks. They are incorporated by successive dehydrogenative polymerization reaction, giving rise to guaiacyl (G), syringyl (S) and hydroxyphenyl (H) units, respectively, in the polymer of lignin (Ma and Xu, 2008). The monolignols are coupled randomly by various interunit linkages, such as aryglycerol-β-ether dimer, resinols, phenylcoumaran, spirodienone, and dibenzodioxin, leading to the irregular three-dimensional reticulated structures of lignin (Buranov and Mazza, 2008; Crestini et al., 2011). The composition of lignin varies with species (Campbell and Sederoff, 1996; Rogers and Campbell, 2004; Bonawitz and Chapple, 2010), plant tissue, cell type (Nakashima et al., 2008; Ruel et al., 2009) and subcellular location (cell wall layers) (Shi et al., 2006; Gierlinger and Schwanninger, 2007; Gou et al., 2008; Ruel et al., 2009). In general, lignin in water-conducting cells of xylem,

which is the major tissue of secondary wall formation, tends to be enriched with the G unit, while high levels of the S unit are found in angiospermous structural fibers (Bonawitz and Chapple, 2010). Lignin of herbaceous plants contains all three lignin units, while the lignin of gymnospermous woody plants (softwood) contains mainly G units and small amounts of H units, and the lignin of angiospermous woody plants (hardwood) is composed of G and S units (Capanema et al., 2004; Capanema et al., 2005).

Differences in genetic background and environment may also result in the incorporation of many other molecular species, such as, ferulates (forming crosslinks between lignin and hemicellulose), coniferaldehyde, and units containing acetate, *p*-coumarate, or *p*-hydroxybenzoate moieties (Ralph et al., 2004; Ralph et al., 2008b). As the lignin units are different in their bonding propensities, the overall structure of a particular lignin polymer, which determines its physical properties, is largely impacted by the composition of lignin polymer (Buranov and Mazza, 2008; Crestini et al., 2011).

In angiospermous plants, the maturation of cell walls is largely dependent on the deposition of S lignin in the secondary cell wall (Li et al., 2003); and it has been shown in *Populous trichocarpa* that S lignin is preferentially deposited in the middle lamella, while the inner cell wall is the preferential site for G lignin (Zhou et al., 2011). The S/G ratio of lignin affects its structural characteristic through the amount of lignin functional groups (such as methoxyl groups; every G and S unit has one and two methoxyl groups, respectively) and the formation of various interunit linkages between the monolignols, which can lead to changes in the three dimensional reticulate structure of lignin polymers (Zeng et al., 2013). For example, an increase in the proportion of coniferyl alcohol increases the number of beta-5-linked dimer, which influences the final composition and structure of lignin (Ma and Xu, 2008). It has been suggested that the sensing

mechanism of plants might be able to recognize not only the altered structure of the cell wall such as reduced contents of cell wall component such as cellulose and lignin but also the difference in lignin composition (mainly S/G ratio) as the causal signal initiating transcriptional feedback to phenylpropanoid pathway (Vanholme et al., 2012a).

In wheat, G and S are the predominant lignin units. Zeng et al. (2013) showed that milled straw lignin fraction and cellulose-digested lignin fraction of wheat straw (*Triticum aestivum*) have S:G:H ratios of 37.5: 60.2: 2.3 and 40.1: 57.8: 2.1, respectively. A similar S:G:H ratio, 30:64:6, was also found in the milled straw lignin of *Triticum durum* (Río et al., 2012). Lignin of wheat straw contains about 110-116 functional methoxyl groups per 100 aromatic rings compared with more than 160 groups in hardwood (Zeng et al., 2013). In addition, the milled straw lignin fraction of these wheat species contained a significant amount of *p*-coumaric and ferulic acids (6% and 9% of the total S+G+H for *T. aestivum* and *T. durum*, respectively), which are known in forming cross-linkage between lignin and cell wall carbohydrates in grasses.

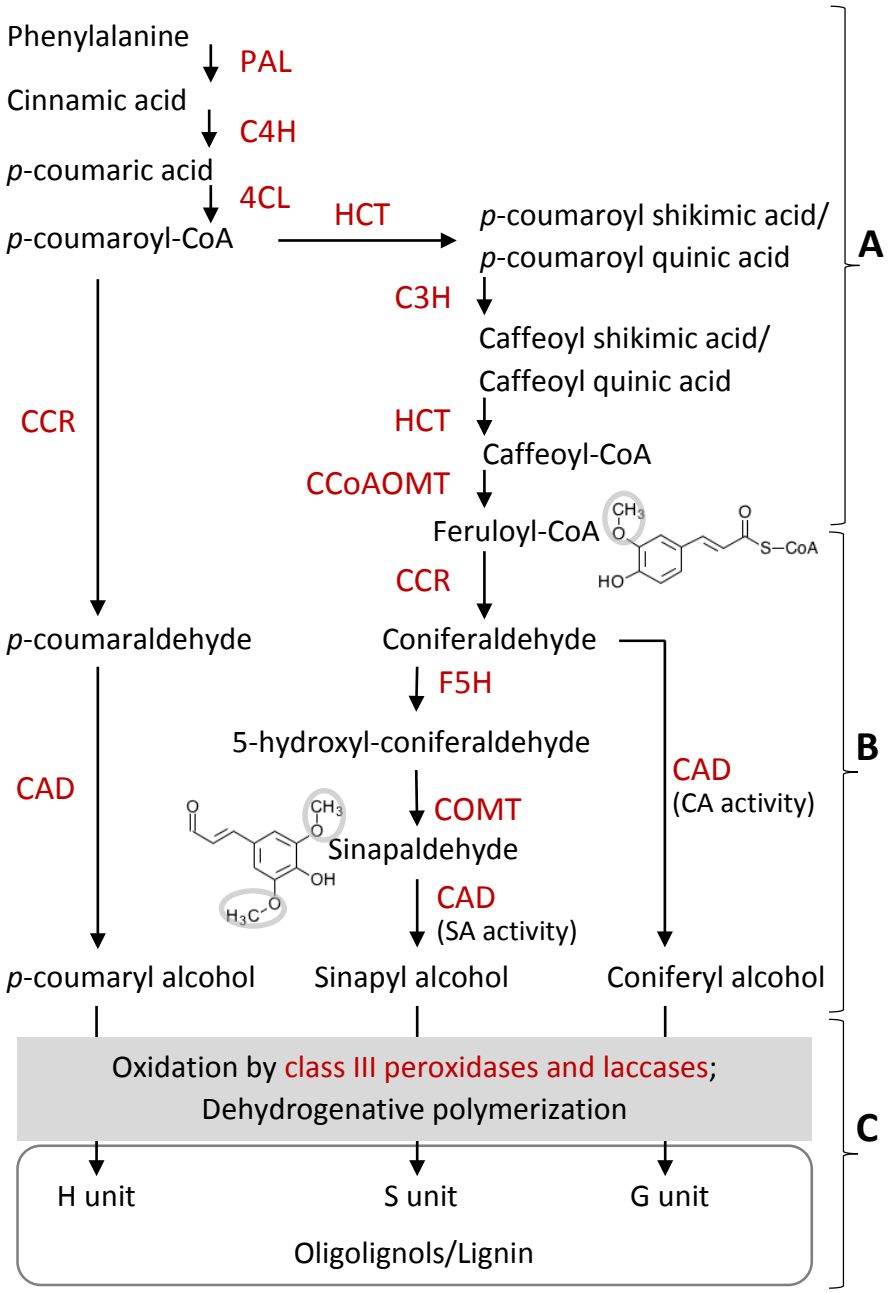

Figure 2.1 Lignin biosynthetic pathway in plants. The monolignols coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol synthesized from phenylalanine through the general phenylpropanoid pathway (**A**) and monolignol-specific pathway (**B**) are oxidized and incorporated to **G** (Guaiacyl), **S** (Syringyl) and **H** (hydroxyphenyl) units, respectively, in the complex and three-dimensional polymer of lignin (**C**). Oligolignols, which are formed during lignin polymerization, are racemic radical coupling products of monolignols. **PAL**, phenylalanine ammonia-lyase; **C4H**, cinnamate 4-hydroxylase; **4CL**, 4-coumarate:CoA ligase; **C3H**, p-coumarate 3-hydroxylase; **HCT**, p-hydroxycinnamoyl-CoA:quinate/shikimate p-hydroxycinnamoyltransferase; **CCoAOMT**, caffeoyl-CoA O-methyltransferase; **CCR**, cinnamoyl-CoA reductase; **F5H**, ferulate 5-hydroxylase; **COMT**, caffeic acid O-methyltransferase; **CAD**, cinnamyl alcohol dehydrogenase.

2.5.4 The monolignol biosynthesis pathway

Monolignols are synthesized from phenylalanine through the general phenylpropanoid and monolignol-specific pathways (Figure 2.1), and the pathway overall involves ten enzymes. Reactions in the general phenylpropanoid pathway, from phenylalanine to feruloyl-CoA, are catalyzed by the phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), *p*-coumarate 3-ydroxylase (C3H) and caffeoyl-CoA O-methyltransferase (CCoAOMT); while the reactions in the monolignol-specific pathway, from feruloyl-CoA to the monolignols, are catalyzed by cinnamoyl-CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeic acid O-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD) (Figure 2.1).

CCoAOMT and COMT are two structurally distinct methyltransferases that are responsible for the methylation of lignin precursors (Figure 2.1). The methyl groups of coniferyl and sinapyl alcohols are methylated in S-adenosylmethionine dependent reactions (Ma and Xu, 2008). While CCoAOMT methylates caffeoyl-CoA to feruloyl-CoA, COMT methylates 5-hydroxyconiferaldehyde to sinapaldehyde (Figure 2.1; Ma and Xu, 2008; Parvathi et al., 2001). The COMT activity may overlap with that of CCoAOMT because COMT, which is supposed to have broad substrate preferences (Ma and Xu, 2008), can also methylate the substrates of CCoAOMT. The CCoAOMT and COMT are believed to have a pivotal role in the monolignol biosynthesis pathway because the methylation of hydroxylated monomeric lignin precursors influences the final proportion of monolignols, and hence the lignin integrity. An increase in proportion of coniferyl alcohol, which is biosynthesized by the branch that does not involve COMT, in the composition of lignin will increase the number of beta-5 linked dimer and thereby

influences the final structure of lignin (Ma and Xu, 2008).

At the last step in the synthesis of monolignols, coniferyl alcohol (for G), sinapyl alcohol (for S), and p-coumaryl alcohol (for H) are resulted from reactions catalyzed by CAD. The recombinant TaCAD1 protein, whose cDNA was isolated form wheat, had a much higher catalytic efficiencies for coniferyl aldehyde (CAD-CA activity) as compared to that for sinapyl aldehyde (CAD-SA activity) (Ma, 2010). This result suggests CAD specific activities, i.e. CAD-CA and CAD-SA, can also influence the final proportion of monolignols.

2.5.5 Lignin biosynthesis and formation of enzyme complexes

The PAL and other enzymes in the phenylpropanoid biosynthetic pathway are nucleated by membrane-bound P450 proteins, and form enzyme complexes or metabolon (Winkel et al., 2004; Jorgensen et al., 2005) that increase metabolic efficiency and regulate pathway activity (Zhang and Liu, 2015). The C4H, next to PAL in the pathway, is a P450 enzyme (Bassard et al., 2012), and most of the cytochrome P450 proteins are localized to the membrane of the endoplasmic reticulum (ER) by their N terminus membrane-targeting peptides (Bayburt and Sligar, 2002). Thus, P450 enzymes can provide nucleation sites for the formation of metabolon. Through co-localization study of tobacco PAL and C4H, Achnine et al (2004) suggested that NtPAL1 binds more strongly than NtPAL2 does to membrane-bound C4H, which might serve as the nucleation site. Furthermore, the association between PAL and the ER membrane has been reported in different plant species (Czichi and Kindl 1977; Hrazdina and Wagner 1985), and this suggests a potential association between PAL and P450 enzymes.

C3H is another P450 enzyme in the phenylpropanoid biosynthetic pathway (Chen et al., 2011), and C3H isoforms are also found to form membrane protein complexes with C4H isoforms

(Chen et al., 2011). In poplar, these complexes are found to be heterodimeric such as C4H1-C3H3 and C4H2-C3H3, and heterotrimeric such as C4H1-C4H2-C3H3 complexes. In Arabidopsis, these heteromers are reported to be mobile within the ER (Bassard et al., 2012). The C3H protein can also form complexes with soluble 4CL. This protein was found to relocate closer to ER when C3H is overexpressed in Arabidopsis (Bassard et al., 2012).

The HCT, an operationally soluble protein, is the first branch-point enzyme for the synthesis of monolignols coniferyl alcohol and sinapyl alcohol (Figure 2.1). Bassard et al. (2012) found that, in Arabidopsis, HCT partially associates with ER; and this enzyme together with 4CL, C4H, and C3H likely form a supramolecular complex. The association of these four consecutive enzymes was found to be loose under normal condition but was enhanced when cell-wall lignification is induced by wounding stress (Bassard et al., 2012). These studies overall suggest that lignin biosynthesis might be regulated at the post-translational level through the formation of protein complexes involving the enzymes in the general phenylpropanoid pathway.

2.5.6 Lignin polymerization

During lignification, monolignols are translocated to the apoplast; the mechanism underlying this translocation is still poorly understood (Bonawitz and Chapple, 2010). Before polymerization, the monolignols and/or lignin units at the elongating end of lignin polymer are single-electron-oxidized to reactive radical species by wall bound laccases and class III peroxidases. The formation of a bond between oxidized monolignol and oxidized unit at the elongating end of lignin polymer, through a process known as bimolecular radical coupling, results in the incorporation of monolignol into the polymer. A bond can also be formed between two oxidized units at the elongating ends of two different lignin polymers, and between two oxidized monolignols

producing dilignols. The number of sites available for bond formation varies with the kind of monolignol/lignin unit and their status, i.e. free or polymerized, which leads to a number of potential coupling patterns. The coupling quenches both oxidized monolignols/lignin units involved, and then another round of oxidation or dehydrogenation is required in order to continue the polymerization process. Instead of being polymerized, oxidized monolignols may oxidize a non-oxidized monolignol through radical transfer reaction (Boerjan et al., 2003; Ralph et al., 2004). It has been suggested that the coupling reactions involved in lignin polymerization are controlled by the availability of monolignols and oxidants, and the surrounding physical conditions, but they are not directly controlled by proteinaceous mechanisms (Hatfield et al., 2001; Ralph et al., 2004; Ralph et al., 2008a). These studies suggest that the content and composition of lignin in plants can be affected under oxidative stress because class III peroxidases, which take part in lignin polymerization, are also antioxidants acting as plant defense against oxidative stress.

2.5.7 The family members of lignin gene

Sixty three protein sequences corresponding to the ten lignin synthesis enzymes have been retrieved from the Arabidopsis database, and the functional domains for each gene family identified (Xu et al., 2009). Using these protein sequences as the query in a blast search against 388,667 annotated protein sequences from 15 species belonging to a symbiotic fungus (1 species), prasinophyte (3 species), bacillariophyte (2 species), chlorophyte (2 species: green algae and alga), bryophyte (1 species: moss), lycophytes (1 species: spike moss), monocot (2 species: sorghum and rice), and dicot (3 species: Arabidopsis, poplar and alfalfa), Xu et al. (2009) found 14 gene homologs for *C3H* and 207 homologs for *CCR* (Table 2.1). The presence of unequal contributions to lignin biosynthesis among members of a gene family or the existence of a predominant

member(s) in each gene family with more than one member has been suggested in Arabidopsis (Raes et al., 2003). For example, the *PAL1* and *PAL2*, *C4H, 4CL1* and *4CL2, CCoAOMT1, CCR1, F5H1, COMT,* and *CAD6* genes have been suggested to be involved in developmental lignification (Boerjan et al., 2003; Costa et al., 2003; Goujon et al., 2003a; Raes et al., 2003; Bonawitz and Chapple, 2010). Mutational studies have indicated that while the total lignin level is reduced in the *c4h, 4cl1, ccoaomt1* and *ccr1* mutants; change in lignin composition is apparent in the *f5h1* and *comt* mutants in which the amount of S-lignin containing oligolignols is decreased (Vanholme et al., 2012a).

Table 2.1 Lignin biosynthetic candidate genes of wheat and other 15 species[a]

Classification/Species	*PAL*	*C4H*	*4CL*	*HCT*	*C3H*	*CCoAOMT*	*CCR*	*F5H*	*COMT*	*CAD*
Symbiotic fungus										
Laccaria bicolor	2	0	5	0	0	1	0	0	0	2
Prasinophyte										
Ostreococcus tauri	0	0	0	0	0	1	0	0	0	3
Ostreococcus lucimarinus	0	0	0	0	0	1	0	0	0	3
Ostreococcus RCC809	0	0	0	0	0	1	0	0	0	2
Bacillariophyte										
Phaeodactylum tricornutum	0	0	1	0	0	1	2	0	0	1
Thalassiosira pseudonana	0	0	1	0	0	0	0	0	0	0
Chlorophyte										
Chlamydomonas reinhardtii	0	0	0	0	0	2	4	0	0	4
Volvox carteri	1	0	0	0	0	2	1	0	0	3
Bryophyte										
Physcomitrella patens	14	4	11	4	1	2	7	0	3	4
Lycophytes										
Selaginella moellendorffii	2	2	26	6	2	8	29	0	28	18
Monocot										
Oryza sativa	14	4	16	9	1	11	55	3	38	5
Sorghum bicolor	8	3	15	4	2	7	44	3	41	14
Triticum aestivum	8	1	2	2	2	5	4	2	2	4
Dicot										
Arabidopsis thaliana	4	1	13	1	3	7	7	2	16	9
Medicago truncatula	4	1	10	6	1	4	18	3	26	21
Populus trichocarpa	6	3	22	7	4	7	40	4	35	21

[a] Sources: Xu et al. (2009) and Bi et al. (2011)

Common wheat (*Triticum aestivum*) is a hexaploid plant in which its genome, AABBDD, is originated from three diploid ancestral species: *Triticum urartu* (AA), *Aegilops speltoides* (BB) and *Aegilops tauschii* (Feldman and Levy, 2005). As a result, most of wheat genes exist as triplicate homeologues encoding a given protein. Transcriptional divergence among the wheat homeologous genes and their organ- and development-specific regulation has been reported (Nomura et al., 2005; Bottley et al., 2006; Shitsukawa et al., 2007; Bottley and Koebner, 2008 Akhunova et al., 2010; Hu et al., 2011; 2013). Through mining of wheat EST database, Bi et al. (2011) identified 32 candidate lignin biosynthesis genes corresponding to the ten gene families (Table 3.1). From this study, *C4H* was found to have only one family member; while *CAD* and *CCR*, each has four family members. The *CCoAOMT* and *PAL* gene families contain five and eight family members, respectively; whereas the other gene families including *4CL, C3H, COMT, F5H* and *HCT*, each has two family members (Table 3.1). Out of the 32 candidate genes, 21 genes, particularly *4CL1, C4H1, C3H1, CAD4, CCR2, F5H1, F5H2, PAL6* and *PAL8*, were found being under strong developmental regulation, and they are suggested to be the most important in lignin deposition in the stem as their expression levels were much higher in this tissue as compared to the other plant parts.

2.5.8 Perturbations of lignin biosynthesis and the whole plant system.

Previous studies have analyzed the effect of genetic modification of lignin biosynthesis using the model plant Arabidopsis (Vanholme et al., 2010b; Vanholme et al., 2012a). Using available mutants corresponding to most of the genes encoding enzymes catalyzing lignin biosynthesis, it has been concluded that mutation in a given lignin biosynthetic gene often has a much greater effect than the expected phenotypes such as change in lignification; exhibiting more consequences

outside of the lignin biosynthetic pathway such as shifts in primary and secondary metabolism (Vanholme et al., 2012a). For example, the suppression of lignin synthesis may cause redirection of metabolic flux toward flavonoids (Millar et al., 2007; Besseau et al., 2007). Comparative gene expression analysis between lignin biosynthesis mutants and the corresponding wild-type plants of Arabidopsis revealed changes in the expression of 675 genes at least in one of the mutants as compared with the wild-type; and this change in gene expression was consistent with alteration in the profile of 97% of the 566 compounds (Vanholme et al., 2012a).

All the recessive mutants derived from the knockout of a single lignin biosynthesis gene, except for *c4h* and *ccr1* that show significant reduction in growth rate and final plant height, do not have obvious morphological phenotypes or developmental shifts. The mutants that produce less lignin including *c4h, 4cl1, ccoaomt1* and *ccr1* exhibit upregulation in the pathways that supply the monolignols (shikimate, methyl-donor, and phenylpropanoid pathways) while the *f5h1* and *comt* mutants that produce the normal amounts of lignin show shifts in lignin composition; reduction in amounts of oligolignols containing S-units (Vanholme et al., 2012a). Moreover, the lignin biosynthesis mutants exhibit altered expression of genes involved in photosynthesis such as genes encoding chloroplast-localized proteins, photosystem I subunit K and photosystem II subunit QA, which showed up-regulation in *c4h, 4cl1, 4cl2* and *ccoaomt1* mutants, and slight down-regulation in *pal1, f5h1* and *comt* mutants (Vanholme et al., 2012a). The reduction of lignin content in the *brown midrib3* (*bm3*) mutant of maize due to mutation in the *COMT* gene was also found to cause alteration in the expression of genes involved in photosynthesis and the synthesis of phenylpropanoid and hemicellulose (Shi et al., 2006).

Genes encoding auxin/indole-3-acetic acid transcriptional regulator family, auxin response factor (ARF) 2 and 4, and indole-3-acetic acid inducible proteins 2, 5, 16, 18, 19, 20 and 32 showed

up-regulation in *pal1* and *f5h1* mutants and down-regulation in *c4h, 4cl1, ccoaomt1* and *ccr1* mutants (Vanholme et al., 2012a). Genes encoding proteins involved in defense response such as receptor kinase, receptor like proteins and disease resistance protein (CC/TIR-NBS-LRR class) exhibit down-regulation in *4cl1* and *ccr1* mutants as compared to the wild-type, however their transcript levels were not altered in other mutants. The abundance of most of the amino acids was found to be higher in the mutants with reduced lignin level such as *c4h*, *4cl1*, *ccoaomt1* and *ccr1* (Vanholme et al., 2012a). These changes in transcriptome and metabolome level between the wild-type and the lignin biosynthetic mutants reflect a system-wide and organized attempt of the plant to compensate for the mutation (Fu et al., 2009; Vanholme et al., 2012a).

2.5.9 Phenotypic buffering in response to lignin perturbation

In some cases plants are able to cope with the effect of a mutation, a phenomenon referred to as phenotypic buffering (Fu et al., 2009). Similar to that observed in the Arabidopsis lignin gene mutants (Vanholme et al., 2012a), no significant changes in plant growth and morphology were apparent in transgenic tobacco plants that exhibit reduced S lignin content due to the suppression of *COMT* using antisense strategy (Ma and Xu, 2008). Although it has been thought that an increase in cellulose content compensates for the lack of lignin (Hu et al., 1999; Li et al., 2003), this hypothesis is still under debate. Jouanin et al. (2000) reported an increase in cellulose content in the stems of poplar trees with reduced lignin levels due to the down-regulation of *COMT*. In contrast, no up-regulation of cellulose biosynthesis genes or increased cellulose content was observed in the developing stems of any of the recessive lignin biosynthesis mutants of Arabidopsis with reduced lignin content (Vanholme et al., 2012a). Overall, their results imply that the biosynthesis of lignin, cellulose and hemicellulose appear to be differentially regulated in

response to lignin perturbation. The interactive effects between lignin and cellulose biosynthesis have also been examined through genetic modification of cellulose biosynthesis genes of Arabidopsis. Cano-Delgado et al. (2003) reported that down-regulation of *AtCESA3* gene of cellulose biosynthesis affects lignification.

2.5.10 Endogenous cues affecting lignin biosynthesis

Lignin biosynthesis is influenced by endogenous/developmental cues such as circadian clock and hormones (Moura et al., 2010). A possible connection between lignin deposition and ethylene production has been reported in Arabidopsis (Zhong et al., 2002). Mutation in a chitinase-like gene (*AtCTL1*) causes ectopic deposition of lignin in cell walls in the pith of inflorescence stems, and overproduction of ethylene in the stem. This mutation also caused intensive distribution of CCoAOMT in some pith cells of stem, which was not observed in the wild-type stem (Zhong et al., 2000). It has been concluded that 1-aminocyclopropane-1-carboxylic acid oxidase plays an important role in the control of the asymmetric ethylene production during gravitational induction of tension wood in poplar stem (Andersson-Gunneras et al., 2003). The common types of tension wood are characterized by having higher and lower amounts of cellulose and lignin, respectively (Qiu et al., 2008). The application of gibberellin to the vertical stems of four species of angiosperm trees can also induce the formation of tension wood on these stems (Funada et al., 2008).

It has been shown previously that the transcript abundance of *PAL1, C4H, CCR1, CAD6, 4CL1* and *F5H1* exhibited two peaks over a diurnal cycle (Rogers et al., 2005). The first peak occurred at about one hour before plants were illuminated, and the second peak at half and four hours after the start of the illumination. When the plants were grown under continuous 24 hour light for 2 days, the transcript abundance of *C4H1, COMT, CCoAOMT1, CCR1* and *CAD6*

exhibited circadian fluctuations, in which the transcript abundance peaks during the subjective night. These results over all suggest that lignin biosynthesis could be under the control of circadian clock, which is independent of the control of diurnal cycle that is the light/dark stimulus.

2.5.11 Exogenous cues affecting lignin biosynthesis

In addition to endogenous/developmental cues, biotic and abiotic environmental factors such as wounding and pathogen infection are known to alter deposition of lignin (Lange et al., 1995; Vance et al., 1980; Bhuiyan et al., 2009a, Bhuiyan et al., 2009b). A study that examined the expression of all the 32 candidate wheat lignin biosynthesis genes in response to infection with fungal pathogens indicated the absence of any significant change in the expression of all the genes examined, except for *PAL1* that exhibited over 3-fold transcriptional activation, in the leaves infected by *Puccina triticina*. However, 17 genes (*4CL1, C3H1, C4H1, CAD1, CAD4, CCoAOMT1, CCoAOMT3, CCoAOMT5,CCR3, HCT1, HCT2, PAL1, PAL2, PAL3, PAL4, PAL5,* and *PAL7*) exhibited over 3-fold transcriptional induction specifically in spikelets after infection with *Fusarium graminearum* (Bi et al., 2011).

Under excessive soil moisture conditions, the deposition of lignin on epidermis or exodermis of root has been shown to increase so as to reduce radical oxygen loss (Hossain and Uddin, 2011). Moreover, there can be an entire change in the concentration of soluble carbohydrate in wheat plants exposed to waterlogging. As only few ATPs are produced from a molecule of glucose during anaerobic metabolism (Sairam et al., 2008), plants accumulate more soluble carbohydrate in the shoot and root (Albrecht et al., 1993), and the increase in the amount of sugar transferred to the roots also leads to increased ratio of root to shoot sugar (Huang and Johnson, 1995). In addition, exposure of plants to prolonged flooding can cause reduction in the

photosynthetic capacity of wheat leaf (Musgrave, 1994; Meisrimler et al., 2014), leading to lack of sugar in the shoot. This is significant as the availability of metabolizable sugars has been shown to positively influence the abundance of lignin (Rogers et al., 2005). Moreover, excessive soil moisture can lead to oxidative stress that may affect lignin deposition. The reduction of photosynthetic activity under excessive soil moisture condition leads to lowered photon utilization, which in turn could result in the production of reactive oxygen species (ROS) (Meisrimler et al., 2014). The plant antioxidative systems, which are involved in the scavenging of the ROS that cause oxidative damages, include antioxidative enzymes such as class III peroxidases (Asada 2006). However, class III peroxidases also take part in the biosynthesis of lignin; and are responsible for monolignol oxidation before the polymerization process (Vanholme et al., 2012a). Under oxidative stress condition, ROS scavenging activity of these enzymes might have more benefit to the plant.

2.6 Lignin biosynthesis genes

2.6.1 Genes involved in the general phenylpropanoid pathway

Genes involved in the general phenylpropanoid pathway include *PAL*, *C4H, 4CL, HCT, C3H*, and *CCoAOMT*, and a number of family members corresponding to these genes have been identified form different plant species including Arabidopsis, alfalfa, poplar, rice, sorghum and wheat (Table 2.1; Xu et al., 2009; Bi et al., 2011). Among these species, rice has largest number of family members; 14 family members of *PAL,* four family members of *C4H,* nine family members of *HCT* and 11 family members of *CCoAOMT*. Poplar consists of more family members of *4CL* (22 family members) and *C3H* (four family members), respectively, than that found in other species.

Repressing the *PAL, C4H, HCT* or *C3H* genes has been suggested as an effective way of

reducing lignin content (Li et al., 2008). Down-regulation of each of the *C4H, HCT, C3H* and *CCoAOMT* genes in alfalfa (*Medicago sativa* L.) led to over 25% reduction in stem lignin content as compared to the control (Chen and Dixon, 2007). In Arabidopsis, the stems derived from *c4h, 4cl1* and *ccaomt1* mutants exhibited lower lignin content as compared to the corresponding stem derived from the wild type (Vanholme et al., 2012a). Although lignin content was not affected in the *pal1* and *pal2* mutants (Vanholme et al., 2012a), it exhibited substantial reduction in the *pal1 pal2* double mutants (Rohde et al., 2004), indicating functional redundancy of *PAL1* and *PAL2* genes.

2.6.2 Genes involved in the monolignol-specific pathway

Genes involved in the monolignol-specific pathway include *CCR, F5H, COMT,* and *CAD*. Family members belonging to each of these genes have also been identified from Arabidopsis, alfalfa, poplar, rice, sorghum and wheat (Table 2.1; Xu et al., 2009; Bi et al., 2011). Different genes appear to have the largest number of family member in specific plant species, for example, CCR has 55 members in rice, F5H has four members in poplar, COMT has 41 members in sorghum, and CAD has 21 members in alfalfa.

Previous studies have characterized two wheat *CCR* genes, *TaCCR1* and *TaCCR2* (Ma and Tian, 2005; Ma, 2007). Although *TaCCR1* exists as a single copy gene, *TaCCR2* has three copies in the wheat genome. *TaCCR1* is suggested to have contribution in lignin biosynthesis because the TaCCR1 protein is mainly expressed in stem while the TaCCR2 expression is localized in the root. Moreover, the kinetic analysis of purified TaCCR1 and TaCCR2 proteins showed that TaCCR1 prefers feruloyl CoA over other possible substrates while TaCCR2 converts all possible substrates with almost similar efficiency (Ma and Tian 2005; Ma, 2007).

In Arabidopsis, *comt* mutant plant showed a normal lignin content with a shift in lignin composition - reduction in the amount of oligolignols containing S units and accumulation of oligolignols containing 5-hydroxyguaiacyl (5H) units derived from the combination of 5-hydroxyconiferyl alcohol with classical monolignols (Vanholme et al., 2012a). The brown midrib mutations identified in maize (*bm3*) (Vignols et al., 1995) and sorghum (*bmr12*) (Dien et al., 2009) have resulted from mutations in the *COMT* gene. The lignin content in the sorghum *bmr12* decreased only 15% while that of *bm3* mutant of maize remained unchanged (Barrière et al., 1994). The maize *BM3* gene has been found to be homologous to the wheat *COMT1* and *COMT2* genes (Bi et al., 2011).

The expression pattern of *COMT* varies with species. In wheat, the expression of *COMT* has been shown to be similar in leaves, stems and roots, suggesting the association of this gene with constitutive lignification process in different vegetative tissues (Ma and Xu, 2008). Similarly, no significant correlation was observed between the expression levels of *COMT* genes and lignin content in different tissues and developmental stage of wheat (Bi et al., 2011). In contrast with these results, *COMT* was found to be highly expressed in roots of maize (Vignols et al., 1995) and stem of ryegrass (McAlister et al., 1998) as compared to other tissues. Comparative analysis of *COMT* expression, COMT activity between wheat cultivars with contrasting stem strength indicated that the association of TaCOMT activity with lignin content is stage specific manner (Ma, 2009). Association of higher COMT activity with elevated lignin content was found in the internodes of a wheat cultivar with increased stem strength as compared to those derived from a wheat cultivar with decreased stem strength at heading and milky stages. No considerable difference in COMT activity as well as lignin content was observed between the two cultivars at elongation stage. Apart from COMT, the production of S unit of lignin is catalyzed by an enzyme

encoded by *F5H*. It has been reported in Arabidopsis that the *f5h1* mutants are similar to the *comt* mutants in the transcript-based clustering as well as in the reduction of the amount of S-unit-containing oligolignols without a significant change in total lignin content (Vanholme et al., 2012a). However, the expression levels of both *F5H1* and *F5H2* genes in wheat have been shown to have a strong positive correlation with lignin content across different plant tissues and developmental stages (Bi et al., 2011).

A number of mutants resulted from mutations in *CAD* gene have been reported in cereal crops including *bm1* of maize (Halpin et al., 1998), *gh2* of rice (Zhang et al., 2006), and *bmr6* of sorghum. These genes have been shown to have high homology with wheat *CAD4* (Bi et al., 2011). The expression of *CAD* is significantly affected by the brown-midrib mutation (*bm1*), which caused substantial reduction in lignin content (Halpin et al., 1998). In wheat, *TaCAD1* has been suggested as the predominant CAD gene in the stem. Comparative analysis of two cultivars with contrasting mechanical stem strength revealed that the transcript abundance of *TaCAD*, the level of TaCAD protein, and the activity of the corresponding enzyme in the internode/stem tissue is closely associated with differences in lignin content (Ma, 2010).

2.6.3 Transcriptional factors regulating lignin biosynthesis

Most of the transcriptional factors that act as regulators of lignin synthesis belong to the MYB family, and all of the lignin biosynthesis genes except *F5H* and *HCT,* are regulated/coregulated through the AC-rich elements corresponding to the MYB transcription factor-binding motif (Zhao and Richard, 2011). The AC-rich element was found in the promoters of *4CL, C3H, CAD, CCoAOMT, CCR* and *PAL*, and more degenerate AC-rich elements are suggested to be present in of the promoters of *C4H* and *COMT* (Zhao and Richard, 2011). In Arabidopsis, a total of 11

transcriptional factors have been reported to act as activators/repressors of the lignin biosynthetic pathway (Zhao and Richard, 2011). The first true lignin-specific transcription factors of Arabidopsis, which only affect lignin biosynthesis, are MYB58, MYB63, and MYB85 (Zhong et al., 2008; Zhou et al., 2009). AtMYB58 directly regulates all of the monolignol genes, except *F5H*, through the AC elements, indicating that the regulation of lignin biosynthesis by such a regulator is global, but not specific to a particular gene, and that the AC-rich elements are necessary for the coordinated regulation of monolignol pathways (Zhao and Richard 2011). The *F5H* gene doesn't contain AC-rich elements in its promoter, and therefore it is not regulated by the MYB58 and MYB63 transcription factors. However, it is directly regulated by NST3 (SND1), a secondary cell wall master switch. Previous study has shown that the NST3 transcription factor also activates the MYB58 and MYB63 (Zhao et al., 2010). In Arabidopsis, a group of NAC transcription factors including NST1, NST2, and NST3 are designated as master switches, as they coordinately regulate the biosynthesis of lignin and two other major components of secondary cell wall, cellulose and xylan (Zhao and Richard, 2011). In addition, it has been reported that the LIM transcription factor regulates lignin biosynthesis pathway in tobacco (Kawaoka et al., 2000).

A number of lignin biosynthesis repressors that belong to a subgroup 4 of the R2R3-MYB have been reported, including AmMYB308 and AmMYB330 from *Antirrhinum majus* (Tamagnone et al., 1998), AtMYB4 and AtMYB32 from Arabidopsis (Jin et al., 2000; Preston et al., 2004), ZmMYB31 and ZmMYB42 from maize (Fornale et al., 2006; Sonbol et al., 2009), and EgMYB1 from *Eucalyptus* (Legay et al., 2007). The AmMYB308 and AmMYB330 repress *4CL*, *C4H,* and *CAD* when overexpressed in tobacco (Tamagnone et al., 1998), and AtMYB4 has been shown to repress *4CL* and *C4H* when overexpressed in Arabidopsis (Jin et al., 2000). AtMYB32 has also been suggested to control lignin pathway negatively through other targets than lignin

biosynthesis genes (Preston et al., 2004). Arabidopsis plants overexpressing ZmMYB42 have been reported to exhibit down-regulation of *4CL*, *C4H, HCT, F5H*, *COMT* and *CAD* genes. Furthermore, ZmMYB31-overexpressing Arabidopsis plants exhibit down-regulation of *COMT* (Fornale et al., 2006; Sonbol et al., 2009), and EgMYB1 represses *Eucalyptus gunnii CCR* and *CAD in vivo* (Legay et al., 2007). Overexpression of wheat MYB4 in tobacco also led to transcriptional repression of the *CAD* and *CCR* genes (Ma et al., 2011). Lignin biosynthesis repressors that belong to other MYB subgroups have been identified from different species such as MYB5a of grapevine (Deluc et al., 2006), MYB21a of *Populus* and MYB52 of Arabidopsis (Karpinska et al., 2004). Moreover, the promoters of *COMT* and *CCoAOMT* can be bound by the knotted1-like homeobox (KNOX) gene of Arabidopsis, *BREVIPEDICELLUS* (*BP*), leading to increased and decreased lignin deposition in *bp* mutants and *BP* overexpressing lines, respectively (Mele et al., 2003). Mutation in *BP* is reported to lead to significantly increased transcript abundance of many lignin genes (Mele et al., 2003).

2.7 Genetic modification of lignin biosynthesis for enhanced lignocellulosic bioethanol production

Many efforts are underway to genetically alter the lignin biosynthetic pathway in order to modify lignin synthesis and composition, and thereby the feedstock quality of lignocellulosic biomass (Chen and Dixon, 2007; Vermerris et al., 2007; Vanholme et al., 2008; Shen et al., 2009; Vanholme 2012b). For example, the sorghum brown midrib (*bmr*) mutants *bmr-6, bmr-12,* and *bmr-18*, have been incorporated into commercial lines creating varieties with lower lignin contents (Sarath et al., 2008). The *bmr-6* mutant is produced by mutation in the CAD gene (Sattler et al., 2009), while *bmr*-12 is a *COMT*-associated mutant (Palmer et al., 2008), and the mutants cause the absence of

active enzymes in plant tissues (Sarath et al., 2008, Sattler et al., 2009). It has been reported by Dien et al. (2009) that lignin content in the biomass of *bmr-6*, *bmr-12,* and *bmr-6-bmr-12* double mutants were 13%, 15%, and 27% lower than that in wild type, respectively. Consistently, the efficiency of conversion of cellulose to ethanol for the biomass was increased by 22%, 21%, and 43% for *bmr-6, bmr12* and *bmr-6-bmr-12* double mutants compared to wild type, respectively. Similarly, an increase of up to 38% in bioethanol yield was obtained from the biomass of switchgrass underexpressing *COMT*. Repression of COMT resulted in only a modest decrease (6-15%) in lignin content, with no considerable change in cellulose content and structure, and overall plant growth and development (Fu et al., 2011a). This increase in bioethanol yield as compared to the control wild type was attributed to the change in the S/G ratio (Fu et al., 2011a).

The relationship between the efficiency of saccharification and lignin content/composition has been studied in alfalfa using transgenic technology (Chen and Dixon, 2007). Down-regulation of selected lignin biosynthetic genes such as *C4H, HCT, C3H* or *COMT* in alfalfa has been shown to lead to reduction of lignin content from 25% to over 50%, as compared to the control, which in turn resulted in 1.5- to 2.8-fold increases in enzymatic saccharification efficiency of alfalfa biomass. Particularly, the saccharification efficiency of biomass derived from alfalfa plants under-expressing the *HCT* gene was 87% of that exhibited by the biomass that had been subjected to acid treatment before the saccharification process. This clearly demonstrates that reduction of biomass content can help to bypass the costly pretreatment step in the conversion of lignocellulosic biomass into bioethanol. Moreover, genetic modification of some transcription factors has been reported to alter lignin content and composition, for example, overexpression of *PvMYB4*, a switchgrass R2R3-MYB transcription factor, resulted in reduced lignin content and ester-linked *p*-CA: FA ratio and a three-fold increased efficiency of sugar release from the biomass (Shen et al., 2012).

Genetic modification of lignin has also been implicated in enhancing the content of other major constituents of lignocellulose such as cellulose. For example, down-regulation of *4CL* in aspen (*Populus temuloides* Michx.) has been reported to lead to a 45% reduction of lignin content and at the same time a 15% increase in the level of cellulose (Hu et al., 1999). Therefore, regulation of lignin and cellulose in a compensatory fashion could be an ideal strategy to meet the optimal balance between biomass quality for bioethanol and other agronomic traits, especially when the biomass remains in the field as a residue. Overall, all the reports discussed above indicate that manipulation of biomass lignin content and composition can have a significant contribution for enhancing the efficiency of generating bioethanol from lignocellulosic biomass.

3. LIGNIN BIOSYNTHESIS IN WHEAT BIOMASS AND ITS RESPONSE TO GENETIC AND ENVIRONMENTAL VARIATIONS

Abstract

Lignin content and/or composition in wheat biomass can be genetically altered to optimize the feedstock quality of the straw while maintaining important field agronomic traits, and this requires identification of regulatory genes. Analysis of the expression of 32 lignin biosynthesis candidate genes revealed the predominance of specific genes including *PAL8, C4H1, 4CL1, HCT1, C3H1, CCoAOMT1, F5H2, COMT2* and *CAD2* in all vegetative tissues assayed, suggesting their significance in lignin formation in wheat biomass. These genes showed higher transcription in the internode/peduncle as compared to other tissues of wheat biomass. Comparative analysis of lignin biosynthesis between three commercial wheat cultivars, namely Harvest, AC Intrepid and Kane, that show difference in their resistance to lodging, indicated a close association between lodging resistance, internode lignin content, and the expression of *4CL1*, *F5H2* and *COMT2* genes, implying that these genes are key determinants of lignin formation in the internode. Furthermore, the study showed absence of a close association between lignin synthesis in the internode and leaf tissues, suggesting their regulation by different mechanisms. Treatment of wheat plants with excessive soil moisture led to reduction in internode and leaf lignin content, and the lignin phenotype in the internode was accompanied by transcriptional repression of *PAL8, CCR2* and *F5H2* genes, and decreased activity of PAL enzyme. Several predominant lignin biosynthesis genes in both internode and leaf tissues were, however, induced in response to excessive soil moisture conditions, and this suggests their regulation by a feedback mechanism.

3.1 Introduction

Lignin is a complex phenolic polymer closely linked with cellulose and hemicellulose, forming an important structural component of plant secondary cell wall. It provides plants with mechanical strength and vascular integrity (Vanholme et al., 2010a), and also plays important roles in conferring tolerance against biotic such as pathogens and abiotic stressors (Dixon et al., 2002; Bi et al., 2011). Recently, increased concerns about climate change and the need to reduce carbon emissions have triggered a growing interest in producing renewable fuels and bioproducts from lignocellulosic biomass (Demura and Ye, 2010). Wheat is one of the most economically important cereal crops globally, and its straw represents a great amount of lignocellulosic crop residue that can be used as a feedstock for sustainable production of biofuel and bioproducts (Sokhansanj et al., 2006; Somerville et al., 2010). However, efficient conversion of lignocellulosic biomass to biofuels is hindered by lignin, which limits the accessibility of plant cell wall polysaccharides to chemical, enzymatic and microbial digestions (Mielenz, 2001, Keating et al., 2006). Genetic alteration of the quantity and quality of biomass lignin has been considered as a viable alternative to mitigate this problem (Li et al., 2008; Simmons et al., 2010; Fu et al., 2011a). However, lignin content in cereal crops such as wheat has been shown to be closely associated with stem mechanical strength, and thereby resistance to lodging (Ma, 2009), one of the major impediments of wheat production as it causes decreases in harvestable yield by up to 80% and quality of grain (Foulkes et al., 2011). Therefore, it is imperative to design tools and approaches that can alter the quantity and quality of wheat biomass lignin without affecting its functions with respect to normal growth, development and field performance of plants. This requires detailed dissection of the molecular mechanisms underlying lignin biosynthesis in wheat.

Lignin is formed by the oxidative coupling of three monolignols that serve as building blocks; coniferyl, sinapyl and p-coumaryl alcohols. These monolignols are synthesized from phenylalanine through the general phenylpropanoid and monolignol-specific pathways (Figure 2.1), and the successive dehydrogenative polymerization reactions give rise to guaiacyl (G), syringyl (S) and hydroxyphenyl (H) units, respectively, that form a complex and three-dimensional lignin polymer (Vanholme et al., 2010a). In addition, differences in species, genetic background and environment may result in the incorporation of other monomers such as hydroxycinnamyl acetates, hydroxycinnamyl *p*-hydroxybenzoates and hydroxycinnamyl *p*-coumarates (Ralph et al., 2004). The lignin biosynthesis pathway overall involves ten enzymes. Reactions in the general phenylpropanoid pathway from phenylalanine to feruloyl-CoA are catalyzed by the phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), p-coumarate 3-ydroxylase (C3H), caffeoyl-CoA O-methyltransferase (CCoAOMT). Whereas cinnamoyl-CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeic acid O-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD) catalyze reactions in the monolignol-specific pathway that convert feruloyl-CoA to the monolignols (Figure 2.1).

Lignin biosynthesis has been studied extensively in the model plant Arabidopsis and other dicot species such as alfalfa and tobacco, and these studies have led to the identification of genes encoding the enzymes catalyzing each step in the pathway (Vanholme et al., 2010a). Given that most of the lignin biosynthetic enzymes have isoforms, they appear to be encoded by gene families (Raes et al., 2003; Xu et al., 2009), and previous studies have demonstrated the physiological roles of these genes in determining lignin deposition and composition (Simmons et al., 2010; Vanholme et al., 2010a). For example, suppression of genes early in the pathway such as *PAL, C4H, HCT*,

C3H and *CCoAOMT* in alfalfa and tobacco led to reduction of total lignin content, while that of *F5H* or *COMT*, which are involved in the synthesis of lignin S unit, resulted in alteration of the lignin S/G ratio with only minimal effect on total lignin content (Li et al., 2008). Repression of *CAD*, encoding an enzyme that catalyzes the last step in the biosynthesis of monolignols, also caused a similar effect; reduction in the S/G ratio with limited effect on total lignin content. Similarly, the *f5h1* and *comt* mutants of Arabidopsis are characterized by decreased amounts S unit containing oligolignols with no effect on the total lignin content, whereas the *c4h*, *4cl1, ccoaomt1* and *ccr1* mutants produce less total lignin as compared to the wild-type (Vanholme et al., 2012a).

It has been shown previously that the transcripts of over half of the 32 candidate wheat lignin biosynthesis genes are highly abundant in the stem tissues as compared to that found in the leaf sheaths and leaf blades during heading (Table 3.1; Bi et al., 2011). The expression patterns of *PAL6, C4H*, *4CL1*, *C3H1*, *CCR2*, *F5H1* and *F5H2* in particular exhibited a significant correlation with tissue lignin content. Furthermore, characterization of the wheat *CCR1*, *COMT1* and *CAD1* genes revealed the presence of higher abundance of *TaCCR1* and *TaCAD1* transcripts, and greater activities of the corresponding enzymes in the stem than in other tissues; while *TaCOMT1* was found to be expressed constitutively in different tissues including stem, leaf and root tissues (Ma, 2007; 2009; 2010). Comparative analysis between lodging resistant and lodging susceptible wheat lines showed the presence of higher transcript abundances of *TaCCR1*, *TaCOMT1* and *TaCAD1*, and higher activities of the corresponding enzymes in the lodging resistant line only following the heading stage; and these results are found to be closely associated with stem lignin content and mechanical strength (Ma, 2007). Despite these results, the molecular mechanism underlying the differential deposition of lignin and stem mechanical strength in wheat is poorly understood.

Table 3.1 Lignin biosynthetic candidate genes of wheat

Genes	Family members
PAL	*PAL1, PAL2, PAL3, PAL4, PAL5, PAL6, PAL7* and *PAL8*
C4H	*C4H1*
4CL	*4CL1* and *4CL2*
HCT	*HCT1* and *HCT2*
C3H	*C3H1* and *C3H2*
CCoAOMT	*CCoAOMT1, CCoAOMT2, CCoAOMT3, CCoAOMT4* and *CCoAOMT5*
CCR	*CCR1, CCR2, CCR3* and *CCR4*
F5H	*F5H1* and *F5H2*
COMT	*COMT1* and *COMT2*
CAD	*CAD1, CAD2, CAD3* and *CAD4*

Wheat production is challenged by a variety of biotic and abiotic factors, including excess soil moisture (transit flooding or water logging), which is projected to become more frequent and more intense as the climate changes. Waterlogging causes reduction in gas diffusion and thereby affecting the availability of oxygen in the rhizosphere, inducing changes in biochemical and metabolic processes (Sairam et al., 2008). One of the main metabolic shifts involves a shift from aerobic to anaerobic respiration, impairing ATP production. Compensation of the resulting energy deficit requires accelerated glycolysis via increased activities of glycolytic and fermentative enzymes, leading to depletion of carbohydrate reserves, a phenomenon referred to as the 'Pasteur effect" (Ricard et al., 1994; Drew, 1997). Consistently, transcriptional activation of glycolytic and fermentative genes has been observed in anoxic rice coleoptiles (Lasanthi-Kudahettige et al., 2007). In contrast with this, root hypoxic conditions have been shown to lead to the accumulation of soluble carbohydrates such as sucrose, and those with storage function such as fructan in both

root and shoot tissues of wheat seedlings (Albrecht et al., 1993), which is inconsistent with the occurrence of enhanced glycolytic and fermentative processes in response to low O_2 conditions. Under long-term hypoxic conditions, large amount of sucrose is found to be partitioned into the synthesis cell wall, mainly cellulose, of wheat roots, leading to changes in cell wall structure (Albrecht and Mustroph, 2003). It is likely that this alteration in cell wall structure serves as one of the strategies to compensate the progressive dissolution of cortical cells for aerenchyma formation, thereby contributing to the maintenance of cell wall function in root tissues under low O_2 stress conditions. Despite these findings, little is known about the response of lignin biosynthesis to excessive soil moisture/flooding.

To gain insights into the molecular mechanism underlying the regulation lignin deposition in different tissues of wheat in response to genetic variations, this thesis investigated lignin biosynthesis in the leaf and internode tissues of three commercial wheat cultivars, namely Harvest, AC Intrepid and Kane using different approaches including gene expression analysis, measurement of the activities of selected enzymes and profiling the major lignocellulosic components. Furthermore, the response of lignin biosynthesis in wheat to excessive soil moisture conditions was examined in the same tissues using cv. Harvest.

3.2 Materials and Methods

3.2.1 Plant material

Three hexaploid wheat cultivars, AC Intrepid, Harvest and Kane, were used for this study. These cultivars were selected based on their resistance to lodging, which has been considered as a measure of mechanical strength in this study. With respect to lodging resistance, cv. Harvest is graded as 'very good' while cvs. AC Intrepid and Kane as 'good' (Saskatchewan Ministry of Agriculture, 2013). Mature dry seeds of these cultivars were imbibed in Petri dishes for three days, after which the germinated seeds were transplanted to one-gallon plastic pots (one seed per pot) containing Supper Mix supplied with 18 g of fertilizers (ACER®nt 13-12-12 consisting of 12% N, 12% P_2O_5, 12% K_2O and micro elements). The plants were grown in a growth chamber at 22°C/20°C (day/night) under a 16/8 h photoperiod until harvest. At the heading stage (Figure 3.1A); two sets of samples were collected. The first set of samples was collected from cv. Harvest, including flag leaf blade (FB), flag leaf sheath (FS), peduncle (PE), first internode (IN-1,), second and third internodes (IN-2&3), and the forth internode (IN-4). The numerical designation of the internodes was based on their relative position to the peduncle in which IN-1 is the youngest internode located next to the peduncle, while IN-4 is the oldest internode located at the base of the stem. The second set of samples was harvested from all the three cultivars, and it consisted of two plant parts: the whole flag leaf (consisting both blade and sheath) and the internode (consisting only the second and third internodes; IN-2&3). Tissues were frozen in liquid nitrogen immediately after harvest and then stored at -80°C until further use.

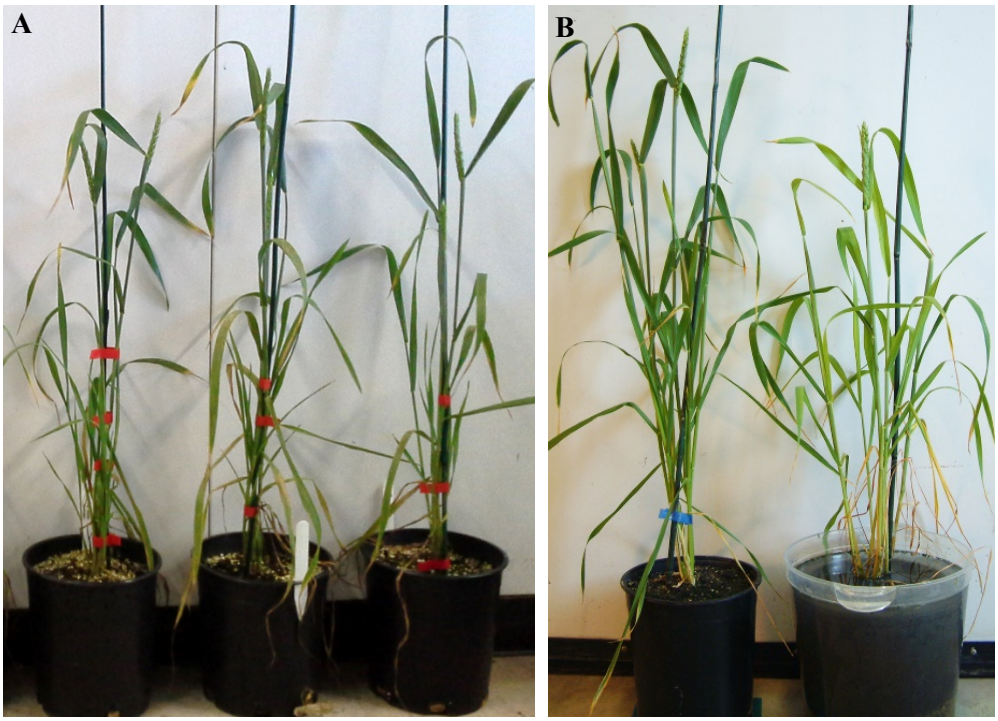

Figure 3.1. Wheat plants of cv. Harvest at heading stage (**A**), and plants treated with and without excessive soil moisture (**B**).

3.2.2 Treatment with excessive soil moisture

Plants of cv. Harvest were grown as described above except that the soil mixture consisted of clay and sand (2:1, v: v). Four weeks after transplanting, a set of 48 plants were treated with excessive soil moisture by submerging the pots containing the plants in 5 L containers filled with water to maintain the water level at 2 cm above the surface of the soil. The water in the bigger container was replenished as required. Concurrently, another set of 48 plants were treated with regular watering every other day. At heading stage, which occurred at 20-22 days in both control and treated plants, the flag leaf and IN-2&3 tissues were harvested in liquid nitrogen and stored at -80^{o}C until further use.

3.2.3 RNA extraction and cDNA synthesis

Total RNA was isolated using TRIzol® Reagent following the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). The RNA samples were digested with DNase (DNA-free Kit; Ambion, Austin, TX, USA) to eliminate genomic DNA contamination. The first strand cDNA was synthesized using iScript^{TM} Reverse Transcription Supermix for (Bio-Rad, Hercules, CA, USA) and 1 µg of total RNA in a total reaction volume of 20 µl. The resulting cDNA samples were diluted 20X before use as a template for real-time quantitative PCR (qPCR).

3.2.4 Real-time quantitative PCR assay

The real time qPCR assays were performed with primers specific to the target lignin biosynthetic and the reference actin genes (Bi et al., 2011; Table 3.1 & 3.2). The specificity of the primers to the corresponding target genes was verified by blast searching target amplicons against GenBank database followed by analysis with RT-PCR. The CFX96 Real-time PCR system was used for to perform the qPCR assay using a 20 µL total reaction volume containing 5 µL of the diluted cDNA as a template, 10 µL of SsoFast EvaGreen Supermix (Bio-Rad), 1.2 µL of 5 µM forward primer (300 nM final concentration), 1.2 µL of 5 µM reverse primer (300 nM final concentration) and 2.6 µL diethylpyrocarbonate (DEPC) treated water. Samples were subjected to the following thermal cycling conditions: DNA polymerase activation at 95^{o}C for 5 min followed by 40 cycles of denaturation at 95^{o}C for 15 s, annealing at 50-66^{o}C (depending on the melting temperature of the primers of the target genes) for 30 s and extension at 72^{o}C for 30 s in duplicate in 96-well optical reaction plates (Bio-Rad). After normalization of the transcript levels of the target genes with that of *β-actin*, the relative transcript levels were expressed using the Livak and Schmittgen (2001) method.

Table 3.2 Sequences of redesigned primers for selected lignin biosynthesis genes

Genes	Unigene ID	EST ID	Type	Primer (5' to 3')
PAL1	TC383948	CJ956144.1	Forward	GCTTGGCAAAGACGGATGTC
			Reverse	AGCGCCAACTACCCATTGAT
PAL8	TC385356	CJ728010.1	Forward	CTCAAGCTCATGTCCTCCACA
			Reverse	TCAGCACCTTCTTCGACACC
C3H2	TC368628	AJ585990	Forward	AAGCCACTGGTTGTCAGGAG
			Reverse	TGAACTCCTGCCCTTCTTCG
CCR2	TC378424	DR739700.1	Forward	GAAACAGCGCGGTTCACGTT
			Reverse	AGGAATGCTGGCAACAAACCC
COMT1	TC369087	DQ223971.1	Forward	GGTTCGCCGCCATGAAGACTA
			Reverse	CAGGTGGATGCATCAGAGAGGTA
COMT2	TC368870	AY226581	Forward	GTCCGCCGATCTGAGAATCTTC
			Reverse	CGACGACACAACCAGTAGAG
CAD1	TC445835	CJ710661.1	Forward	TTACCTACCAGCTCCAGCCA
			Reverse	TCTGGAATTGCACGCACCAA

3.2.5 Measurement of enzyme activity

Protein concentration and enzyme activity were determined spectrophotometrically (Ultrospec 3100 *pro*, Artisan Scientific, Champaign, Illinois, USA). Total protein extraction, and the CAD-CA and CAD-SA activity assays were carried out as described in Zhang et al. (2006). Briefly, frozen plant tissue (~ 200 mg for flag leaf tissue or ~300 mg for internode tissues) was ground into fine powder, mixed with protein extraction buffer followed by incubation at 4^{o}C for 2.5 h. The concentration of the resulting protein in the extract was measured using Quick Start^{TM} Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). The CAD-CA and CAD-SA activities were examined by monitoring the conversion of coniferyl alcohol, a substrate for CAD-CA, and sinapyl alcohol, a substrate for CAD-SA, to their respective products at 400 nm using the total protein extract (100 µg) in 500 µL reaction buffer. The PAL activity of the protein extract was assayed as

described in Edwards and Kessmann (1992) in which the formation of cinnamic acid from L-phenylalanine by the total protein (100 µg for flag leaf or 10 µg for internode) in 1 mL reaction buffer was monitored at 290 nm for up to 2 h. The activity for each enzyme was expressed as nKatal (referring to enzyme activity for converting 1 nmol of the substrate per second) per gram of total protein.

3.2.6 Lignocellulose measurement

Frozen leaf and internode tissue samples were freeze-dried, and then the amounts of lignin and acid detergent fiber (ADF) in each sample were analyzed using the AOAC Official Method 973.18 (Latimer, 2012). Whereas the neutral detergent fiber (NDF) content was determined using the AOAC Official Method 2002.04 (Latimer, 2012). Cellulose content was calculated as the difference between ADF and lignin; while the hemicellulose content as the difference between NDF and ADF.

3.2.7 Statistical analysis

Significant difference between sample means was tested using LSD test or t-student test at $P<0.05$.

3.3 Results

3.3.1 Expression of lignin biosynthetic genes in different wheat tissues

Differential expression patterns across the different tissues were exhibited by each candidate lignin biosynthetic gene, ranging from 3-fold for *CAD4* to 100-fold for *PAL8* (Table 3.3 and Appendix 1). Except *CCR1*, *COMT2* and *CAD2*, all the candidate genes showed higher transcription in the peduncle and internode tissues than in the flag leaf blade (FB) and flag leaf sheath (FS). With respect to *CCoAOMT* and *F5H* gene families, all members were predominantly expressed in the peduncle, which also exhibited higher levels of *PAL6, PAL8, HCT2, C3H1, COMT1,* and *CAD4* transcripts than observed in the other tissues. Comparative analysis of the different internode sections revealed that most of the lignin genes exhibited higher levels of expression in IN-2&3 and/or IN-4. The genes that were highly expressed in the internodes, mainly in IN-4, included *PAL1, PAL2, PAL3, PAL4, PAL5, PAL7, C4H1, 4CL1, 4CL2, HCT1, CCR2, CCR3, CCR4, CAD1* and *CAD3*. While the two members of the *COMT* gene family were found to be highly expressed in the IN-1, no differential expression of *CAD2* and *PAL8* was apparent across the different internode sections. The *CCR1* and *CAD2* genes exhibited significantly higher transcription in the FB than in other tissues considered, while the highest transcription of *COMT2* was found in the FS tissue. Over half of the genes analyzed in this study showed significantly higher levels of expression in the FS than in the FB (Table 3.3 and Appendix 1), noticeably *PAL8, CCoAOMT4*, *CCR2,* and *COMT1*, which showed over 6-fold higher expression in FS than in the FB.

In order to gain insights into their contribution to lignin biosynthesis, we also performed comparative analysis of the expression of members in each gene family. Our analysis revealed the presence of a specific member consistently expressed at a higher level than other members across

the different tissues (Table 3.3 and Appendix 1). The *PAL8, 4CL1, CCoAOMT1, F5H2* and *COMT1* appeared to be the most predominant genes in all tissues as compared to other members in their respective gene family (Table 3.3 and Appendix 1). With respect to *HCT* and *CAD* gene families, the *HCT1* and *CAD2* genes showed higher transcription than the other family members in all tissues assayed, except in the PE where *HCT2* and *CAD4* appeared to be the most predominant ones (Appendix 1). Tissue specific predominance of a specific gene family member was also observed for *CCR* in which *CCR1* was predominantly expressed in FB and FS, while *CCR2* in all other tissues.

Table 3.3 Relative transcript levels of predominant lignin biosynthesis genes in various different tissues of wheat cv. Harvest[a]

Gene	FB	FS	PE	IN-1	IN-2&3	IN-4
PAL8	0.83 ±0.071d	9.05 ±0.798c	89.25 ±19.53a	54.50 ±6.706b	54.57 ±11.98b	33.66 ±6.983b
C4H1	0.38 ±0.033d	0.91 ±0.039c	2.68 ±0.395b	3.67 ±0.606b	9.57 ±1.200a	11.22 ±3.709a
4CL1	1.00 ±0.088d	2.04 ±0.076c	7.61 ±1.099b	7.13 ±0.571b	12.51 ±2.022a	16.84 ±2.830a
HCT1	0.58 ±0.034d	0.70 ±0.058d	2.53 ±0.544b	1.54 ±0.196c	3.09 ±0.397b	5.42 ±0.600a
C3H1	0.12 ±0.028e	0.21 ±0.031de	1.24 ±0.057a	0.87 ±0.115b	0.55 ±0.125c	0.46 ±0.033cd
CCoAOMT1	1.52 ±0.170e	3.32 ±0.579d	32.73 ±5.275a	11.98 ±2.506c	19.56 ±3.506bc	21.89 ±4.771b
CCR1	11.11 ±0.209a	6.64 ±0.358b	0.29 ±0.033f	1.59 ±0.306c	0.78 ±0.020d	0.45 ±0.066e
CCR2	0.17 ±0.015e	1.56 ±0.073d	3.77 ±0.784c	5.93 ±0.294b	8.44 ±0.848a	7.82 ±1.088a
F5H2	0.84 ±0.043e	1.39 ±0.039d	9.20 ±1.670a	6.09 ±0.968ab	3.38 ±1.047c	4.23 ±0.838bc
COMT2	15.51 ±1.129b	36.04 ±4.195a	16.09 ±2.857b	31.56 ±4.397a	14.18 ±1.635b	12.63 ±0.673b
CAD2	13.49 ±1.100a	9.33 ±0.208b	0.93 ±0.088d	2.76 ±0.661cd	2.85 ±0.355cd	3.54 ±0.630c

[a] Transcript levels were expressed relative to that of *4CL1* in flag leaf, which was set to value of 1. Data are means of 2-3 independent biological replicates ± SE. Means followed by different letters within each gene show statistically significant difference at $P<0.05$. FB, flag leaf blade; FS, flag leaf sheath; PE, peduncle; IN-1, the first internode; IN-2&3, the second and third internode; IN-4, the fourth internode (The numerical designation of internodes was based on their relative position to the peduncle in which IN-1 is the youngest/the one next to the peduncle while IN-4 is the oldest internode of the stem).

Overall, the *PAL8, C4H1, 4CL1, CCoAOMT1, CCR1, CCR2, F5H2, COMT2* and *CAD2* genes appeared to be predominant in the flag leaf and/or internode tissues as compared to the other genes studied. As a result, these genes were considered for further comparison of their expression in the whole flag leaf and internode tissues across the three cultivars.

3.3.2 Expression of lignin biosynthesis genes in three wheat cultivars

All the lignin genes considered as predominant in wheat, except *CCR1* and *CAD2*, exhibited significantly higher transcript abundance (1.4- to 3.5-fold) in the flag leaf tissue of cv. AC Intrepid than that observed in the other two cultivars, Kane and Harvest. Flag leaf derived *CCR1* and *CAD2* of cv. AC Intrepid also exhibited higher expression than the corresponding genes in cv. Harvest; although the expression level of the two genes in cv. Harvest was similar to that observed in cv. Kane (Figure 3.2). No differential expression of all predominant genes was evident between the flag leaf of cv. Kane and cv. Harvest.

The transcription of all the genes that appeared to be predominant in the internode of cv. Harvest was either higher than or equal to that observed in the internodes of cv. Kane and/or cv. AC Intrepid (Figure 3.3). The *4CL1, F5H2* and *COMT2* genes exhibited significantly higher transcript abundance in the internodes of cv. Harvest as compared to that found in the other two cultivars. Although not statistically significant, higher transcription of *CCR2* was also observed in the internode of cv. Harvest. The *C4H1* and *CCoAOMT1* genes also exhibited significantly higher expression in the internode of cv. Harvest than that of cv. AC Intrepid, although the two genes are expressed similarly in the internodes of cv. Harvest and Kane. No differential expression was apparent for *PAL8* and *CAD2* across the three cultivars.

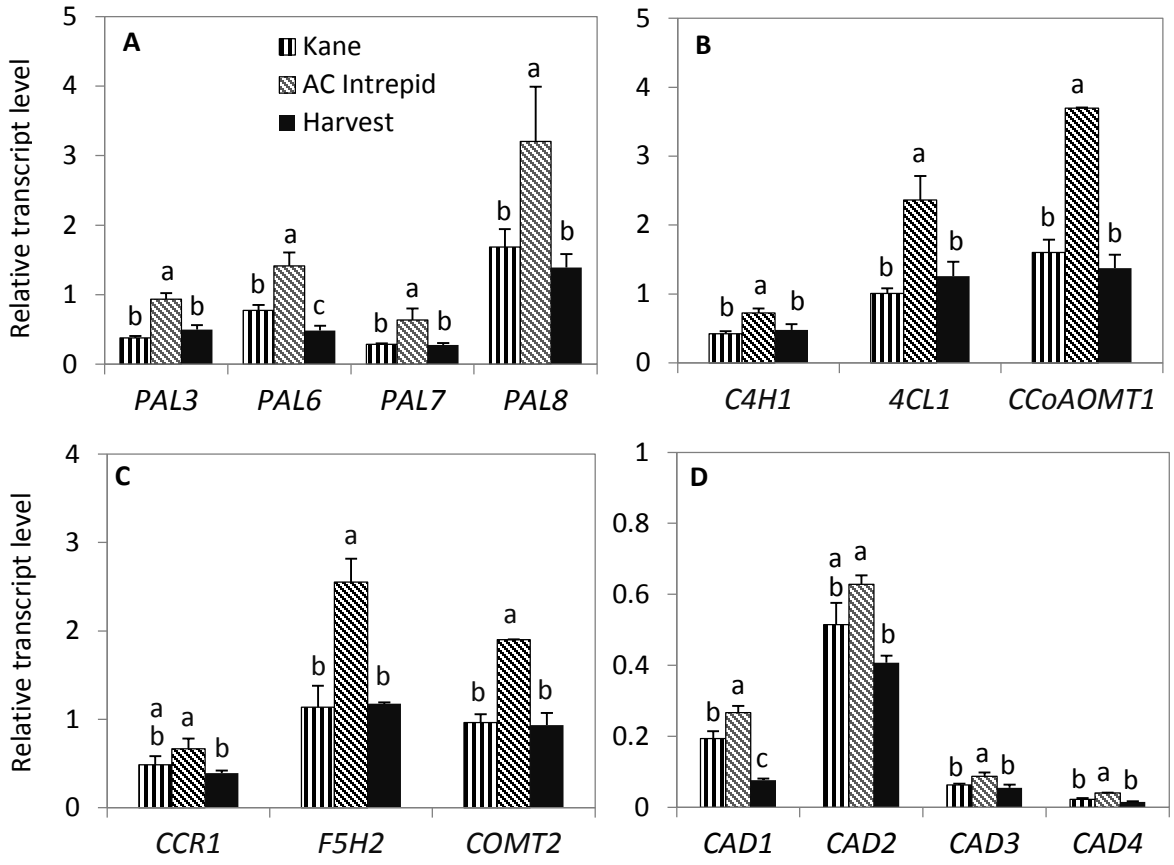

Figure 3.2 Relative transcript levels of lignin biosynthetic candidate genes in flag leaf of wheat cultivars Kane, AC Intrepid and Harvest. Genes involved in the general phenylpropanoid pathway (**A, B**) and genes in the monolignol specific pathway (**C, D**). Transcript levels were expressed relative to that of 4CL1 in cv. Kane, which was set to value of 1. Data are means of 2-3 independent biological replicates ± SE. Different letters within each gene show statistically significant difference in transcript level at $P<0.05$.

While *F5H2* showed higher expression in the internode of cv. AC Intrepid than that of cv. Kane, no differential expression of *4CL1*, *CCR2* and *COMT2* was evident between the two cultivars. Our analysis revealed that *C4H1* also showed differential expression between the internodes cvs. AC Intrepid and Kane; exhibiting higher expression in cv. Kane. Furthermore, slightly higher expression of *CCoAOMT1* was observed in the internode of cv. Kane than that of cv. AC Intrepid.

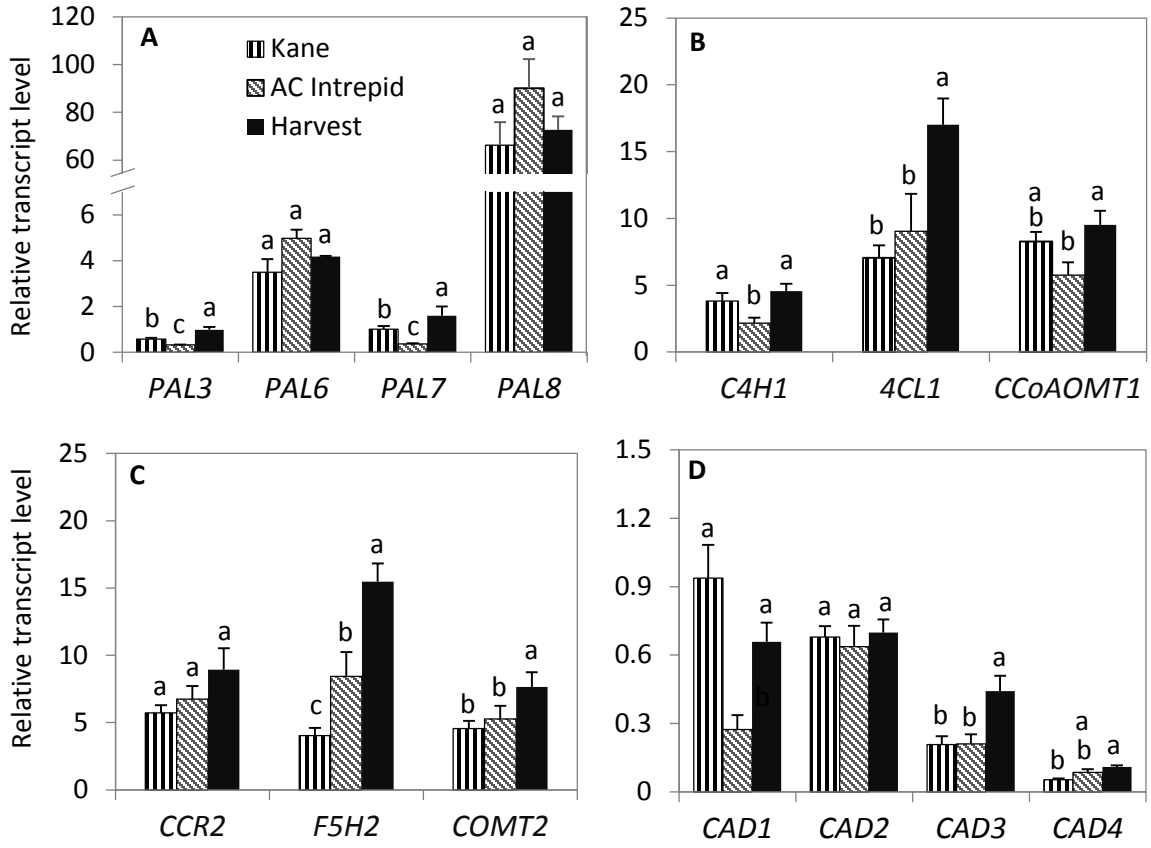

Figure 3.3 Relative transcript levels of lignin biosynthetic candidate genes in internode of wheat cultivars Kane, AC Intrepid and Harvest. Genes involved in the general phenylpropanoid pathway (**A, B**) and genes in the monolignol specific pathway (**C, D**). Transcript levels were expressed relative to that of *4CL1* in cv. Kane, which was set to a value of 1. Data are means of 2-3 independent biological replicates ± SE. Different letters within each gene show statistically significant difference in transcript level at $P<0.05$.

3.3.3 Effect of excessive soil moisture on the expression of lignin biosynthetic genes

Excessive soil moisture significantly induced the transcription of 18 out of the 32 genes analyzed in the flag leaf as compared to that in the untreated control; the transcription of the remaining 13 genes remained unaffected (Figure 3.4A). Genes that exhibited ≥ 2-fold transcriptional activation in response to excessive soil moisture include *PAL8, C4H1, 4CL1, HCT2, CCoAOMT1, CCoAOMT2, CCoAOMT4, CCR2, CCR3, F5H2, COMT1, COMT2, CAD1,* and *CAD4* of which the *PAL8, 4CL1, CCoAOMT1, F5H2* and *COMT2* genes are among those considered as the predominant lignin biosynthesis genes in wheat. Although the expressions of *CCoAOMT2* and *CCR3* showed drastic increases (over 7-fold) in response to excessive soil moisture, the basal expression of these two genes in the control untreated plants was very low.

The expression internode derived lignin biosynthesis genes in response to excessive soil moisture appeared to have different pattern from that observed in the flag leaf tissue (Figure 3.4). Excessive soil moisture led to transcriptional repression of *PAL8, 4CL2, CCR2, F5H1, F5H2*, and *COMT1* genes, of which *4CL2* and *COMT1* are the most affected ones as their transcript levels was decreased by over 2-fold as compared to that found in the control samples. On the other hand, the transcription of all *PAL* genes, except *PAL6* and *PAL8*, was strongly activated by flooding, in particular the transcription of *PAL1* and *PAL2* was induced by over 50-fold as compared to that in the control. However, the basal expression of these genes in the internodes of untreated control plants was very low. Other genes that exhibited over 2-fold transcriptional activation in response to excessive soil moisture include *C4H1*, *CCoAOMT1, CCoAOMT2, CCoAOMT3, CCoAOMT5, CCR3* and *CCR4*. The transcription of six genes including *PAL6, 4CL1, C3H1, COMT2, CAD2,* and *CAD3* remained unaffected by treatment with excessive soil moisture.

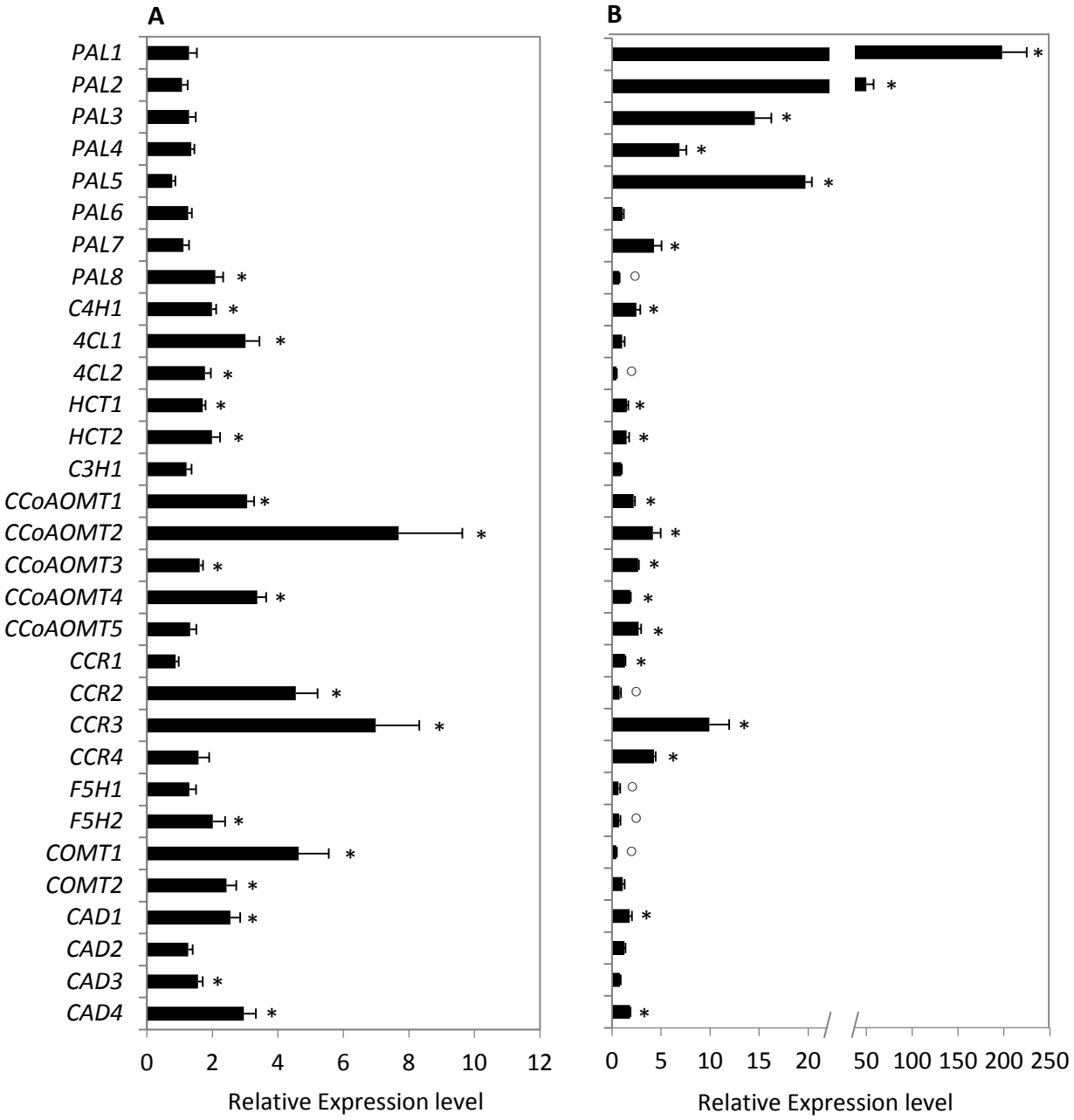

Figure 3.4 Relative transcript levels of lignin biosynthetic candidate genes in the flag leaf (**A**) and internode (**B**) of wheat plants cv. Harvest treated with excessive soil moisture. Transcript levels of each gene in the treated tissues were expressed relative to that detected in the corresponding control, which was set to a value of 1. Data are means of 3-4 independent biological replicates ± SE. The * and ◦ symbols indicate statistically significant higher and lower expression levels, respectively, as compared to that of the control at $P<0.05$.

The effect of excessive soil moisture on the transcription of several lignin biosynthesis genes appeared to be tissue specific. The transcription of *PAL8, 4CL1, 4CL2, CCR2, F5H2, CAD3, COMT1,* and *COMT2* was activated by excessive soil moisture only in the flag leaf; the transcription of these genes in the internode was either repressed or remained unaffected (Figure 3.4). In contrast, excessive soil moisture induced transcriptional activation of *PAL1, PAL2, PAL3, PAL4, PAL5, PAL7, CCoAOMT5, CCR1,* and *CCR4* was found specifically in the internode; and the transcription of these genes in the flag leaf tissue appeared not to be affected by excessive soil moisture.

3.3.4 Enzyme activities in the three wheat cultivars

In all three cultivars, the activity of internode derived PAL and CAD was over 4-fold greater than that found in the flag leaf (Figure 3.5). The activity of flag leaf derived PAL and CAD-CA appeared to be the highest in cv. AC Intrepid followed by cv. Kane and then by cv. Harvest. Significant difference in the activity of CAD-SA derived from the flag leaf was observed among the three cultivars in which the highest activity was recorded in cv. Kane followed by cv. AC intrepid and then cv. Harvest.

The activity of internode derived PAL was found to be higher in cv. AC Intrepid than that of the other two cultivars; the internode of cv. Kane showed slightly higher PAL activity than the internode of cv. Harvest. The internode CAD-SA activity also showed higher activity in cv. AC Intrepid than the other two cultivars, which exhibited a similar level of CAD-SA activity. The internode CAD-CA activity, on the other hand, did not show any substantial difference among the three cultivars.

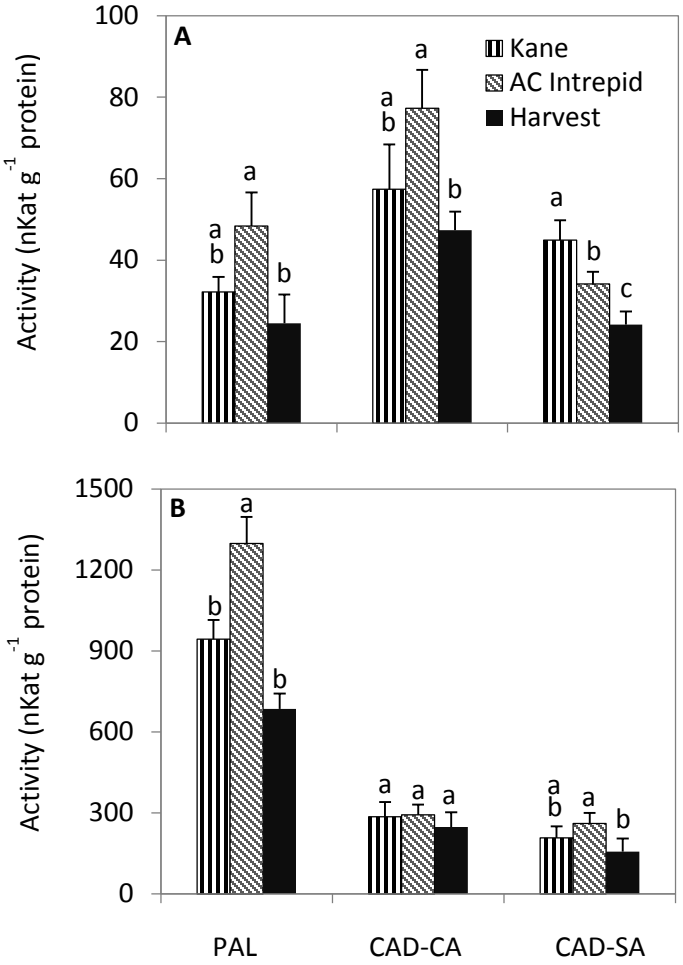

Figure 3.5 Activities of PAL, CAD-CA and CAD-SA in flag leaf (**A**) and internodes (**B**) of three wheat cultivars at heading stage. Data are means of 2-3 independent biological replicates ±SE. Different letters within each enzyme show statistically significant difference in activity at $P<0.05$.

Excessive soil moisture led to a significant increase in the activities of PAL, CAD-CA and CAD-SA in the flag leaf (Figure 3.6A), while causing a significant reduction in the activities of internode derived PAL and CAD-SA. The activity of internode CAD-CA remained unaffected by excessive soil moisture (Figure 3.6B).

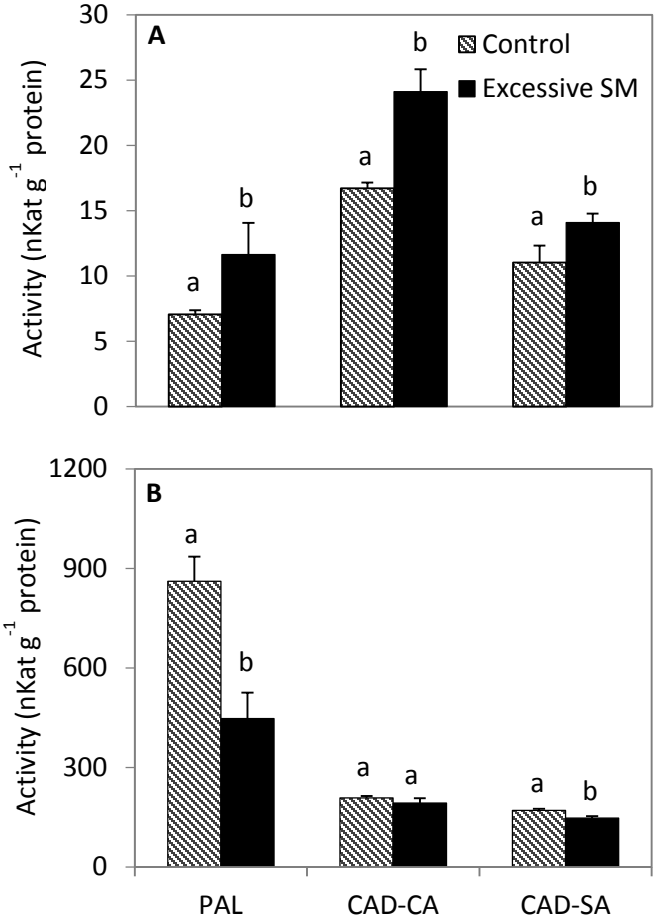

Figure 3.6 Changes in the activities of PAL, CAD-CA and CAD-SA in flag leaf (**A**) and internodes (**B**) of wheat cv. Harvest in response to treatment with excessive soil moisture (SM). Data are means of 2-3 independent biological replicates ±SE. Different letters within each enzyme show statistically significant difference in activity at $P<0.05$.

3.3.5 Content of lignin, cellulose and hemicellulose

Across all the cultivars studied, lignin and cellulose contents of the internode are higher than that of flag leaf. Cellulose content of the internode was 51 to 65% higher than that of the flag leaf; while lignin content of the internode was 73 to 101% higher than that found in the flag leaf. Both the internode and the flag leaf tissues contained a similar amount of hemicellulose (Figure 3.7).

The flag leaf lignin content of cv. AC Intrepid was significantly higher (over 14%) than that of cv. Kane. Although not statistically significant, the flag leaf of cv. AC Intrepid also contained ~7% more lignin than that of cv. Harvest, and the flag leaf of cv. Harvest was found to contain over 7% more lignin than the flag leaf of cv. Kane. The three wheat cultivars did not show differences in flag leaf cellulose content. The hemicellulose content of the flag leaf was also similar between cvs. Kane and AC Intrepid; however, it was 7% higher in cv. Kane than in cv. Harvest, and ~5% higher in cv. AC Intrepid than in cv. Harvest.

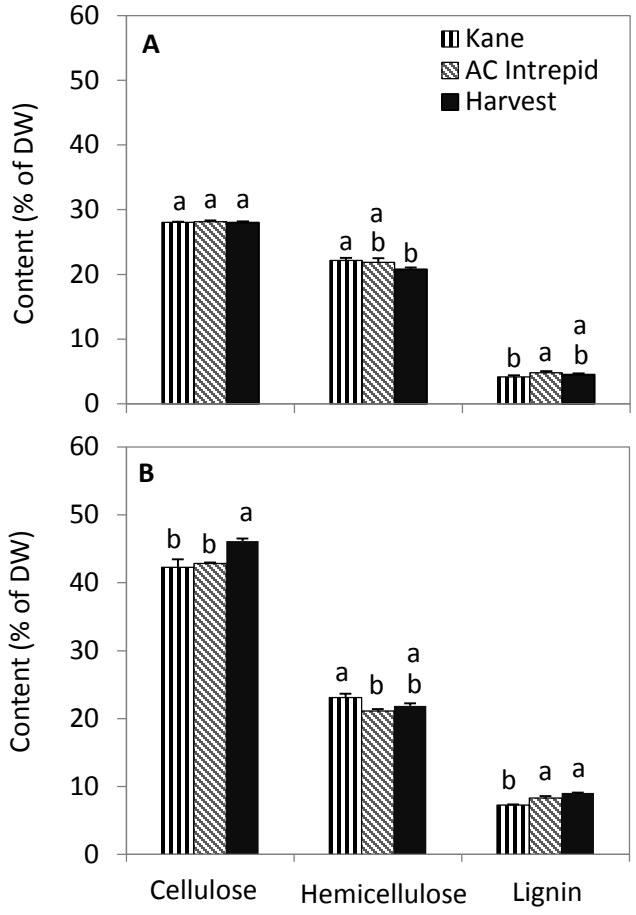

Figure 3.7 Cellulose, hemicellulose and lignin contents of the flag leaf (**A**) and internodes (**B**) of three wheat cultivars. Data are means of 2-3 independent biological replicates ±SE. Different letters within each lignocellulosic constituent show statistically significant difference in activity at $P<0.05$.

Internode lignin content of cv. Harvest was over 8% and 23% higher than that observed in cvs. AC Intrepid and Kane, respectively (Figure 3.7B). The internode lignin content of cv. AC Intrepid was over 13% higher than that found in cv. Kane. Internode cellulose content of cv. Harvest was also ~ 8% and over 9% higher than that of cvs. AC Intrepid and Kane, respectively; but no difference was observed between that of cv. Kane and cv. AC Intrepid. Hemicellulose content of the internode derived from cv. Harvest was almost similar to that of cv. AC Intrepid, while the internode of cv. Kane contained ~6% and ~10% more hemicellulose than cv. Harvest and cv. AC Intrepid, respectively.

Excessive soil moisture caused changes in lignocellulosic composition in both flag leaf and internode tissues. It led to 60% decrease in flag leaf lignin content as compared to that derived from the control untreated plants, while inducing 5% and 30% increases in cellulose and hemicellulose contents, respectively (Figure 3.8A). In the internode, excessive soil moisture significantly reduced the amounts of lignin (36%) and cellulose (20%). Internode hemicellulose content remained unaffected by excessive soil moisture (Figure 3.8B).

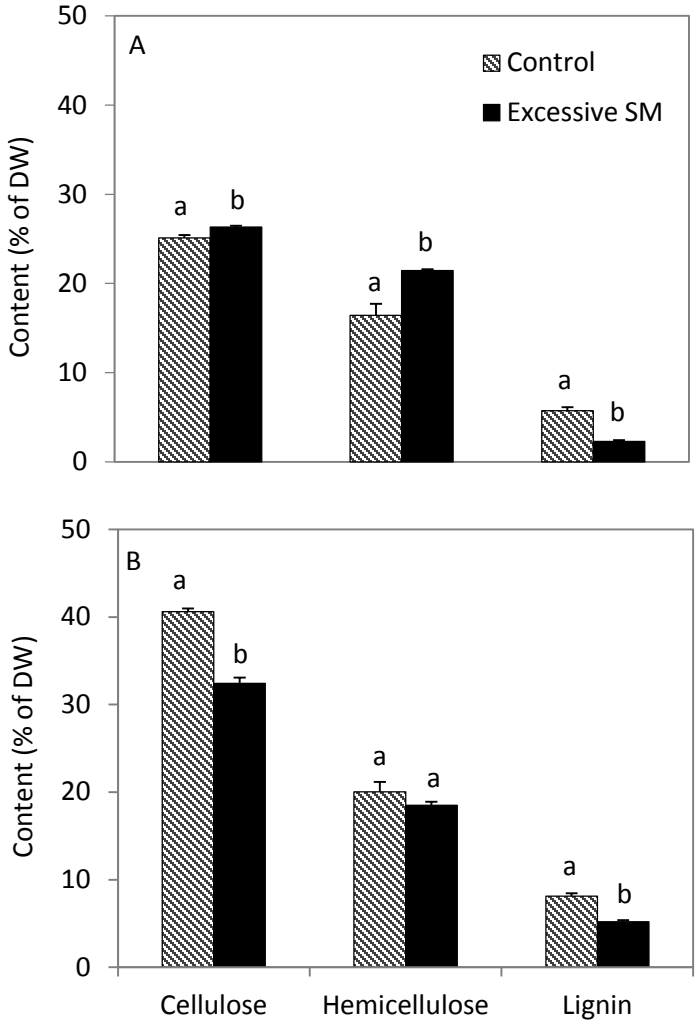

Figure 3.8 Changes in the contents of cellulose, hemicellulose and lignin in the flag leaf (**A**) and internodes (**B**) of wheat cultivar Harvest in response to treatment with excessive soil moisture (SM). Data are means of 3-4 independent biological replicates ±SE. Different letters within each lignocellulosic constituent show statistically significant difference in activity at $P<0.05$.

3.4 Discussion

This study investigated the transcriptional regulation of lignin biosynthesis genes in different wheat vegetative tissues and compared the transcription patterns of genes/gene family members identified as predominant between wheat varieties graded agronomically as "very good" and "good" in terms of resistance to lodging. Furthermore, excess soil moisture induced changes in gene transcription; enzyme activities and lignin formation were examined.

Relative to the tissues derived from the flag leaf, higher transcription for most of the candidate genes, except for *CCR1*, *COMT2* and *CAD2*, was apparent in the internode and peduncle tissues of cv. Harvest (Table 3.3 and Appendix 1), suggesting the prevalence of lignin synthesis in these tissues. Consistently, the internode tissue exhibits greater activity of PAL and CAD (Figure 3.5), and higher lignin content than that observed in the flag leaf (Figure 3.7). Within the stem, older internodes appear to have increased expression for most of the candidate genes as compared to the corresponding younger internodes. In agreement with this finding, the expressions of selected lignin biosynthesis genes and lignin content have been reported to increase with internode age (Bi et al., 2011).

From our results, it also appears that a specific member in each gene family exhibit predominance consistently across the tissues examined, except that two members of the *CCR* family exhibited tissue specificity; *CCR1* is predominant in the flag leaf while *CCR2* in the peduncle and internode tissues (Table 3.3). Consistently, the transcripts of *CCR2* have been shown to be highly abundant in the internode of a wheat cultivar H4564 (Ma, 2007), which is genetically different from the one considered in this study. Overall our study identified *PAL8, C4H1, 4CL1, HCT1, CCoAOMT1, CCR1, CCR2, F5H2, COMT2,* and *CAD2* as predominant genes in wheat biomass; and it is more likely that these genes contribute to the majority of the respective enzyme

activities, and thereby lignin formation. Our result is consistent with that reported by Bi et al. (2011), and some lignin biosynthesis genes have also been identified as being predominant in Arabidopsis tissues (Raes et al., 2003). The presence of statistically significant positive correlation in transcription between the predominant genes in each family and the other (non-predominant) members across the different tissues, such as those of *PAL8* (with *PAL6* [r = 0.88]), *CCoAOMT1* (with *CCoAOMT3* [r = 0.95], *CCoAOMT4* [r = 0.96], or *CCoAOMT5* [r = 0.83]), *CCR2* (with *CCR3* [r = 0.70]), *F5H2* (with *F5H1* [r = 0.83]) and may suggest the existence of additive transcriptional effect and their control by a common regulatory mechanism.

The *COMT* gene along with *F5H* catalyzes successive reactions that represent a specific monolignol pathway, converting coniferaldehyde to sinapaldehyde (SA), which in turn produces S unit of lignin by the action of SA recognizing form of CAD (Vanholme et al., 2010a). Given that mutations in the *F5H* and *COMT* genes have been shown to reduce the amount of oligolignols containing S units but not the total lignin content (Vanholme et al., 2012a), the upregulation of *COMT2* in the flag leaf sheath and in the youngest internode tissue may suggest that the two tissues contain higher amount of S unit lignin than the other flag leaf and stem tissues studied. Relative that found in the internode/peduncle tissues, the transcripts of *CCR1* and *CAD2* genes are highly abundant in the flag leaf tissue, which is characterized by lower lignin content (Table 3.3; Appendix 1), and this result may lead us to suggest that these genes encode enzymes catalyzing non-rate limiting steps. However, previous genetic studies in other species have shown that mutation in *CCR1* and *CAD2* results in decreased lignin content (Dien et al., 2009; Vanholme et al., 2012a), implying their regulation by a posttranscriptional mechanism. In agreement with this, a higher CAD-SA: CAD-CA activity ratio was detected in the internode (0.63) than in the flag leaf (0.51) (Figure 3.5). The existence of a significant positive correlation ($r=0.98$, $P<0.001$) between

the two genes across the different tissues might indicate their regulation by a common mechanism, as observed for their transcription, which appears to be regulated by the same transcription factor, TaMYB4 (Ma et al., 2011).

To get insights into the role genetic variation may have on lignin formation in wheat biomass, we performed comparative analysis of gene expression, the activity of selected lignin biosynthetic enzymes and lignocellulosic profiles across three commercial wheat cultivars that show difference in their resistance to lodging; cv. Harvest is designated agronomically as more resistant to lodging than AC Intrepid and Kane. The higher internode lignin content in cv. Harvest as compared to that of cvs. AC Intrepid and Kane (Figure 3.7B), suggests a close association between stem strength and stem lignin content (Ma 2009; 2010; Wu et al., 2011; Wang et al., 2012). The internode lignin content of cv. Harvest was also found to be closely associated with the expression of *4CL1*, *CCR2, F5H2* and *COMT2*, suggesting the significance of these genes as predominant mediators of variation in internode lignin content, and thereby stem mechanical strength in these cultivars. Consistently, a close association of variations in wheat stem lignin content with differential transcriptions of *CCR* and *COMT* genes and activities of the corresponding enzymes has been observed (Ma, 2007; 2009). As *F5H2* and *COMT2* have been reported to play major role in altering lignin composition, the ratio of lignin units (S/G) in particular, our results might also imply the presence of higher S/G ratio in the internode lignin of cv. Harvest as compared to the other two cultivars.

Comparison between cvs. Harvest and AC Intrepid also suggest a close association between the transcription patterns of *C4H1* and *CCoAOMT1*, and lignin content in the internode. However, the expression of these genes in the internode of cv. Harvest exhibits a similar pattern with that of cv. Kane irrespective of differences in resistance to lodging and internode lignin

content between the two cultivars, implying their control by genetic divergence induced post-transcriptional mechanisms.

Relative to the other two cultivars, higher flag leaf lignin content was found in cv. AC Intrepid (Figure 3.7A), and this is accompanied by enhanced transcription of all of the genes analyzed and increased activities of PAL and CAD-CA (Figures 3.2 and 3.5A). Given that it exhibited significantly higher internode cellulose content, and similar/lower leaf lignin content than the other two cultivars (Figure 3.7), cv. Harvest can be targeted for genetic alteration of lignin content/composition for improving the feedstock quality of wheat straw while maintaining sufficient stem mechanical strength against lodging. However, biomass feedstock quality is also affected by the ratio of lignin units (Vanholme et al., 2012b), thus it is important to characterize this feature of the wheat cv. Harvest biomass. Previous study by Zeng et al. (2013) has shown that G and S are the predominant lignin units in wheat straw, which exhibits a S:G:H ratios of 37.5: 60.2: 2.3.

Lignin biosynthesis is also influenced by several environmental cues including low temperatures, water deficit, excessive light, and abnormal concentration of mineral element in the soil (Escamilla-Trevino et al., 2010; Moura et al., 2010; Moura-Sobczak, 2011). Plants exposed to excessive soil moisture conditions accumulate more soluble carbohydrate in their shoot and root tissues (Albrecht et al., 1993), and transport increased amount of sugar to the root (Huang and Johnson, 1995). Such changes might have a significant effect on the level of structural carbohydrate polymers such as cellulose, hemicellulose and lignin in growing vegetative tissues (Usadel et al., 2008; Lastdrager et al., 2014). Consistently, the lignin contents of both internode and flag leaf tissues were substantially reduced in response to treatment with excessive soil moisture (Figure 3.8). This along with the significant reduction of cellulose level in the internode

tissue under excessive soil moisture condition led to a decrease in the total amount of structural carbohydrates. However, in the flag leaf tissue, the decrease in lignin content was compensated by significant increases in the levels of cellulose and hemicellulose. Prolonged flooding reduces leaf photosynthetic capacity in wheat (Musgrave, 1994; Meisrimler et al., 2014), and thereby the amount of photoassimlates required to support growth and maintenance. In agreement with this, the wheat plants treated with excessive soil moisture for 3 weeks displayed yellowish green leaves (Figure 3.1B), which is an indication of a decrease in chlorophyll content and photosynthesis, and this effect was reflected by a significant reduction in the contents of cellulose and lignin in the internode tissue where these structural carbohydrates are the primary constituents. Furthermore, the increased demand for soluble sugars, particularly glucose, in flood-treated plants (Huang and Johnson 1995; Huang et al., 1997) might direct a greater proportion of phosphoenol pyruvate to other pathways such as glycolysis rather than to the shikimate pathway, which produces phenylalanine, an important substrate for the first reaction of lignin biosynthesis (Vanholme et al., 2012a). Since the biosynthesis of structural components such as lignin is an energy-consuming and irreversible process (Amthor, 2003; Zhao and Dixon, 2011), reduction in the accumulation of lignin in the water logged plants might be one of the energy/carbon conserving strategies employed by plants under such stress conditions (Ricard et al., 1994; Drew, 1997).

Among the genes identified as predominantly expressed in wheat biomass, transcriptional repression of *PAL8, CCR2* and *F5H2* was apparent specifically in the internode tissue of plants treated with excessive soil moisture, suggesting their significance in modulating tissue specific lignin deposition and composition under stress conditions. Consistently, the activity of internode derived PAL was decreased in the internode. In contrast to the lignin content phenotype, increased transcription of several predominant lignin biosynthesis genes was evident in both internode

(*C4H1, HCT1* and *CCoAOMT1*) and flag leaf (*PAL8, C4H1, 4CL1, HCT1, CCoAOMT1, F5H2* and *COMT2*) in response to excessive soil moisture conditions (Figure 3.4A, B). Concurrently, PAL activity was increased in the flag leaf tissues derived from plants grown under excessive soil moisture (Figure 3.6A). Thus, our results might suggest that some of the lignin biosynthesis genes in wheat are under feedback regulation. Although not predominant, the *CAD1*, *CAD3* and *CAD4* genes also exhibited transcriptional activation in flag leaf tissues of plants treated with excessive soil moisture, and this was accompanied by a considerable increase in the activities of both CAD-CA and CAD-SA (Figure 3.6A).

4. GENERAL DISCUSSION AND CONCLUSION

Meeting the growing demands for food and energy due to population growth will be a global challenge in near future. Cereal crops are one of the major crops in the world, for example the total area under wheat cultivation is over 210 million hectares (FAO, 2014). The world population is projected to reach 9.6 billion by 2050 (United Nations, 2013), and by that time about 3 billion tons of cereals would be needed for both food and animal feed (FAO, 2009b). It predicted that the global energy consumption by the year 2050 will increase by up to 61% of that consumed in 2010, and fossil fuels will still remain as the major source of energy (World Energy Council, 2013). It is considerable for the world energy safety because the world fossil fuel would be exhausted by 2088 (USCIA, 2014). These predictions implicate the need for global efforts to meet the ever increasing demands for both food and energy without affecting the quality of our environment (IPCC 2014). Given that cereals are among the major crops cultivated in many countries, efficient production of bioethanol from cereal crop residues such as wheat straw that do not compete with food and feed can have a significant contribution with this respect. This is because the residues of cereal crops are non-edible biomass often left in the field except that a small proportion is used for soil conservation, and animal feed and bedding.

One of the major factors hindering the commercial success of producing bioethanol from lignocellulosic biomass is the presence of lignin as a structural component in plant cell wall/biomass (Burton and Fincher, 2014). Lignin confers recalcitrance to plant biomass used as feedstock for the production of bioethanol and bioproducts. Therefore, genetic reduction of biomass lignin content or alteration of its composition such as the S/G and *p*-CA/FA ratios, without affecting the agronomic performance of plants, has been considered as a viable strategy to reduce biomass recalcitrance for efficient and cost-effective bioethanol production. For example, genetic

manipulation of lignin has been shown to improve saccharification efficiency and bioethanol yield in a number of plant species including sorghum (Dien et al., 2009), alfalfa (Chen and Dixon, 2007), switchgrass (Fu et al., 2011a; Fu et al., 2011b; Shen et al., 2012) and aspen (Hu et al., 1999). These findings implicate that the feedstock quality of wheat straw can also be enhanced through alteration of either the amount and/or the composition of lignin. However, this requires a detailed understanding of the molecular and genetic mechanisms underlying the synthesis of lignin and its composition in wheat biomass.

In this study, we first characterized the expression patterns of 32 candidate lignin biosynthetic genes in different wheat tissues using a commercial wheat cultivar, cv. Harvest. It appears from our results that the *4CL1, C3H1, C4H1, CAD2, CCoAOMT1, COMT2, F5H2, HCT1* and *PAL8* are expressed predominantly in all of the tissue examined. However, a tissue specific predominance was observed for *CCR* family members; *CCR1* being highly abundant in flag leaves while *CCR2* in peduncles and internodes. All of the predominant genes, with the exceptions of *CAD2* and *COMT2,* exhibited several fold higher levels of transcript in the internodes than in the flag leaves. Consistent with these results, higher PAL activity and higher lignin content were measured in the internode as compared to flag leaves. The lack of correlation between the expression levels of *CAD2* and *COMT2*, and lignin content across all the tissues examined may implicates that the *CAD2* and *COMT2* genes are under posttranscriptional regulation.

The second part of this thesis performed comparative analysis of the expression of predominant wheat lignin genes, activities of selected enzymes and lignocellulosic profiles in the internode and flag leaf tissues of three Canadian wheat cultivars, Harvest, Kane, and AC Intrepid. Of the three cultivars, cv. Harvest is more resistant to lodging than cvs. Kane and AC Intrepid, and consistently it exhibited higher internode lignin content as compared to cvs. Kane and AC Intrepid.

This result of lignin profile is closely associated with the differential expression patterns of *4CL1, COMT2* and *F5H2* genes, suggesting the importance of these genes in inducing variation in stem lignin content among the three cultivars.

The final part of the thesis investigated the response of lignin biosynthesis in wheat to excessive soil moisture using cv. Harvest as an experimental material. Treatment of four-week-old wheat plants with excessive soil moisture for three weeks greatly reduced lignin content in both flag leaf and internode tissues. The transcriptional repression of *PAL8, CCR2* and *F5H2* in the internodes of wheat plants treated with excessive soil moisture suggests the significance of these genes in modulating tissue specific lignin deposition under stress conditions. The decreased expression of internode derived *PAL8* was also shown to be accompanied by reduced PAL activity. In contrast, the transcription of several predominant lignin biosynthesis genes was enhanced in both flag leaves and in internodes by the treatment, implying the regulation of these genes by a feedback mechanism.

In conclusion, the finding in this study provides important insights into our understanding of the molecular mechanisms underlying lignin formation in response to genetic variation and environmental factors. Further study of the posttranscriptional regulation of the genes identified as playing a role in mediating variation in lignin content across the different cultivars would provide additional useful information to develop tools that can facilitate the development of wheat cultivars with improved feedstock quality.

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APPENDIX 1. SUPPLEMANTARY TABLES

Table S1. Relative transcript levels of lignin biosynthesis candidate genes in different tissues of wheat cv. Harvest[a]

Gene	FB	FS	PE	IN-1	IN-2&3	IN-4
PAL1	0.34 ±0.084b	0.02 ±0.004c	0.01 ±0.001d	0.25 ±0.059b	2.18 ±0.249a	2.40 ±0.288a
PAL2	0.01 ±0.001c	ND	ND	0.03 ±0.006b	0.18 ±0.021a	0.22 ±0.029a
PAL3	0.68 ±0.044bc	0.43 ±0.062c	0.58 ±0.086bc	0.87 ±0.185b	2.15 ±0.222a	2.82 ±0.437a
PAL4	0.26 ±0.045cd	0.16 ±0.023d	0.36 ±0.097c	0.75 ±0.131b	2.76 ±0.217a	3.22 ±0.227a
PAL5	0.26 ±0.031c	0.12 ±0.009d	0.16 ±0.025d	0.32 ±0.047c	1.48 ±0.155b	2.35 ±0.295a
PAL6	0.54 ±0.034d	1.58 ±0.162c	7.92 ±0.595a	6.51 ±0.702b	6.95 ±1.067ab	7.28 ±0.758a
PAL7	0.32 ±0.033e	0.28 ±0.014e	0.49 ±0.081d	0.89 ±0.043c	3.80 ±0.442b	5.71 ±0.653a
PAL8	0.83 ±0.071d	9.05 ±0.798c	89.25 ±19.53a	54.50 ±6.706b	54.57 ±11.98b	33.66 ±6.983b
C4H1	0.38 ±0.033d	0.91 ±0.039c	2.68 ±0.395b	3.67 ±0.606b	9.57 ±1.200a	11.22 ±3.709a
4CL1	1.00 ±0.088d	2.04 ±0.076c	7.61 ±1.099b	7.13 ±0.571b	12.51 ±2.022a	16.84 ±2.830a
4CL2	0.10 ±0.012d	0.25 ±0.029c	0.51 ±0.065ab	0.63 ±0.056a	0.35 ±0.088bc	0.46 ±0.037ab
HCT1	0.58 ±0.034d	0.70 ±0.058d	2.53 ±0.544b	1.54 ±0.196c	3.09 ±0.397b	5.42 ±0.600a
HCT2	0.19 ±0.018d	0.31 ±0.023d	2.77 ±0.723a	0.85 ±0.089c	1.35 ±0.103b	2.18 ±0.404ab
C3H1	0.12 ±0.028e	0.21 ±0.031de	1.24 ±0.057a	0.87 ±0.115b	0.55 ±0.125c	0.46 ±0.033cd
C3H2	ND	ND	ND	0.01 ±0.001	ND	ND
CCoAOMT1	1.52 ±0.170e	3.32 ±0.579d	32.73 ±5.275a	11.98 ±2.506c	19.56 ±3.506bc	21.89 ±4.771b
CCoAOMT2	0.02 ±0.001c	0.05 ±0.001c	0.03 ±0.004a	0.02 ±0.004ab	0.02 ±0.001c	0.02 ±0.003bc
CCoAOMT3	0.72 ±0.043d	0.94 ±0.067cd	6.35 ±1.810a	1.24 ±0.128c	3.15 ±0.555b	5.04 ±1.470ab
CCoAOMT4	0.27 ±0.018e	1.62 ±0.321d	11.95 ±0.466a	5.30 ±0.890c	4.75 ±0.674c	7.05 ±1.038b
CCoAOMT5	0.48 ±0.077e	0.99 ±0.081d	2.29 ±0.371ab	1.26 ±0.025cd	1.64 ±0.186bc	3.02 ±0.538a
CCR1	11.11 ±0.209a	6.64 ±0.358b	0.29 ±0.033f	1.59 ±0.306c	0.78 ±0.020d	0.45 ±0.066e
CCR2	0.17 ±0.015e	1.56 ±0.073d	3.77 ±0.784c	5.93 ±0.294b	8.44 ±0.848a	7.82 ±1.088a
CCR3	0.03 ±0.003cd	0.01 ±0.004e	0.02 ±0.003de	0.06 ±0.003bc	0.22 ±0.081ab	0.56 ±0.009a
CCR4	0.02 ±0.009b	0.01 ±0.006b	0.01 ±0.001b	0.03 ±0.023b	0.03 ±0.008b	0.12 ±0.002a
F5H1	0.44 ±0.125c	0.37 ±0.065cd	4.56 ±0.798a	0.80 ±0.085b	0.20 ±0.031d	0.55 ±0.085bc
F5H2	0.84 ±0.043e	1.39 ±0.039d	9.20 ±1.670a	6.09 ±0.968ab	3.38 ±1.047c	4.23 ±0.838bc
COMT1	0.01 ±0.000f	1.28 ±0.265c	6.06 ±1.163a	2.82 ±0.318b	0.34 ±0.090d	0.16 ±0.023e
COMT2	15.51 ±1.129b	36.04 ±4.195a	16.09 ±2.857b	31.56 ±4.397a	14.18 ±1.635b	12.63 ±0.673b
CAD1	0.14 ±0.012c	0.10 ±0.026c	0.38 ±0.090b	0.61 ±0.117b	1.81 ±0.232a	1.61 ±0.342a
CAD2	13.49 ±1.100a	9.33 ±0.208b	0.93 ±0.088d	2.76 ±0.661cd	2.85 ±0.355cd	3.54 ±0.630c
CAD3	1.32 ±0.072b	2.05 ±0.083a	0.16 ±0.001c	0.95 ±0.215b	2.02 ±0.174a	2.55 ±0.286a
CAD4	0.84 ±0.101abc	1.15 ±0.104ab	1.36 ±0.374a	1.10 ±0.250ab	0.70 ±0.039bc	0.42 ±0.045c

[a] Transcript levels were expressed relative to that of *4CL1* in flag leaf, which was set to value of 1. Data are means of 2-3 independent biological replicates ± SE. Means followed by different letters within each gene show statistically significant difference at $P<0.05$. FB, flag leaf blade; FS, flag leaf sheath; PE, peduncle; IN-1, the first internode; IN-2&3, the second and third internode; IN-4, the fourth internode (The numerical designation of internodes was based on their relative position to the peduncle in which IN-1 is the youngest/the one next to the peduncle while IN-4 is the oldest internode of the stem), nd=not detected.

Table S2. Analysis of variance for relative transcript levels of *PAL3* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.04747	0.02373	1.26	0.335
Cultivar	5	0.48443	0.09689	5.13	0.021
Residual	8	0.15107	0.01888		
Total	15	0.68297	0.04553		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.26

Average LSD: 0.28

Maximum LSD: 0.33

Table S3. Analysis of variance for relative transcript levels of *PAL6* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.06013	0.03007	1.09	0.38
Cultivar	5	1.07975	0.21595	7.86	0.006
Residual	8	0.21975	0.02747		
Total	15	1.35962	0.09064		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.31

Average LSD: 0.34

Maximum LSD: 0.40

Table S4. Analysis of variance for relative transcript levels of *PAL7* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.029933	0.014966	1.54	0.266
Cultivar	5	0.194406	0.038881	4.01	0.034
Residual	9	0.087364	0.009707		
Total	16	0.311702	0.019481		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.18

Average LSD: 0.19

Maximum LSD: 0.21

Table S5. Analysis of variance for relative transcript levels[a] of *PAL8* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.01391	0.00695	0.42	0.67
Cultivar	5	0.2355	0.0471	2.85	0.091
Residual	8	0.13224	0.01653		
Total	15	0.38165	0.02544		

[a]Using log transformed data

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.24

Average LSD: 0.27

Maximum LSD: 0.31

Table S6. Analysis of variance for relative transcript levels of *C4H1* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.028935	0.014468	1.82	0.224
Cultivar	5	0.458859	0.091772	11.52	0.002
Residual	8	0.063735	0.007967		
Total	15	0.551529	0.036769		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.17

Average LSD: 0.18

Maximum LSD: 0.21

Table S7. Analysis of variance for relative transcript levels[a] of *4CL1* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.089383	0.044692	7.75	0.017
Cultivar	5	0.175213	0.035043	6.08	0.017
Residual	7	0.040356	0.005765		
Total	14	0.304952	0.021782		

[a]Using log transformed data

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.14

Average LSD: 0.17

Maximum LSD: 0.18

Table S8. Analysis of variance for relative transcript levels of *CCoAOMT1* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	2.7373	1.3686	12.37	0.004
Cultivar	5	7.2208	1.4442	13.05	0.001
Residual	8	0.885	0.1106		
Total	15	10.8431	0.7229		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.63

Average LSD: 0.68

Maximum LSD: 0.77

Table S9. Analysis of variance for relative transcript levels[a] of *CCR1* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.024684	0.012342	1.39	0.303
Cultivar	5	0.201039	0.040208	4.53	0.029
Residual	8	0.070982	0.008873		
Total	15	0.296705	0.01978		

[a]Using log transformed data

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.18

Average LSD: 0.19

Maximum LSD: 0.22

Table S10. Analysis of variance for relative transcript levels of *F5H2* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	1.10883	0.55442	9.01	0.016
Cultivar	5	2.43757	0.48751	7.92	0.013
Residual	6	0.36936	0.06156		
Total	13	3.91577	0.30121		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.50

Average LSD: 0.56

Maximum LSD: 0.61

Table S11. Analysis of variance for relative transcript levels of *COMT2* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.31209	0.15605	5.97	0.026
Cultivar	5	1.95424	0.39085	14.95	< 0.001
Residual	8	0.20916	0.02615		
Total	15	2.47549	0.16503		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.30

Average LSD: 0.33

Maximum LSD: 0.37

Table S12. Analysis of variance for relative transcript levels of *CAD1* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.0039024	0.0019512	2.62	0.152
Cultivar	5	0.0761225	0.0152245	20.47	0.001
Residual	6	0.0044626	0.0007438		
Total	13	0.0844875	0.006499		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.054

Average LSD: 0.064

Maximum LSD: 0.067

Table S13. Analysis of variance for relative transcript levels[a] of *CAD2* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.005676	0.002838	0.46	0.649
Cultivar	5	0.203846	0.040769	6.56	0.01
Residual	8	0.049733	0.006217		
Total	15	0.259256	0.017284		

[a]Using log transformed data

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.15

Average LSD: 0.16

Maximum LSD: 0.18

Table S14. Analysis of variance for relative transcript levels of *CAD3* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.0004625	0.0002312	2.28	0.165
Cultivar	5	0.0052943	0.0010589	10.44	0.002
Residual	8	0.0008113	0.0001014		
Total	15	0.006568	0.0004379		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.019

Average LSD: 0.021

Maximum LSD: 0.023

Table S15. Analysis of variance for relative transcript levels of *CAD4* in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.00006857	0.00003428	1.84	0.238
Cultivar	5	0.00077577	0.00015515	8.32	0.011
Residual	6	0.00011188	0.00001865		
Total	13	0.00095622	0.00007356		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.0086

Average LSD: 0.0103

Maximum LSD: 0.0115

Table S16. Analysis of variance for relative transcript levels[a] of *PAL3* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.003723	0.001862	0.24	0.791
Cultivar	5	0.475295	0.095059	12.32	0.001
Residual	8	0.061743	0.007718		
Total	15	0.540761	0.036051		

[a]Using log transformed data

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.17

Average LSD: 0.18

Maximum LSD: 0.20

Table S17. Analysis of variance for relative transcript levels of *PAL6* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	3.034	1.517	1.27	0.332
Cultivar	5	4.659	0.932	0.78	0.591
Residual	8	9.561	1.195		
Total	15	17.254	1.15		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 2.1

Average LSD: 2.2

Maximum LSD: 2.5

Table S18. Analysis of variance for relative transcript levels[a] of *PAL7* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.038158	0.019079	2.19	0.175
Cultivar	5	0.803046	0.160609	18.42	< 0.001
Residual	8	0.069758	0.00872		
Total	15	0.910961	0.060731		

[a]Using log transformed data

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.18

Average LSD: 0.19

Maximum LSD: 0.22

Table S19. Analysis of variance for relative transcript levels of *PAL8* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	707.3	353.6	1.26	0.334
Cultivar	5	3330.2	666	2.38	0.132
Residual	8	2241.5	280.2		
Total	15	6278.9	418.6		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 31.5

Average LSD: 34.4

Maximum LSD: 38.6

Table S20. Analysis of variance for relative transcript levels of *C4H1* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	3.6667	1.8333	2.33	0.153
Cultivar	5	20.076	4.0152	5.1	0.017
Residual	9	7.079	0.7866		
Total	16	30.8218	1.9264		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 1.6

Average LSD: 1.7

Maximum LSD: 1.9

Table S21. Analysis of variance for relative transcript levels[a] of *4CL1* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.06712	0.03356	2.75	0.124
Cultivar	5	0.64229	0.12846	10.51	0.002
Residual	8	0.09774	0.01222		
Total	15	0.80716	0.05381		

[a]Using log transformed data

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.21

Average LSD: 0.23

Maximum LSD: 0.27

Table S22. Analysis of variance for relative transcript levels[a] of *CCoAOMT1* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.00756	0.00378	0.3	0.75
Cultivar	5	0.17349	0.0347	2.72	0.091
Residual	9	0.11474	0.01275		
Total	16	0.29579	0.01849		

[a]Using log transformed data

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.21

Average LSD: 0.22

Maximum LSD: 0.24

Table S23. Analysis of variance for relative transcript levels of *CCR2* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	11.17	5.585	1.77	0.224
Cultivar	5	55.473	11.095	3.52	0.048
Residual	9	28.335	3.148		
Total	16	94.979	5.936		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 3.3

Average LSD: 3.4

Maximum LSD: 3.7

Table S24. Analysis of variance for relative transcript levels of *F5H2* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	5.268	2.634	0.65	0.546
Cultivar	5	280.734	56.147	13.78	< 0.001
Residual	9	36.661	4.073		
Total	16	322.663	20.166		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 3.7

Average LSD: 3.9

Maximum LSD: 4.3

Table S25. Analysis of variance for relative transcript levels[a] of *COMT2* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.00416	0.00208	0.14	0.874
Cultivar	5	0.26657	0.05331	3.51	0.049
Residual	9	0.13652	0.01517		
Total	16	0.40725	0.02545		

[a]Using log transformed data

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.23

Average LSD: 0.24

Maximum LSD: 0.26

Table S26. Analysis of variance for relative transcript levels of *CAD1* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.10578	0.05289	4.52	0.049
Cultivar	5	0.72521	0.14504	12.4	0.001
Residual	8	0.09357	0.0117		
Total	15	0.92456	0.06164		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.20

Average LSD: 0.22

Maximum LSD: 0.25

Table S27. Analysis of variance for relative transcript levels[a] of *CAD2* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.04456	0.02228	0.48	
Cultivar	5	0.04624	0.00925	0.2	0.956
Residual	10	0.46575	0.04658		
Total	17	0.55656			

[a]Using log transformed data

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.39

Average LSD: 0.39

Maximum LSD: 0.39

Table S28. Analysis of variance for relative transcript levels of *CAD3* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.042087	0.021043	6.98	0.015
Cultivar	5	0.115949	0.02319	7.69	0.005
Residual	9	0.027151	0.003017		
Total	16	0.185187	0.011574		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.10

Average LSD: 0.11

Maximum LSD: 0.12

Table S29. Analysis of variance for relative transcript levels of *CAD4* in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.0001505	0.0000753	0.27	0.775
Cultivar	5	0.0064755	0.0012951	4.58	0.046
Residual	6	0.0016975	0.0002829		
Total	13	0.0083236	0.0006403		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.034

Average LSD: 0.040

Maximum LSD: 0.044

Table S30. Analysis of variance for activity of PAL in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	761.3	380.7	2.99	
Cultivar	5	1504	300.8	2.36	0.116
Residual	10	1274.5	127.4		
Total	17	3539.8			

Least significant difference (LSD) at $P \leq 0.05$ is 20.6

Table S31. Analysis of variance for activity of CAD-CA in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	1371.37	685.69	12.62	
Cultivar	5	2104.08	420.82	7.74	0.003
Residual	10	543.47	54.35		
Total	17	4018.92			

Least significant difference (LSD) at $P \leq 0.05$ is 13.4

Table S32. Analysis of variance for activity of CAD-SA in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	874.66	437.33	8.36	0.009
Cultivar	5	801.7	160.34	3.06	0.069
Residual	9	470.9	52.32		
Total	16	2147.27	134.2		

Least significant difference (LSD) at $P \leq 0.05$ is 14.0

Table S33. Analysis of variance for activity of PAL in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	94701	47350	1.46	
Cultivar	5	994139	198828	6.15	0.007
Residual	10	323451	32345		
Total	17	1412291			

Least significant difference (LSD) at $P \leq 0.05$ is 327

Table S34. Analysis of variance for activity of CAD-CA in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	71303.7	35651.9	73.23	
Cultivar	5	4474.5	894.9	1.84	0.193
Residual	10	4868.2	486.8		
Total	17	80646.5			

Least significant difference (LSD) at $P \leq 0.05$ is 40.1

Table S35. Analysis of variance for activity of CAD-SA in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	63751.2	31875.6	63.78	
Cultivar	5	24248	4849.6	9.7	0.001
Residual	10	4998	499.8		
Total	17	92997.2			

Least significant difference (LSD) at $P \leq 0.05$ is 40.1

Table S36. Analysis of variance for content of cellulose in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.7509	0.3755	2.93	0.105
Cultivar	5	2.1925	0.4385	3.42	0.052
Residual	9	1.1541	0.1282		
Total	16	4.0975	0.2561		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.66

Average LSD: 0.69

Maximum LSD: 0.75

Table S37. Analysis of variance for content of hemicellulose in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	8.14	4.07	10.47	0.004
Cultivar	5	10.4252	2.085	5.36	0.015
Residual	9	3.4991	0.3888		
Total	16	22.0643	1.379		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 1.15

Average LSD: 1.21

Maximum LSD: 1.31

Table S38. Analysis of variance for content of lignin in the flag leaf of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.5364	0.2682	2.53	0.134
Cultivar	5	2.964	0.5928	5.6	0.013
Residual	9	0.9533	0.1059		
Total	16	4.4538	0.2784		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.60

Average LSD: 0.63

Maximum LSD: 0.69

Table S39. Analysis of variance for content of cellulose in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	10.102	5.051	4.51	0.044
Cultivar	5	26.439	5.288	4.72	0.022
Residual	9	10.083	1.12		
Total	16	46.624	2.914		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 1.96

Average LSD: 2.05

Maximum LSD: 2.23

Table S40. Analysis of variance for content of hemicellulose in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	5.4204	2.7102	3.89	0.061
Cultivar	5	11.7727	2.3545	3.38	0.054
Residual	9	6.2678	0.6964		
Total	16	23.4609	1.4663		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 1.54

Average LSD: 1.61

Maximum LSD: 1.76

Table S41. Analysis of variance for content of lignin in the internode of the three wheat cultivars

Source	Degree of Freedom	Sum of Squares	Mean of Squares	Variation Ratio	F Probability
Replicate	2	0.6596	0.3298	1.35	0.308
Cultivar	5	9.65	1.93	7.88	0.004
Residual	9	2.2032	0.2448		
Total	16	12.5128	0.7821		

Least significant difference (LSD) at $P \leq 0.05$

Minimum LSD: 0.91

Average LSD: 0.96

Maximum LSD: 1.04

APPENDIX 2. SUPPLEMANTARY FIGURES

Figure S1. The correlation between relative transcript levels of *PAL8 and PAL6*

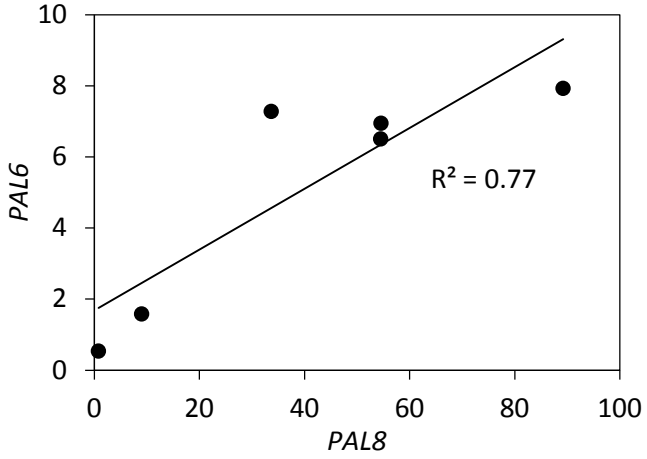

Figure S2. The correlation between relative transcript levels of *CCoAOMT1* and *CCoAOMT3 / CCoAOMT4 / CCoAOMT5*.

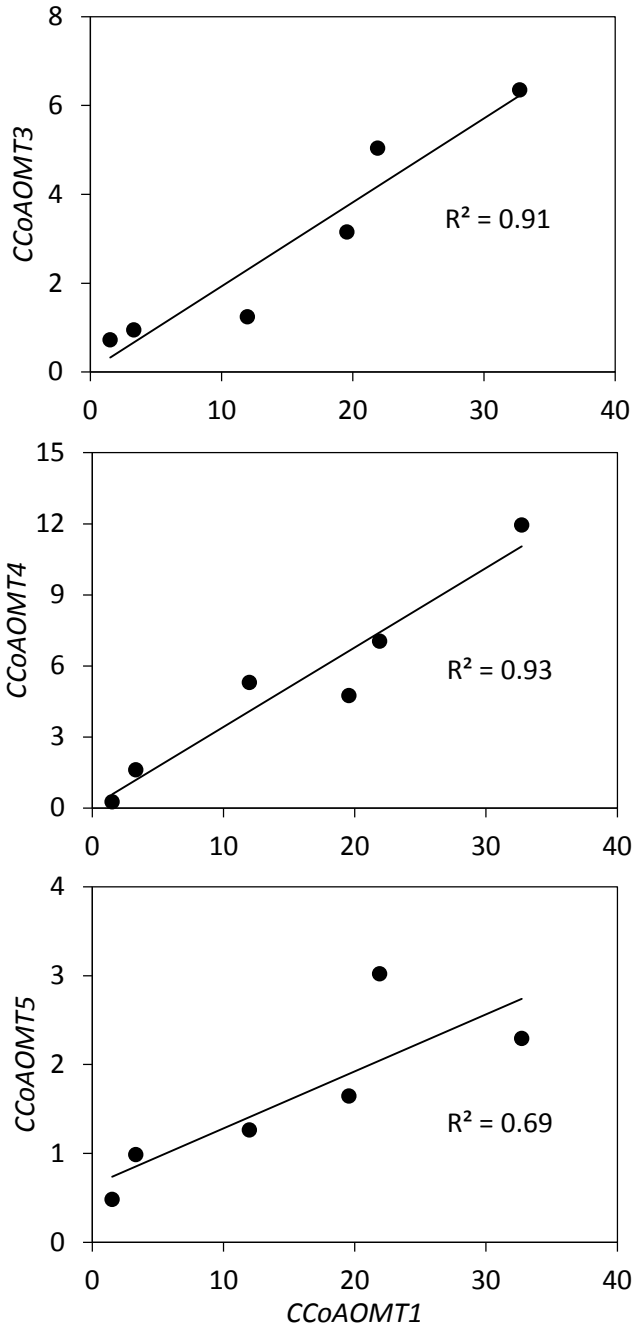

Figure S3. The correlation between relative transcript levels of *CCR2 and CCR3*.

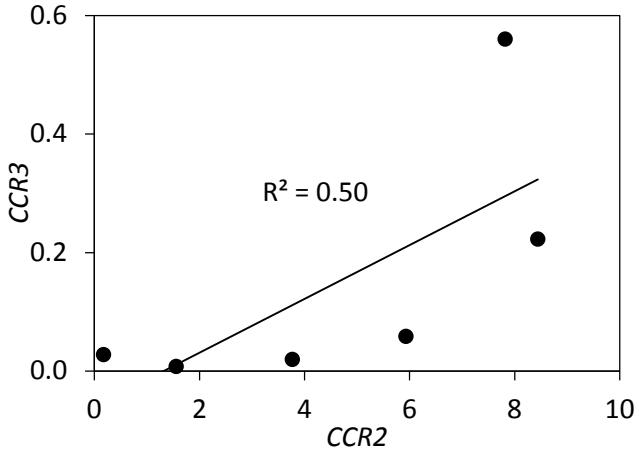

Figure S4. The correlation between relative transcript levels of *F5H2 and F5H1*.