Cellulose content variation and underlying gene families in bread wheat

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LIST OF ABBREVIATIONS

AFLPs	Amplified fragment length polymorphism
AX	Arabinoxylan
BLAST	Basic local alignment search tool
BSMV	Barley stripe mosaic virus
cDNA	Complementary deoxyribonucleic acid
CEF	Cellulose elementary fibril
CesA	*Cellulose synthase A*
CIMMYT	International Maize and Wheat Improvement Center
CSC	Cellulose synthase complex
Csl	*Cellulose synthase-like*
C-SR	Class-specific region
CSS	Chromosome Survey Sequence
DFMs	Direct Functional Markers
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
EST	Expressed Sequence Tag
FPKM	Fragments Per Kilo base of transcript per Million mapped reads
FarmCPU	Fixed and Random Model Circulating Probability Unification
FMs	Functional Markers
GAPIT	Genomic Association and Prediction Integrated Tool
GAX	Glucuronoarabinoxylan

GBS	Genotyping by sequencing
GH	*Glycosyl Hydrolase*
GS	Genomic selection
GT	Glycosyltransferase
GTMs	Gene Target Markers
GWAS	Genome-wide association study
HRS	Hard Red Spring
HWS	Hard White Spring
IFM	Indirect Functional Markers
IWGSC	International wheat genome sequencing consortium
LD	linkage disequilibrium
MAF	Minor allele frequency of
NGS	Next Generation Sequencing
PCR	Polymerase chain reaction
P-CR	Plant-conserved regions
PCW	Primary cell wall
PDS	*Phytoene desaturase*
PIECE	Plant Intron-Exon Comparison and Evolution database
PNW	Pacific North West
QTL	Quantitative trait loci
RAPDs	Random Amplified Polymorphic DNA
RDM	Random markers
RFLPs	Restriction fragment length polymorphism

RNA	Ribonucleic Acid
RNAi	RNA interference
SCW	Secondary cell wall
SNP	Single nucleotide polymorphism
SRS	Soft Red Spring
SSR	Simple Sequence Repeat
SWS	Soft white spring
TaCesA	*Triticum aestivum Cellulose synthase A*
TILLING	Targeting Induced Local Lesions IN Genomes
TMDs	Transmembrane domains
UDP	Uridine diphosphate
UGT	UDP-glucuronosyltransferase
VIGS	Virus-induced gene silencing
ZnF	Zinc-finger

ABSTRACT

Synthesis and remodelling of various cell wall components play a vital role in plant development, architecture and innate immunity. Plant cell walls are mainly composed of cellulose and hemicellulose which produce a bulk of renewable biomass vital for food, feed and biofuels. Cellulose in the primary and secondary cell wall of plants is synthesised by the family of genes called *CesA* (*Cellulose synthase A*). This study is a first report about the distinctive structural and functional motifs of primary and secondary cell wall synthesis genes. Using publicly available genomic databases and resources, 22 *TaCesA* genes located on A, B and D genomes of hexaploid wheat were identified. Cellulose in secondary cell walls is synthesised by three genes (*TaCesA4*, *TaCesA7*, and *TaCesA8*) co-expressing in the mature stem tissues of bread wheat. But the relative transcript abundance was found to be higher for *TaCesA4* genes, which indicates its major role in the secondary cell wall cellulose synthesis. We employed the virus-induced gene silencing (VIGS) approach to functionally characterize *TaCesA4* gene through silencing its three homoeologs (*TaCesA4A, TaCesA4B,* and *TaCesA4D*) collectively in bread wheat. Silenced plants showed a significant reduction in transcript abundance and cellulose content in the stem tissues. However, the anatomy of stem cross sections of silenced plants did not show any evidence of abrupt changes in the secondary cell wall of stems at the booting stage. A panel of 265 diverse wheat lines was evaluated for natural variation of cellulose content that was linked to the SNP genotyping data through genome-wide association studies (GWAS). This analysis led the identification of novel genes (*β-tubulin* and *UDP-glycosyl transferase*) associated with cellulose biosynthesis in wheat. In addition, *Cellulose synthase-like* (*Csl*) genes of wheat were explored. These genes have been known for the regulation/synthesis of hemicelluloses such as heteromannan, xyloglucan, heteroxylans, and mixed-linkage glucan. A total of 108 *Csl* genes were identified based on the

family specific Pfam conserved domains. Tissue-specific expression and phylogeny of *Csl* genes were also elucidated. Taken together, genome- wide exploration of *CesA* & *Csl* genes and their association with cellulose and hemicellulose biosynthesis offer a valuable resource for designing high yielding wheat varieties possessing appropriate lignocellulosic traits.

RÉSUMÉ

La synthèse et la remodelage des divers composants des parois cellulaires jouent un rôle important dans le développement, l'architecture et l'immunité innée des plantes. Les parois cellulaires sont principalement composées de cellulose et d'hémicellulose, lesquelles représentent une quantité importante de biomasse dans les aliments pour humains et bétail autant que dans les biocombustibles. La cellulose présente dans les parois cellulaires primaires et secondaires est synthétisée par des gènes de la famille *CesA* (*Cellulose synthase A*). Cette étude est la première à décrire les motifs structurels et fonctionnels caractéristiques de ces gènes de synthèse de parois cellulaires primaires et secondaires. Utilisant des ressources génétiques disponibles, 22 gènes *TaCesA* situés sur les génomes A, B et D du blé hexaploïde furent identifiés. La cellulose dans les parois cellulaires secondaires est synthétisée par trois gènes (*TaCesA4*, *TaCesA7* et *TaCesA8*) qui sont coexprimés dans les tissus matures des tiges de blé. Cependant, les transcrits du gène *TaCesA4* étaient plus abondants, ce qui indique l'importance élevée de ce gène pour la synthèse de la cellulose dans les parois cellulaires secondaires. Par biais d'une technique silençage de gène induit par virus (VIGS), nous avons caractérisé la fonctionnalité du gène TaCesA4 en désactivant tous ses trois homologues (*TaCesA4A*, *TaCesA4B* et *TaCesA4D*) dans le blé. Les plantes avec les gènes ainsi désactivés montrèrent une réduction significative en abondance des transcrits et en quantité de cellulose présente dans les tissus de leurs tiges. Cependant, l'anatomie des sections transversales des plantes aux gènes désactivés ne montrèrent aucune évidence de changements dramatiques dans les parois secondaires des cellules des tiges au phase de reproduction. Un ensemble de 265 diverses lignées de blé fut évalué pour caractériser la variation naturelle de la teneur en cellulose. Ces différences furent ensuite comparées avec des données de génotypage de polymorphismes mononucléotidiques par biais d'une étude d'association pangénomique. Cette analyse mena à

l'identification de nouveaux gènes (*β-tubulin* et *glycosyl transférase UDP*) associés avec la biosynthèse de la cellulose dans le blé. Des gènes du blé similaires à ceux de la cellulose, *Cellulose synthase-like* (*Csl*), furent aussi explorés. Ceux-ci ont déjà été reconnus pour leur rôle dans la régulation et la synthèse des hémicelluloses tels que le l'hétéromannane, le xyloglucane, les hétéroxylanes, et les glucanes à liaisons mixtes. Un total de 108 gènes de *Csl* fut identifié grâce aux domaines Pfam conservés spécifiques à cette famille, et la phylogénie et l'expression au niveau des tissus de ceux-ci furent ensuit analysées. L'analyse en profondeur de l'architecture génétique de la biosynthèse de la cellulose et de l'hémicellulose offre un atout précieux pour l'amélioration végétale et les modifications génétiques des variétés de blé en but d'obtenir une production de biomasse désirable tout en conservant une résistance suffisante envers de divers stresses.

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PREFACE AND CONTRIBUTION OF THE AUTHORS

a. Preface

This thesis work is presented in a manuscript-based format. During the course of my PhD studies, I have conducted comprehensive analysis of the genetic variation in cellulose content in bread wheat and genes underlying this. I explored the genes involved in the synthesis of cellulose and hemicellulose of cell wall through genetic, genomics and bioinformatics approaches. The thesis contains four different studies revolving around cell wall- associated genes. In study 1, *cellulose synthase* (*CesA*) genes were identified and analysed for their structure, function and evolution in wheat, study II involves the functional characterization of secondary cell wall specific *CesA4* gene using virus-induced gene silencing (VIGS), study III was performed to estimate the cellulose content of wheat straw and its genetic control in diverse wheat varieties, and final study IV was the Genome-wide analysis of the *Cellulose synthase-like* (*Csl*) gene family in bread wheat. The results of these studies have been presented in Chapter III, IV, V and VI respectively.

The following features of this study are considered as distinctive contributions to knowledge:

- Identification of *CesA* genes in wheat and their structural, functional and evolutionary studies will lead to designing cultivars suitable for both food and fuel.
- Functional validation of SCW-forming *CesA4* gene extrapolates differential functional role in higher plants
- Discovery of novel genes and/or SNPs associated with cellulose content in wheat could be helpful in devising molecular- assisted selection strategies for enhancing the culm strength, lodging resistance and wheat stem sawfly tolerance.

- Genome-wide identification and expression studies of poorly understood *cellulose synthase-like* (*Csl*) genes underscore their role in various polysaccharide biosynthetic processes in plants

b. Contribution of the authors

This thesis involves four studies (Chapter III to VI) printed in the form of four manuscripts as per the thesis preparation guidelines provided by McGill. The research work presented here has been completely outlined by me under the guidance of my supervisor Dr Jaswinder Singh. I have performed all the experiments in the greenhouse and laboratory set up and conducted genetic, genomic, bioinformatics analyses. Under the supervision of Dr Jaswinder Singh, I have analysed the data, wrote manuscripts and the thesis. He helped in troubleshooting, provided constructive comments, suggestions and financial support to conduct the experiments. He has thoroughly edited all the manuscripts and incorporated his suggestions.

The first manuscript (Chapter III) is co-authored by Kanwarpal S. Dhugga, Kulvinder Gill, and Jaswinder Singh. Dr Dhugga thoroughly edited the manuscript and added his valuable thoughts, Dr Gill shared the ideas of representation of bioinformatics analysis. The second manuscript (Chapter IV) was co-authored by Kanwarpal S. Dhugga, Raj Duggavathi, Kulvinder Gill and Jaswinder Singh. Dr Gill provided the training for VIGS and Dr Dhugga and Dr Duggavathi provided their expert advises performing the experiments. The third manuscript (Chapter V) was co-authored by Xu Zhang, Amita Mohan, Prashant Vikram, Sukhwinder Singh, Kanwarpal S. Dhugga, Zhiwu Zhang, Kulvinder Gill and Jaswinder Singh. Prashant Vikram, and Sukhwinder Singh provided the genotyping data, Xu Zhang and Amita Mohan helped in the creating the SNP data and GWAS analysis. Dr Dhugga provided the protocols for cellulose content

analysis, Dr Zhang and Dr Gill provided their expert advice and suggestions to interpret the results. The fourth manuscript (Chapter VI) was co-authored by Kanwarpal S. Dhugga and Jaswinder Singh. Dr Dhugga again provided his expert advice and edited the manuscript.

Chapter I: General introduction

The cell wall is the robust outermost layer of plant cells that covers the plasma membrane (Keegstra 2010). In the living cells, these walls not only encase the protoplasm but also act as complex and dynamic compartments with diverse and subtle functions (Fry 2004). They play a major role in plant growth, development, physical strength and innate immunity (Cosgrove 2000). Polysaccharide composition of cell walls makes them fundamentally different from cell membranes that are made up of proteins and phospholipids (Fry 2001). Cell walls usually laid down soon after the mitosis surrounding the dividing daughter cells. The thickness of the walls usually increases with the deposition of new microfibrils on the inner face of the developing cell wall (Cosgrove 2005).

Cell walls are classified into primary and secondary walls (Burton and Fincher 2014a). Primary cell walls are laid around the plasma membrane just after the cell division, allowing the cells to increase in size as they grow (Thomas et al. 2013). Whereas, secondary cell wall usually develops inner to the primary cell wall after the cell stops growing (Zhong and Ye 2014). Secondary cell walls provide greater mechanical strength to the cells and often surrounds the xylem vessels and lignin-rich woody tissues (Boerjan et al. 2003). The composition of the cell wall is fractionated into three polysaccharide classes: cellulose, hemicellulose and pectins (Achyuthan et al. 2010). In addition to these components, the cell wall matrix also contains some proteins, lignin, cutin and suberin infiltered between the microfibrils (Fry 2004).

Cell wall polymers are the end products of solar energy transformation by plants through photosynthesis. Total dry matter of plants including carbohydrate polymers (cellulose, hemicellulose and pectins) and aromatic polymers (lignin) is called lignocellulosic biomass (Guerriero et al. 2016). Beyond their fundamental significance associated with overall plant

physiology, lignocellulosic cell walls represent the most abundant renewable carbon source for biofuels and biomaterial industries. Over 90% of the global plant biomass is lignocellulose which accounts for about 200×10^9 tonnes/year, of which $8–20 \times 10^9$ tonnes remains accessible every year (Saini et al. 2015).

Wheat, a major staple food of the world, which also produces a large amount of lignocellulosic straw (1–3 tonnes/acre annually), is currently an important target crop for the synthesis of bioproducts (Saini et al. 2015). However, synthesis of cell-wall components, genetic diversity and their association with polysaccharide composition are not well understood in wheat. Identification and characterization of these genes is a prerequisite in designing the crops for more desirable harvests.

Cellulose (a homopolymer of glucose) and hemicellulose (heteropolymer of pentoses and hexoses) are the major components of the lignocellulose and are synthesised by the genes of a large superfamily known as *Glycosyltransferase 2* (*GT2*) (Breton et al. 2006; Kaur et al. 2016). Within this superfamily, there are two distinct multigene families that encode the catalytic subunits for the synthesis of cellulose and hemicellulose. The group of genes that involve in the synthesis of cellulose at the plasma membrane are called *Cellulose synthase A* (*CesA*) (McFarlane et al. 2014). On the other hand, hemicelluloses are synthesised by *Cellulose synthase-like* (*Csl*) genes located in the Golgi membranes (Pauly et al. 2013).

In addition to the *CesA* genes, the genes of *Glycosyl Hydrolase 9* (*GH9*) family and sucrose synthase (Fujii et al. 2010) have been reported to be involved in the synthesis of cellulose and cell expansion (Szyjanowicz et al. 2004; Lei et al. 2014; Vain et al. 2014). This explains the complexity of the cellulose synthesis process in the plant. Many of cell wall-related genes have been reported in case of model species Arabidopsis (Turner and Somerville 1997; Arioli et al. 1998; Taylor et al.

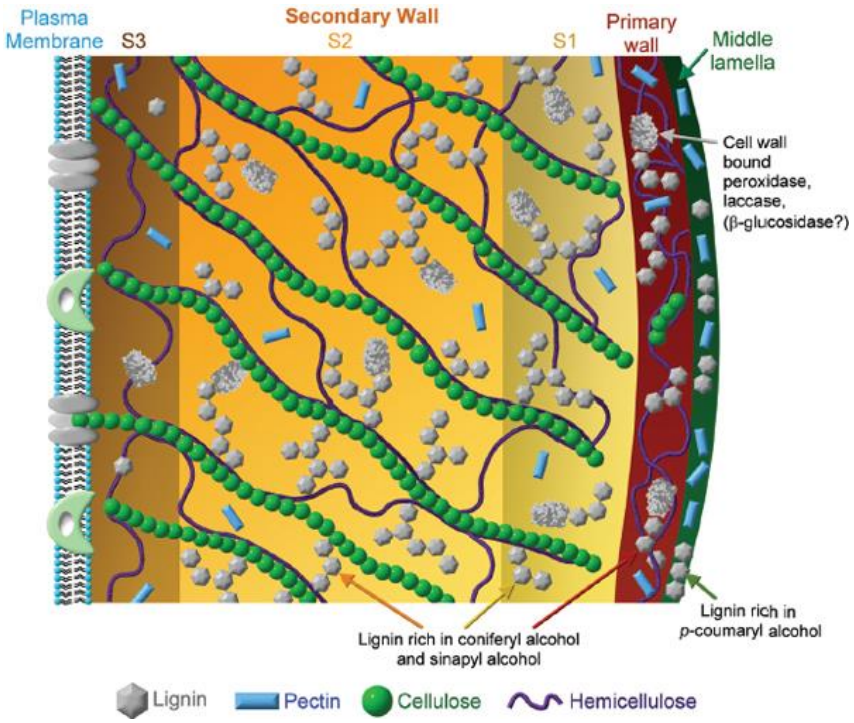
1999; Desprez et al. 2007b) and other cereals such as rice (Wang et al. 2010a), maize (Appenzeller et al. 2004), brachypodium (Coomey and Hazen 2015) and barley (Schwerdt et al. 2015)

However, bread wheat is lagging behind in the understanding of cell wall genetic architecture due to its complex and large genome size (17 Gb) (Krasileva et al. 2017). In addition to that, the first version of the chromosome-based draft genome sequence of bread wheat (*Triticum aestivum*) has been made available to the public recently (https://www.wheatgenome.org). Reference genotype Chinese Spring has been used to sequence the whole genome by international Wheat Genome Sequencing Consortium (IWGSC) (Consortium 2014).

Recent progress in sequencing efforts and availability of extensive genomic resources have permitted the identification and isolation of candidate genes of interest. But the functional validation of these genes is a major challenge for the researchers. There are many ways to characterise genes in model crops and crops with small genome size, such as chemicals, T-DNA, stable transformation through RNAi (Chen et al. 2014). Although some of these approaches are also available for wheat but are very laborious, time-consuming and expensive. A rapid and less expensive tool has recently developed in wheat called virus-induced gene silencing (VIGS) (Stratmann and Hind 2011; Bennypaul et al. 2012a; Baenziger et al. 2014).

Therefore, bioinformatics approaches coupled with functional genomics tools such as VIGS can enable the rapid exploration of structure and function of cell wall related genes in wheat. Being a crucial polysaccharide for plants and humans, exploring whole genome targets is vital to uncover the complex mechanism of cellulose synthesis in plants. Genome-wide association studies (GWAS) has emerged as an effective way to find the novel gene-trait associations. Moreover, the screening of diverse wheat genotypes for cellulose will provide the basis of genetic manipulation of lignocellulose and stalk strength.

Fig 1.1 Schematic showing the components of plant cell wall. Adapted from Achyuthan et al. 2010.



1.1 General hypothesis

We hypothesised, cellulose content in bread wheat cultivars varies greatly which is associated with cellulose synthase, cellulose synthase like and other related genes

1.2 General objectives

I: Identification of *Cellulose synthase* (*CesA*) genes to understand their structure, function and evolution in wheat

II: Functional characterization of secondary cell wall specific *CesA* gene using virus-induced gene silencing (VIGS)

III: Estimation of the cellulose content of wheat straw and its genetic control in diverse wheat varieties

IV: Genome-wide analysis of the *Cellulose synthase-like* (*Csl*) gene family in bread wheat

Chapter II. Literature review

2.1 Future energy requirements

With the increase in global population, depleting energy sources are among the biggest concerns for humanity (Scholey et al. 2016). Use of fossil fuels as an energy source over the years is a major factor in global warming and increase in greenhouse gas emissions (Strezov and Evans 2014). Additionally, fossil fuels are a finite resource and the process of fossil fuel formation is very slow, therefore one cannot survive by solely relying on this fuel (Moriarty and Honnery 2016). There is a need for renewable energy resources to overcome the danger of depleting non-renewable energy resources and to conserve the environment. Concerns of fuel depletion and environmental safety have attracted governments and scientists to search for alternatives to fossil fuels to secure future energy requirements (Perera 2016). Lignocellulose, the most abundant renewable biomass on the earth, has tremendous potential for conversion into biofuels (Broom et al. 2013). It is estimated that 10^{11} tonnes of cellulose are synthesised each year through the process of photosynthesis (Carroll et al. 2012). Research in the field of biofuels from lignocellulose feedstock is growing to meet the future energy requirements and check greenhouse gas emissions.

2.2 Lignocellulosic materials as bioethanol

Agricultural residues such as stems, stalks, and straws are the most abundant sources of renewable lignocellulosic biomass that can be efficiently converted to bioethanol (Hood 2016). Lignocellulosic biomass is less competitive, cheaper and has no influence on the growing demand for human food (Gabhane et al. 2014). A large part of agricultural lignocellulosic biomass comes from world's major crops such as maize, wheat, rice, and sugarcane (Chandra et al. 2012) Plant biomass is mainly composed of cell walls and quality of biomass is determined by the type of cell

walls (Sorek et al. 2014). There are two types of cell walls: primary cell walls and secondary cell walls. Deposition of primary cell wall takes place during the cell division and expansion stage whereas the secondary cell wall is deposited on the cell after expansion ceases until the cell dies (Carroll et al. 2012). All of the cell wall components cellulose, hemicellulose, pectin, lignin, and minerals, are collectively known as lignocellulose (Guerriero et al. 2016). The composition of lignocellulose varies depending on the species, cell type, environmental conditions and developmental stages of the plant (Sorek et al. 2014).

2.3 Structure and composition of lignocellulose

Lignocellulosic material is a complex network of three major cell wall components: cellulose, hemicelluloses and lignin along with other minor components. In general lignocellulose of wheat straw is comprised of cellulose (~30-40%), hemicelluloses (~20-35%) and lignin (~15-25%) (Ruiz et al. 2013). The composition of lignocellulose plays a crucial role in determining its biodegradation to bioethanol. To improve the efficiency of biofuel production, there is a need to explore the composition of lignocellulose and its genetic regulation.

2.3.1 Cellulose

Cellulose, the major component of plant cell walls, consists of a linear chain of β (1→4) linked glucan (poly glucose) units. Cellulose elementary fibril (CEF) is the fibril synthesised by the cellulose synthase complex (CSC) and the bundle of these CEF are called macrofibrils. It is probable that CSC containing more than 24 isoforms of cellulose synthase can synthesise about 36-chain CEFs. Microfibrils are morphological units that can be either CEF associated with hemicelluloses or a small macrofibril. The cellulose microfibril is 2 to 50 nm in size. Physiochemical properties of cellulose varies according to the degree of polymerization, a number

of chains and the orientation of the chains which are packed together (Ding et al. 2013). Cellulose is insoluble in water and most organic solvents because of intra and intermolecular hydrogen bonding which results from its free alcoholic groups (Shaveta et al. 2014). The size of cellulose microfibrils can vary in different tissues depending upon the degree of polymerization, from 500 to 15,000 glucose molecules. Cellulose microfibrils are generally bonded to hemicellulose through hydrogen bonds (Sorek et al. 2014).

2.3.2 Hemicellulose

In addition to cellulose, there is another cell wall component made up of several heteropolymers that are called hemicellulose. Hemicellulose is made up of diverse linear and branched polysaccharides and their composition varies widely depending on tissue and species. They mainly contain a β-(1, 4)-linked glucan, xylan, galactan, mannan, or glucomannan backbone branched with glycosyl residues. In addition to these components, mixed linked (1-4), (1-3) β-glucans are also abundant in some grass species (Sorek et al. 2014). Due to the presence of heterogenous substituents or other linkages in their polymer backbone, the structure of hemicellulose is amorphous and can be easily hydrolysed as compared to cellulose. These polysaccharides can interact with cellulose chains through hydrogen bonds (Pauly et al. 2013). Hemicellulose acts as a cross-linking agent between cellulose bundles, lignin and proteins through covalent or non-covalent bonding (Sorek et al. 2014).

Xylans are the most important hemicellulose and second most abundant polymer in the plant kingdom. Glucuronoarabinoxylan (GAX) is the major hemicellulose of monocot plants' secondary cell wall (Ong et al. 2014). Agricultural crops such as sorghum, sugar cane, corn stalks are all potential sources of xylans. Xylan occurs up to 70% of the weight in some tissues of grasses and

cereals (Ebringerová et al. 2005). Another important hemicellulose class Arabinoxylans (AXs), are most commonly found in the cell walls of cereal grains (Girio et al. 2010).

2.3.3 Lignin

Lignin is one of the major components of cell walls and is responsible for making them rigid, impermeable, and resistant to microbial attack and oxidative stress. Lignin makes biomass insoluble, therefore higher the lignin content lowers the digestibility of a given biomass (Eudes et al. 2014). It is the second most abundant polymer in nature after cellulose and is comprised of amorphous, heteropolymer of phenylpropane units. Lignin in most of the angiosperm species is composed of the phenylpropanoids p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) in different proportions (Penning et al. 2014). Lignin forms a covalent bond with hemicelluloses and occupies the spaces in the cell wall between cellulose and hemicellulose (Sorek et al. 2014).

2.4 Biofuels and plant cell walls

Plant cell walls possess polysaccharides that are a huge source of possibly fermentable sugars. Cell wall polysaccharides are catching industry attention for their use in the production of various bioproducts (Burton and Fincher 2014b). Natural variability of different components of cell wall provides an opportunity to select biomass for specific applications (Ciesielski et al. 2014). Among the major cell wall biopolymers, cellulose is the key fermentable sugar, but the productivity of biofuels is highly influenced by hydrolysis of cellulose and hemicellulose. Breakdown of these polysaccharides into fermentable sugars (Saccharification) is the major step that determines the efficiency of biofuel production. Lignocellulosic biomass in its innate shape is recalcitrant to enzymatic degradation because of complex cellulose-hemicellulose network and lignin cross-linking (Douche et al. 2013). Distortion of this interaction between lignin, cellulose and

hemicellulose needs a pre-treatment step that increases the cost of converting the feedstock to biofuel. The efficiency of pre-treatment largely depends upon the presence of covalent linkages among cell wall components, the strength of hydrogen bonding between cellulose and hemicellulose, thickness of the cell wall, and accessibility of cellulose for the breakdown. Therefore optimisation of biosynthesis of cell wall components is imperative to increase the efficiency of enzymatic hydrolysis for lignocellulosic feedstock (Ong et al. 2014). Several mutant studies have been performed to identify genes involved in cellulose and hemicelluloses biosynthesis, however, our current knowledge of mechanisms involved in cell wall polysaccharides biosynthesis is still rudimentary (Burton and Fincher 2012). Understanding cell wall characteristics and its natural variability will allow the creation of biomass specifically designed for efficient biofuel production.

2.5 Functional significance and synthesis of key cell wall components

Cell walls are the most abundant renewable source on earth and play a major role in providing physical strength and innate immunity to plants (Sarkar et al. 2009; Endler and Persson 2011). Plant cell walls consist mainly of cellulose, along with different proportions of hemicellulose and lignin. Among all the components of the cell wall, cellulose is the major target of the biofuel industry and most plentiful carbohydrate and a biopolymer in nature.

2.5.1 Genetics of cellulose synthesis

The cellulose in primary and secondary cell walls of plants is synthesised by a multigene family called *cellulose synthase* (*CesA*). In higher plants, the *CesA* gene family is primarily responsible for the synthesis of cellulose. In herbaceous plants, the secondary cell wall is deposited inside the primary cell wall. Genes involved in secondary cell wall thickening are important candidates to

study the genetic variability between diverse genotypes and are valuable in breeding programmes (Tian et al. 2014). Plant *cellulose synthases* (*CesAs*) belong to a large enzyme family called glycosyltransferase 2 (GT2), which is responsible for the creation of β-linkages between the glucose molecules in cellulose (Richmond 2000). Cellulose synthesis takes place at the Golgi membrane through the action of different isoforms of *CesA* that are specific to primary and secondary cell wall celluloses. CESAs are intrinsic membrane proteins whose catalytic domains extend into the cytoplasm (Rayon et al. 2014). They are found as rosettes, or cellulose synthase complexes (CSC), which are composed of a hexagonal structure of six protein subunits. A recent study predicted that each of the six subunits of cellulose synthase complex is composed of 4-6 enzymatically active CESAs that lead to the formation of an elementary microfibril. This microfibril is made of 24/36 glucan chains which are arranged in a rectangular form, with eight sheets of three chains to each sheet (Burton and Fincher 2014b). Multiple CESA proteins catalyse the synthesis of cellulose microfibrils through the polymerization of glucan chains and are involved in the crystallisation process (Li et al. 2014b). UDP-glucose acts as a substrate for a single-step CESA-catalysed reaction that polymerises the glucose residues (Liu et al. 2012). The structure and composition of cellulose microfibrils in primary and secondary cell walls determine cell wall elasticity and plant growth.

Higher plant CESAs are predicted to be comprised of eight transmembrane domains that form a pore in the plasma membrane to extrude newly synthesised cellulose. A zinc finger domain on the cytoplasmic amino terminal of CESAs is thought to be involved in the protein-protein interaction and dimerization of CESA proteins (Kaur et al. 2016). Motifs are the functional units of proteins and their discovery is important for the analysis of functional variability in different genes. The highly conserved motif 'CXXC' is present within this domain and distinguishes *CesA*

genes from *cellulose synthase-like* (*Csl*) genes (Richmond 2000). The zinc finger domain is followed by an acidic amino acid-rich region called the hypervariable region (Fig 4). A central domain between the second and third transmembrane domains possess most of the conserved residues of glycosyltransferases. Three conserved aspartic acid residues (D1, D2, and D3) and a QXXRW motif present in the central domain act as signature residues in all species and are probably involved in substrate binding, acceptor binding, and catalysis (Li et al. 2014).

2.5.2 *Cellulose Synthase-Like* (*Csl*) genes and their importance

Hemicellulose in plants is synthesised by a superfamily of genes called *cellulose synthase-like* (*Csl)* genes. These genes encode the catalytic subunit of enzymes required for hemicellulose synthesis. These genes possess the "D, D, D, QXXRW" motif that is characteristic of glycosyltransferases. *Csl* genes share sequence similarity with *CesA* genes; 30 to 50 *Csl* genes can be found in different plant species (Hazen et al. 2002) There are 30 known *Csl* genes in *Arabidopsis* and about 37 in rice (Hazen et al. 2002; Somerville et al. 2004). *Csl* genes are classified into nine subfamilies (*CslA–CslH* and *CslJ*). Among which, the *CslD* subfamily, is conserved in all land plants. In addition, it shares the highest sequence similarity with *CesA* genes. This reveals their fundamental role in plant development. Two groups of *Csl* genes, *CslF* and *CslH,* have evolved independently in grasses and are responsible for the biosynthesis of (1-3),(1-4)-β-D-glucan (Burton et al. 2011b). A third group *CslJ* had been recently identified as grass specific (Farrokhi et al. 2006). Arabidopsis which does not make (1-3), (1-4)-β-D-glucan shows small amounts of β-D-glucan when the gene from rice was heterologously expressed (Burton et al. 2006a). Comparative genomic analysis has revealed seven *CslF* family members in barley i.e., *HvCslF3*, *HvCslF4, HvCslF6, HvCslF7, HvCslF8*, *HvCslF9* and *HvCslF10* (Burton et al. 2008).

In barley, the *CslF* gene family is located in the genomic region corresponding to a major QTL involved in the synthesis of mixed linked glycans (Burton et al. 2006a). Transcription profiles of *CslF3* to *CslF10* have been detected in barley using 16 different tissues (Burton et al. 2008). Results showed the variable expression pattern of different *CslF* genes in different types of tissues. Relatively higher expression of *CslF3* and *CslF7* was detected in stem and peduncle tissues of barley; prompting the additional analysis of the involvement of specific *CslF* genes in the synthesis of (1-3), (1-4) β-glucan (Burton et al. 2011b). To date, the functional role of *CslF4*, *CslF6* and *CslH* in the synthesis of (1-3), (1-4) β-glucan has been demonstrated (Schreiber et al. 2014b). Taketa et al. (2012) reported the role of *CslF6* genes in β-glucan biosynthesis using β-glucanless mutants (Taketa et al. 2012) and the role of these genes have additionally, functionally characterised in wheat grain (Nemeth et al. 2010).

The subfamilies *CslA*, *CslC*, and *CslD* are found in all land plants, while the *CslB* and *CslG* subfamilies are present in dicots (Dhugga 2012). Four members of *CslA* group are involved in the synthesis of mannan and/or glucomannan. Expression profiling of seed development in guar (*Cyamopsis tetragonolobus*) shows that the *CslA* gene is responsible for mannan synthesis (Dhugga et al. 2004b; Liepman et al. 2005). Reverse genetic approaches in *Arabidopsis* have revealed that the *CslA* family is responsible for glucomannan biosynthesis (Goubet et al. 2009). Recent studies indicated the role *CslD* family in mannan synthesis (Verhertbruggen et al. 2011; Yin et al. 2011). Heterologous expression studies in the case of *Pichia* revealed that the *CslC* genes are involved in the synthesis of 1-4-β-glucan backbone of xyloglucan and some other polysaccharides (Cocuron et al. 2007). Despite much progress in the identification and functional analysis of *CesA/Csl* gene families in plants, there are no *(CesA)* and very few (*Csl*) case studies in wheat on these gene families.

2.6 Wheat straw and its potential as biofuel

Wheat (*Triticum aestivum*) is an important food crop all over the world. It is cultivated in around 115 nations, with an annual grain harvest of nearly 700 million tonnes (Zhang et al. 2014a). Global production of wheat straw is approximately 355 million tonnes every year which has potential to yield about 104 gigaliters of bioethanol (Saini et al. 2015). Wheat straw is composed of leaf and stem residues that remain after the harvesting of grain. It is comprised of 50-60% internodes, 15-30% leaves, and 10% nodes (Motte et al. 2014). Most of this straw is discarded as waste or burnt in the fields in developing countries. This creates big environmental and economic issue that could be otherwise used as a powerful resource of energy or source of biofuels (Shaveta et al. 2014). Canada is a major wheat producer; being ranked 6th on a global scale (Zhang et al. 2012). In the Canadian prairies, wheat, barley, oat, and flax grain production resulted in 37 million tonnes (Mt) of straw annually. Wheat alone contributes 25 Mt of straw. All this straw is not always available for industrial purposes as 0.75 t/ha to 1.5 t/ha of straw is required for soil conservation, depending on soil type. Also, 13-15 Mt of straw is required for livestock. However, 15 Mt of straw remains available for industrial purposes, that varies largely between 27-2.3 Mt (Sokhansanj et al. 2006; Tumuluru et al. 2014). Biofuel from wheat straw has been considered the most effective way to reduce the greenhouse effect and to generate energy from abundant biomass (Qureshi et al. 2013). Currently, the complexity in the structure of wheat lignocellulose makes the process of ethanol production less efficient. Current varieties of wheat have not been designed for cellulosic biofuel production. However, great potential exists at genetic and genomics level to alter lignocellulose composition of wheat and other grasses (Ong et al. 2014). Our current knowledge is limited in respect to the genetic and phenotypic variation of lignocellulosic biomass. Inclusive understanding

of cell wall components is necessary for the complex process of converting lignocellulose into biofuel.

2.7 Importance of wheat and its genetics

Wheat originated about 10,000 years ago in the Near Eastern Fertile Crescent (Faris 2014). Wheat provides 20% of total calories in the average human diet and feeds 40% of the world population (Gupta et al. 2008). Wheat, an allohexaploid, has the genome size of ~17 Gb of which ~80-90% are repetitive sequences. In hexaploid wheat, three homeologous sets of seven chromosomes are distributed in three A, B and D subgenomes. These subgenomes were originally derived from three diploid species, *Triticum urartu* (AA), an unknown close relative of *Aegilops speltoides* (BB), and *Aegilops tauschii* (DD). Tetraploid wheat *Triticum turgidum* L. (2n=4x=28; AABB) originated about 0.5 million years ago (MYA) through the first hybridization event between the ancestral species: *Triticum urartu* (2n=2x=14) and *Aegilops speltoides* (2n=2x=14). About 8,000 years ago, a second hybridization event between Tetraploid wheat and a wild relative: *Aegilops tauschii,* which contributed the DD subgenome, resulted in *Triticum aestivum* with the (2n = 6x = 42) AABBDD genome (Choulet et al. 2014b). It still behaves as a diploid because of the action of homologous pairing through the action of *Ph* genes. However, subgenome B possess a higher number of gene loci as compared to the A and D subgenomes. Gene sequences on subgenomes A, B and D of hexaploid wheat have more than 99% identity with their respective diploid progenitors (Mayer et al. 2014). It has been reported that present day genome of hexaploid wheat is resulted from multiple rounds of hybrid speciation (homoploid and polyploid) (Marcussen et al. 2014).

2.8 Molecular markers in wheat

2.8.1 Random Markers (RDMs)

Random markers (RDMs) are derived arbitrarily from polymorphic sites in genomic DNA and cDNA (Gupta and Rustgi 2004) and are developed using restriction enzyme based methods. The most commonly used random DNA markers are RFLPs (Restriction fragment length polymorphism), SSRs (Simple sequence repeat) and AFLPs (Amplified fragment length polymorphism). Sequence information is required for SSRs, SNPs but not for RFLPs, RAPDs (Random Amplified Polymorphic DNA), AFLPs etc. SSR and SNPs (single-nucleotide polymorphism) are markers of choice for molecular breeding (Salgotra et al. 2014) and play an important role in crop improvement. For example, they are used for gene introgression through marker assisted backcrossing/marker-assisted recurrent selection, germplasm characterization, diversity analysis, identifying polymorphisms, construction of molecular maps, QTL analysis, gene tagging, map-based cloning, and phylogenetic analysis (Varshney et al. 2007).

2.8.2 Gene Target Markers (GTMs)

Due to the availability of high-throughput sequencing platforms and genomic information, there was a shift in trends from RDM to GTMs and functional Markers (FMs); located in or near the gene of interest (Poczai et al. 2013). GTMs are developed from polymorphic sites within genes, that may or may not be involved in phenotypic trait variations (Varshney et al. 2007). These markers can also tag untranslated regions of genes (Poczai et al. 2013). These markers are developed through sequencing, expression profiling, sequence comparisons, or synteny studies (Andersen and Lubberstedt 2003).

2.8.3 Functional Markers (FMs)

Genome-wide sequencing provides a platform to mine molecular markers (Muthamilarasan et al. 2013). Functional markers are the polymorphic sites within genes that are functionally validated for phenotypic variations (Salgotra et al. 2014). These makers are further classified into two groups: indirect functional markers (IFMs) and direct functional markers (DFMs); depending upon whether the proof for their role in phenotypic trait variation is indirect or direct. Functional markers can be derived from non-redundant EST databases either by direct mapping or database mining for markers such as EST-SNP (Mochida and Shinozaki 2010). GTMs and FMs allow the detection of nucleotide diversity in the genes controlling agronomic traits. These markers are useful in predicting the genetic relationship, as well as the functional diversity of the genes in relation to adaptive variation. In contrast to RDMs, these markers are transferable to related species or genera (Varshney et al. 2007).

2.9 Comparative genomics

Comparative genomics is based on collinearity and synteny of genes or chromosomes in diverse species descended from a common ancestor (Poursarebani et al. 2013). Grass species such as rice, oats, barley, and wheat and *Brachypodium*, are derived from common ancestors, therefore, gene order in these species is highly conserved. Among all these grass species, wheat has most recently split from barley (Bolot et al. 2009). Comparative analysis shows that wheat chromosome groups 2, 3, 4, 5, 6, and 7 are syntenic to barley chromosomes 2H, 3H, 4H, 5H, 6H, and 7H respectively (Cho et al. 2006). Comparative genomics studies provide us with information about orthologous gene functions from different species that are expected to produce similar phenotypes. With the progress of sequencing facilities and the availability of whole genome sequences for major cereals

such as rice, maize and barley, it is now possible to identify genes and predict their functions in cereal crops with more limited sequencing information. Comparative genomics predicts gene function by exploring genomics and post-genomics associations for the genes, either within or between plant species and prokaryotes. Biochemical functions can also be determined using 3D structures (Bradbury et al. 2013). The availability of large-scale genomic information and conserved synteny between various grass species provides an opportunity to explore gene function and structure (Mochida and Shinozaki 2013; Molnár et al. 2016; Devos et al. 2017).

Sequence comparison using online resources such as ensemblplants (http://plants.ensembl.org/index.html) (Bolser et al. 2015), gramene (http://www.gramene.org/ and (https://phytozome.jgi.doe.gov/pz/portal.html) (Goodstein et al. 2014) are important comparative, functional genomics analysis tool for crop plants (Monaco et al. 2014). Comparative analysis was performed taking *Arabidopsis* as a model to identify the Sm family of RNA-binding proteins in rice and maize (Chen and Cao 2014). A *Phytoene synthase (Psy)* gene was identified and cloned in wheat using its ortholog from maize, using *in silico* cloning (He et al. 2008). And an Ortholog (*TaGW2)* of a gene involved in grain development in rice has been similarly identified in wheat (Su et al. 2011). A comparative genomic analysis resulted in the introgression of *Yr5* resistance, a major resistance component against yellow rust (McGrann et al. 2014).

2.10 Functional genomics in wheat

Functional genomics is a wide approach for predicting functions and interactions of genes and their products. With the advancement of genome sequencing platforms, large numbers of plant genomes have been fully sequenced. A large-scale genomic information needs to be characterised by assigning functions to individual genes and exploring the role of non-coding sequences.

Integration and analysis of the genomic data are currently the biggest challenges (Mittler and Shulaev 2013).

Several reverse genetics tools such as transposons mutagenesis, T-DNA insertion, RNA interference (RNAi), and Targeting Induced Local Lesions IN Genomes (TILLING) enable researchers to study specific genes and their functions (Chen et al. 2014). The introduction of the maize *Ac-Ds* transposable element system as a transposon tagging tool into heterologous species offers unprecedented opportunities to link genes with function by creating and characterising mutant alleles (Singh et al. 2012). Similarly, virus-induced gene silencing (VIGS) has been considered as a rapid and cost-effective functional analysis tool for complex crop species such as wheat to suppress the expression of homeologous genomes (Stratmann and Hind 2011; Baenziger et al. 2014).

2.10.1 Gene silencing approach through RNA interference

RNA interference (RNAi)-induced gene silencing is the post-transcriptional degradation of m-RNA. It is a robust functional genomics tool to suppress the expression of three homologous genes in wheat. It can be efficiently utilised for silencing multigene families and homoeologous genes in polyploids with functional redundancy. RNA interference (RNAi) induced phenotype is stably inherited, that makes it very important tool for functional analysis of genes in wheat (Baenziger et al. 2014). A gene controlling grain traits was functionally characterised through RNAi (Hong et al. 2014). A recent study led to the downregulation of gliadins wheat lines through RNAi that can be useful for production of gluten free products for the celiac community (Gil-Humanes et al. 2014).

2.10.2 Virus-induced gene silencing in wheat

VIGS has emerged as a powerful tool for plant functional genomics. VIGS involve the silencing of target gene/genes as a part of plant defence mechanism against viral attack. This is a fast and cost-effective alternative to the polyploid crops where stable transformation through RNAi is difficult to perform (Senthil-Kumar and Mysore 2011). Infection of plants by a virus engineered with fragments of the gene of interest activates the post-transcriptional gene silencing as an innate defence response. VIGS can be performed with or without the availability of sequence information as reverse and forward genetic tool for functional analysis of genes (Ramegowda et al. 2014). This technique is based on the spread of the virus in a plant upon inoculation. Multiplication of recombinant cloned virus with incorporated plant gene sequence led to the complete or partial loss of gene function through post-transcriptional gene silencing. Suppression of gene expression and phenotypic changes can be observed in VIGS treated plants (Lee et al. 2012). VIGS provides an opportunity to clone genes in genetically complex organisms such as wheat, using the candidate gene approach. Barley stripe mosaic virus (BSMV)-based VIGS system can be used to silence three homoeologous copies of each gene in wheat (Bennypaul et al. 2012b; Buhrow et al. 2016; Zhang et al. 2016).

2.11 Genomics-integrated breeding

The modern era of -omics (functional genomics, comparative genomics) and high throughput marker technology provides an opportunity to understand the functions of genes with small effects that underlie most of the important traits (Madramootoo 2015). Genome-wide markers have potential to capture all additive effects for selection of desirable genotypes. Emerging genomic-integrated breeding technologies are revolutionising the understanding of mechanisms of complex quantitative traits in time/cost efficient manner.

2.11.1 Genome-wide association (GWA)

Association mapping is an advanced tool to detect the genes/QTLs based on phenotypic and genotypic associations. It is an important strategy to identify genes underlying variations in quantitatively inherited traits. It is based on the principle of linkage disequilibrium (LD). In simple terms, linkage/LD is the deviation from Mendel's 2nd law, which explains the independent assortment of two different loci. The phenomenon of association between two loci is called linkage when the common ancestor is within the recorded pedigree. Whereas, when the common ancestor is the recorded pedigree, it is known as LD (Laird and Lange 2011). Whole genome scanning for LD between mapped marker loci and traits of interest is called Genome-wide Association (GWA). Genome-wide markers have the potential to capture additive effects and thereby aid in the selection of desirable genotypes (Neumann et al. 2010).

There are a number of factors influencing association mapping studies, such as genetic marker coverage, a number of individuals studied, and linkage disequilibrium (Cockram et al. 2010). Marker density on a genomic map should be higher than the extent of LD, which in turn depends upon the population structure, genetic diversity and number of recombination events that have occurred and have restructured that diversity (Brachi et al. 2011).

A recent study integrated the approach of sequence-based GWAS and functional genome annotation displayed the potential of matching complex traits to their causal polymorphisms in rice (Huang *et al*., 2012). Modern maize breeding techniques have shown a remarkable increase in its productivity in the last few decades. As maize is such a diverse crop, genome-wide genetic variation pattern among various maize lines has been studied extensively. In a GWAS maize study, two candidate genes were identified that were associated with yield-related traits measured under water-stress conditions (Hu and Xiong, 2013). Nested association mapping (NAM) population of

25 RIL families was generated for quantitative trait analysis in maize (McMullen et al. 2009). Genome-wide association (GWA) study of the maize nested association mapping (NAM) panel was performed to determine the genetic basis of quantitative leaf architecture traits and identification of some of the important genes (Tian et al. 2011). Genome-wide association studies (GWAS) found a strong association between genetic loci and 14 agronomic traits in the population of *Oryza sativa* subspecies indica (Huang et al. 2010). Genetic architecture of (aluminium) Al tolerance and Al tolerance loci in rice was identified through bi-parental QTL mapping and GWAS (Famoso et al. 2011). A recent GWA study showed the involvement of *Glycosyltransferases* (*GT*) and *Glycoside hydrolases* (*GH*) along with *Cellulose synthase A* (*CesA*) in the culm cellulose content of barley (Houston et al. 2015)

2.11.2 Genomic selection (GS)

The genomic selection was first introduced by (Meuwissen et al. 2001) as a recent advancement in molecular breeding technology for the study of quantitative traits. Quantitative traits are controlled by a large number of genes, with a cumulative effect of each gene on the trait. This approach uses whole genome molecular markers (high-density markers and high throughput genotyping) to develop a prediction model for estimating a breeding value for each individual (Crossa et al. 2011). The availability of full genome sequences through NGS (Next Generation Sequencing) has provided high throughput molecular markers (Jonas and de Koning 2013).

GS based on LD can be applied to the populations having extensive phenotypic data over the years to dissect complex traits. This process also avoids the generation of special mapping populations (Xu et al. 2013). In contrast to few major genes/QTLs, thousands of molecular markers possessing strong LD with the trait of interest are used for GS. A number of simulation studies in

various crops such as wheat, maize, oil palm (Bernardo and Yu 2007; Wong and Bernardo 2008; Bassi et al. 2016), and forages (Simeao Resende et al. 2014) illustrated higher genetic gain through GS as compared to MAS or phenotypic selection. GS predicts the breeding values based on phenotyping and genotyping of only a small training population and selection is based on the genotyping of breeding population at early stages without phenotyping (Battenfield et al. 2016; Michel et al. 2016).

CONNECTING STATEMENT FOR CHAPTER III

Chapter III entitled Novel structural and functional motifs in *Cellulose synthase A* (*CesA*) genes of bread wheat (*Triticum aestivum*, L.) authored by Simerjeet Kaur, Kanwarpal S. Dhugga, Kulvinder Gill, and Jaswinder Singh was published in "*PLOS ONE*" *.

Based on the literature review in chapter II, Cellulose is the key fermentable sugar found as the major proportion of plant cell walls. Cellulose in the primary and secondary cell wall of plants is synthesised by the family of genes called *CesA* (*Cellulose synthase A*) (Haigler et al. 2016). The structure, function, and evolution of *CesAs* are poorly understood in wheat. This study is a first report about the distinctive structural and functional motifs of primary and secondary cell wall synthesis genes in wheat. Using available genomic resources, this study in chapter III describes the identification of 22 *TaCesA* genes located on A, B and D genomes of hexaploid wheat. A thorough analysis was performed to investigate their structure, motif & domain architecture, evolution and expression patterns. Newly identified motifs were found to act as signature residues for the specificity of different *CesA* genes. A detailed information about these genes is discussed in chapter III.

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Chapter III. Novel structural and functional motifs in *Cellulose synthase A* (C*esA*) genes of bread wheat (*Triticum aestivum*, L.)

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3.1 Abstract

Cellulose is the primary determinant of mechanical strength in plant tissues. Late-season lodging is inversely related to the amount of cellulose in a unit length of the stem. Wheat is the most widely grown of all the crops globally, yet information on its *CesA* gene family is limited. We have identified 22 *CesA* genes from bread wheat, which include homoeologs from each of the three genomes, and named them as *TaCesAXA*, *TaCesAXB* or *TaCesAXD*, where X denotes the gene number and the last suffix stands for the respective genome. Sequence analyses of the CESA proteins from wheat and their orthologs from barley, maize, rice, and several dicot species (Arabidopsis, beet, cotton, poplar, potato, rose gum and soybean) revealed motifs unique to monocots (Poales) or dicots. Novel structural motifs CQIC and SVICEXWFA were identified, which distinguished the CESAs involved in the formation of primary and secondary cell wall (PCW and SCW) in all the species. We also identified several new motifs specific to monocots or

dicots. The conserved motifs identified in this study possibly play functional roles specific to PCW or SCW formation. The new insights from this study advance our knowledge about the structure, function and evolution of the *CesA* family in plants in general and wheat in particular. This information will be useful in improving culm strength to reduce lodging or alter wall composition to improve biofuel production.

3.2 Introduction

Cellulose is the primary determinant of mechanical strength in plants (Appenzeller et al. 2004; Ching et al. 2006). It is also the world's most abundant renewable carbon source (Dhugga 2001; Dhugga 2007). In plants, the secondary cell wall is deposited inside the primary wall and, because of its greater thickness, it generally constitutes a majority of the vegetative biomass (Tian et al. 2014). The primary cell wall is deposited during cell division and expansion stages, whereas the secondary cell wall begins to form as cell expansion approaches cessation (Carroll et al. 2012). Cellulose in plants is synthesised by multimeric protein complexes, which consist of hexameric, rosette-like structures in the plasma membrane (McFarlane et al. 2014). Individual members of each of the hexameric components are referred to as *Cellulose synthase A* (*CesA*), where the letter *A* stands for the catalytic subunit (Dhugga 2001). Arabidopsis (*Arabidopsis thaliana*) genome contains at least 10 *CesA* genes, which cluster into six groups (Richmond 2000; Richmond and Somerville 2000; Hamann et al. 2004). Mutational genetics established that six of the ten genes each had a nonredundant function in primary or secondary cell wall (PCW or SCW) formation. Three of the genes, *AtCesA1*, *AtCesA3*, and *AtCesA6*, are involved in PCW formation and another three, *AtCesA4*, *AtCesA7* and *AtCesA8* in SCW formation (Endler and Persson 2011; Hill et al. 2014). The remaining genes, *AtCesA2, 5* and *9*, are partially redundant with *AtCesA6* (Desprez et al. 2007a; Persson et al. 2007). *AtCesA10* remains uncharacterized. Maize and rice possess 13

and 11 *CesA* genes, respectively (Wang et al. 2010a; Zhang et al. 2014b). Barley has nine genes, of which *HvCesA1, HvCesA2, and HvCesA6* make PCW, and *HvCesA4, HvCesA7,* and *HvCesA8* from SCW (Houston et al. 2015). *HvCesA3, 5* and *9* are different from both the groups because of their unique tissue-specific transcript levels (Burton et al. 2004). Mapping studies from Arabidopsis, maize and rice revealed that the members of the *CesA* gene family were spread across the genome although some genes were clustered together (Holland et al. 2000; Wang et al. 2010a).

Plant CESAs belong to family 2 of glycosyltransferases (GT2), which catalyse beta linkage between the glycosyl residues. CESAs are intrinsic plasma membrane proteins with their catalytic domains extending into the cytoplasm (Rayon et al. 2014). Each of the six subunits of a cellulose synthase complex (CSC) is believed to be composed of 6 enzymatically active CESAproteins. Each of the CESA proteins catalyses the synthesis of an individual β-1, 4-linked chain (Morgan et al. 2013; Slabaugh et al. 2014). Multiple chains extruded from the CSC then polymerise through hydrogen bond formation into a microfibril outside the plasma membrane.

A CESA protein of higher plants possesses eight transmembrane domains (TMDs), which are believed to form a pore in the plasma membrane to allow extrusion of the newly synthesised glucan chain. Two zinc-finger domains (ZnF), which are highly homologous to the RING-finger motif, are present on the cytoplasmic face close to the amino terminus (Kurek et al. 2002). The central or the catalytic domain is located between the second and third TMDs (Li et al. 2014a). Three aspartyl residues (referred to as D1, D2, and D3) and a QXXRW motif in the catalytic domain of the CESA proteins are conserved across all the species studied thus far. The D1 and D2 residues are believed to coordinate UDP binding while D3 provides a catalytic base for glucan chain extension (Saxena et al. 1995) The QXXRW motif acts as a binding site for the terminal disaccharide of the glucan (Morgan et al. 2013).

Motifs are the conserved groups of residues in proteins, which can be associated with structural and functional variability across species. A highly conserved motif, CXXC, which is located within the ZnF, distinguishes CESAs from the CSL (cellulose synthase-like) proteins (Richmond 2000; Richmond and Somerville 2000). Crystal structure of the cellulose synthase subunit A (BcsA) and accessory protein BcsB of *Rhodobacter sphaeroides* demonstrated the involvement of a single catalytic site in the formation of the β-1,4-glycosidic bond of the glucan chain (Morgan et al. 2013). Computationally predicted model of GhCESA1 revealed two class-specific regions (C-SR-I and C-SR-II), which distinguished different CESAs, and two plant-conserved regions (P-CR), which were absent in the bacterial BcsA but highly conserved in all the plant CESAs (Ranik and Myburg 2006; Sethaphong et al. 2013; Lin et al. 2014; Slabaugh et al. 2014). The P-CR might be potentially involved in the multimerization of the plant CESA polypeptides, leading to the formation of rosettes. C-SRs are probably responsible for regulating cellulose synthesis at different developmental stages.

The *CesA* gene family has not yet been compiled from wheat, the most widely grown crop in global agriculture. Functional classification of the *CesA* genes in cereal crops has proved helpful in associating various genes with culm or stalk strength (Appenzeller et al. 2004; Houston et al. 2015). In this report, we present the *CesA* gene family from wheat. To understand the involvement of the different *CesAs* in primary or secondary wall formation in grasses or dicot plants, we have identified unique sequence motifs. Sequence comparisons of the PCW and SCW *TaCesA* genes were performed at both the DNA and protein levels. Phases of intron evolution were predicted and compared between the groups of the *TaCesA* genes involved in the formation of PCW or SCW. Unique motifs were identified among the representative monocot and dicot species. RNA-seq

expression profiling of the *TaCesA* genes revealed unique, homoeolog-specific expression patterns in different tissues.

3.2.1 Hypothesis Genes involved in cellulose synthesis in primary and secondary cell walls possess unique structural and functional motifs

3.2.2 Objective I. *In silico* identification of true orthologs of *CesA* genes in wheat

3.2.3 Objective II. Comparative analysis of structural and functional conservation between genes involved in cellulose synthesis in primary and secondary cell walls

3.3 Methods and materials

3.3.1 Identification of *CesA*s in wheat and their true orthologs from different species

The conserved cellulose synthase domains from barley CESA proteins was used as a query to perform the tBLASTn search with Chromosome Survey Sequence (CSS) (http://plants.ensembl.org/Triticum_aestivum/Info/Index) generated by the International Wheat Genome Sequencing Consortium (IWGSC) (Mayer et al. 2014). Availability of whole genome sequence of barley (http://webblast.ipk-gatersleben.de/barley/) made it possible to isolate full-length barley *CesA* sequences (Burton et al. 2004). Genome databases of *Triticum urartu* and *Aegilops tauschii*, A and D genome progenitors of wheat, respectively, were also explored to identify full-length *CesA* genes for the sequences missing in hexaploid wheat. The homoeologs were first identified from Ensembl Plant database followed by amino acid sequence alignment for the presence of conserved motifs and domains. Highly variable class-specific regions (C-SRs) present in different *CesAs* were used to differentiate the homoeologous genes from each other (Fig 1).

Orthologs of various *CesA* genes were identified through alignment of the wheat *CesA*s with those from Arabidopsis, barley, rice and maize. The ortholog of each gene was selected based on the sequence identity and query coverage, presence of all domains and motifs similar to the query sequence, Amino acid content/size and distance among various new motifs identified in this study relative to the query sequence. Arabidopsis, rice and maize *CesA* sequences were retrieved from Phytozome v9.1: Home (http://www.phytozome.net/) (Goodstein et al. 2012).

3.3.2 Gene structure analysis

Although in this study we identified 22 *TaCesA* genes, comparative studies for gene structure were performed only for the genes that were specific for PCW and SCW cellulose synthesis. Based on analysis of the orthologs, *TaCesA4, 7* and *8* were characterised as one-to-one orthologs of SCW-specific, *TaCesA1, 2,* and *6* as PCW-specific, and *TaCesA3, 5* and *9* as partially redundant to the PCW *CesAs*. The homoeologous copies of each gene shared 95-99% sequence identity in addition to all the motifs and domains. Therefore only one copy among the three homoeologs was used for comparative analysis. Intron-exon boundaries and translation start and stop sites were predicted through alignments of full-length genomic copies of *TaCesA* genes with their corresponding cDNA sequences. The introns and exons were drawn to scale for all the genes as indicated by the cDNA-genomic sequence comparisons. Phases of intron evolution were predicted using Plant Intron-Exon Comparison and Evolution database (PIECE) (http://wheat.pw.usda.gov/piece/) (Wang et al. 2013)

3.3.3 Protein structure and motif identification

Amino acid sequence similarity of TaCESA protein sequences was determined by multiple sequence alignment (http://www.genome.jp/tools/clustalw/). Colour Align Conservation tool (http://www.bioinformatics.org/sms2/color_align_cons.html) was used to differentiate the conserved patterns of aligned sequences. Conserved domains and motifs were identified by manual search in the aligned sequences.

3.3.4 Phylogenetic analysis

22 newly identified wheat CESA proteins were used to deduce their phylogenetic relationships. Protein sequences for *Arabidopsis thaliana* (AtCESA), *Beta vulgaris* (BvCESA), *Eucalyptus grandis* (EgCESA), *Glycine max* (GmCESA), *Gossypium hirsutum* (GhCESA), *Hordeum vulgare* (HvCESA), *Oryza sativa* (OsCESA), *Populus trichocarpa* (PtCESA), *Solanum tuberosum* (StCESA), *Zea mays* (ZmCESA) were retrieved from NCBI (www.ncbi.nlm.nih.gov) (Kaur et al. 2013). An unrooted phylogenetic tree was constructed with bootstrap analysis over 1000 replicates, using the Neighbor-Joining method using the MEGA6 program (Saitou and Nei 1987; Tamura et al. 2013). Evolutionary distances were computed using Poisson correction method (Zuckerkandl and Pauling 1965). All positions containing gaps and missing data were eliminated.

GenBank accession numbers for CESA amino acid sequences used to generate the phylogenetic tree are: AtCESA1, AF027172; AtCESA2, AF027173; AtCESA3, AF027174; AtCESA4, AB006703; AtCESA5, AB016893; AtCESA6, AF062485; AtCESA7, AF088917; AtCESA8, AL035526; AtCESA9, AC007019; AtCESA10, At2G25540; ZmCESA1, AF200525; ZmCESA2, AF200526; ZmCESA3, NP_001292792.1; ZmCESA4, AF200528; ZmCESA5, AF200529; ZmCESA6, AF200530; ZmCESA7, AF200531; ZmCESA8, AF200532; ZmCESA9, AF200533; ZmCESA10, AY372244; ZmCESA11, AY372245; ZmCESA12, AY372246;

ZmCESA13, KJ874174; OsCESA1, AF030052; OsCESA2, D48636, OsCESA3, BAD30574; OsCESA4, AK100475; OsCESA5, BAD30574; OsCESA6, XM_477282; OsCESA7, XM_477282; OsCESA8, XM_477093; OsCESA9, XM_477093; OsCESA10, LOC_O-*4*2g29300; OsCESA11, LOC_OS06g39970; HvCESA1, AY483150; HvCESA2, AY483152; HvCESA3, AY483151; HvCESA4, AY483154; HvCESA5/7, AY483153; HvCESA6, AY483155; HvCESA8, AY483156; HvCESA9, AK367031; PtCESA6, XP_002319002; EgCESA5, XP_010063196; StCESA3, XP_006354075; GmCESA2, XP_003531396; GhCESA5, AFB18634 and BvCESA2, XP_010678670.

3.3.5 RNA-seq expression profiling of *TaCesA* genes

Gene expression profiling for 21 of the wheat *CesA* genes was performed using publicly available RNA-seq data from two different databases (http://wheat-urgi.versailles.inra.fr/Seq-Repository/RNA-Seq) at McGill University and Genome Quebec Innovation Center. First dataset was a non-oriented library with five wheat organs analysed in duplicates at three development stages for each of the organs. The five organs taken into consideration with respect to developmental stages were root (at seedling, three leaves, and meiosis stages), leaf (seedling, three tillers, and 2 days after anthesis), stem (spike at 1 cm, 2 nodes, and anthesis), spike (2 nodes, meiosis, and anthesis) and grain (2, 14, and 30 days after anthesis). The second dataset was the oriented library with five wheat organs (root, leaf, stem, spike, and grain) with five conditions pooled for 4 lines per organ (Pingault et al. 2015).

The abundance of transcripts from RNA-Seq data was reported using the estimated counts quantified by a programme Kallisto (v0.42.1) (Bray et al. 2015). Counts-per-million reads were obtained using Bioconductor's edgeR (Robinson et al. 2010). Ward's linkage method was applied

to the matrix of Pearson's correlation distances to for cluster analysis. Heat map of the candidate transcripts was reported by log2 counts per million (CPM) standard deviation (Bolger et al. 2014).

3.4 Results

3.4.1 Identification and mapping of *CesA* gene family in wheat

We queried the Chromosome Survey Sequence (CSS) (http://plants.ensembl.org/Triticum_aestivum/Info/Index) generated by the International Wheat Genome Sequencing Consortium to identify the orthologs of various *CesA* genes from bread wheat corresponding to the barley *CesA* sequences [32]. Twenty-two *TaCesA* genes were isolated, six of which were partial (S1 Text). The identified genes were named following the nomenclature of barley, which shares synteny with wheat. To simplify the nomenclature, we attached a suffix corresponding to the specific wheat genome identifier (A, B, or D) at the end of the gene number. For example, *CesA1* in genomes A, B, and D is named as *TaCesA1A*, *TaCesA1B*, and *TaCesA1D*, respectively. As expected, we found three copies for a majority of the nine *CesA* orthologs corresponding to the barley genes. For *CesA6, 7*, and *8* we were able to find only two *CesA* homoeologs. Only one copy was identified for *TaCesA9*. The missing homoeolog of *CesA6* belonged to the D genome but we obtained it from the D genome progenitor *Aegilops tauschii*. The *TaCesA7* homoeolog, which was absent in the A genome, was recovered from the A genome progenitor *Triticum urartu*. We were unable to find the A genome copy of *TaCesA8* from bread wheat as well as the A genome donor, *Triticum urartu*. The three homoeologous copies of each of the *CesA* genes shared 95-99% sequence identity. Different *CesA* genes within a species possessed two highly variable class-specific regions (C-SR-I and C-SR-II) that differentiated them from each other. The wheat orthologs of the CESA proteins of other species exhibited a similarity of 70-80% at the amino acid level with Arabidopsis and 90-

95% with rice and barley. The *TaCesA* genes ranged from 4044 to 5251 bp in length and contained 9-13 introns. The ensembl IDs of all the newly identified wheat *CesA* genes are given in Table 1.

The newly identified wheat *CesA* genes were mapped to respective chromosomes based on the physical mapping information available in the wheat IWGSC survey sequence annotation database (http://www.wheatgenome.org/). As expected the chromosomal locations of different *CesA* genes followed the trend reported earlier in the syntenic species barley (Burton et al. 2004). *TaCesA4A, B,* and *D* mapped in respective genomes to chromosome 1; *TaCesA7B* and *D* to chromosome 3; and *TaCesA8B* and *D* to chromosome 5. Similarly, the homoeologs of *TaCesA1*, *2*, *3*, *5*, and *6* mapped to chromosomes 2, 5, 5, 1 and 6 of the respective genomes. However, *TaCesa9B* mapped to chromosome 2B, unlike its ortholog from barley, which is located on chromosome 6. The approximate location of *TaCesA* genes and their homoeologs is presented in Table 1.

3.4.2 DNA sequence comparison of primary and secondary cell wall *TaCesA* genes

On average, a PCW forming gene was longer than the one involved in SCW formation. The longest gene, *TaCesA6,* was 5251 base pairs (bp) and the shortest, *TaCesA4,* was 3923 bp in length. The size variations among different *CesA* genes arose mainly from the number and length of introns (Table 2). *TaCesA1*, *2*, and *6* had 13 introns each, whereas *TaCesA4*, 7 and *8* had 7, 12, and 9 introns, respectively (Fig 2).

The introns in PCW *TaCesA1*, *2*, and *6* accounted for 1732-2026 bp of the genes, approximately double that of the 791 and 879 bp for the SCW *TaCesA4* and *8* genes. One of the SCW genes, *TaCesA7*, possessed a large total intronic region of 2095 bp, which was similar to the PCW *TaCesA* genes. Exonic regions in all the PCW forming genes (~3.2 kb) were similar in length

to those of the SCW forming genes (2.9-3.2kb). Exon-intron boundaries were random in all the genes studied, which was in contrast to the conserved boundaries reported in other species (Endo et al. 2002). The PCW and SCW genes, across groups, were 45-52% similar. Sequence similarity within the PCW and SCW groups was 54-56% and 46-63% respectively.

3.4.3 Evolution of introns in *TaCesA* gene family

Three different phases of intron evolution were predicted. Phase 0, 1, or 2 referred to the insertion of an intron between two consecutive codons, between the first and the second base or second and the third base of a codon, respectively (Csuros et al. 2011). In PCW *TaCesA* genes, all of the introns had identical phase distributions: introns 1, 3, 7, 8, 9, 10, 12, and 13 occurred in 0 phase, introns 2, 4, and 11 were in phase 1, and introns 5 and 6 occurred in phase 2. In contract, SCW *TaCesA* genes exhibited variable patterns of intron phase distribution. Introns 2, 5, 6, and 7 in *CesA4* had 0 phase distribution, introns 1 and 3 had 1, and intron 4 had a phase distribution of 2. *TaCesA7* also had introns with all three types of phase distribution; introns 2, 6, 7, 8, 9, 11, 12 were in phase 0, introns 1, 3, and 10 in phase 1, and introns 4 and 5 in phase 2. *CesA8* similarly had introns 1, 4, 5, 6, 8, and 9 in phase 0, introns 2 and 7 in phase 1, and intron 3 in phase 2 (Fig 3). The largest proportion of introns (57-66%) in all the studied genes was found to be in phase 0, followed by phase 1 (22-28%) and phase 2 (11-16%).

3.4.4 Amino acid variability of predicted TaCESA proteins

The predicted size of PCW and SCW TaCESAs ranged between 1075-1091 and 991-1055 amino acids (AA), respectively. To identify group-specific changes in primary and secondary cell wall CESA proteins, AA sequences from all TaCESAs were aligned. All the complete CESA proteins

possessed the already known, specific CESA domains, such as a ZnF (CX2-CX14-ACX2-CX4-CX2-CX7-GX3-CX2-C) near the N-terminus of the derived amino acid sequence (S2 Text). All the TaCESAs possessed eight TMDs; two towards the N-terminus and six near the C-terminus, as well as the conserved D, DXD, D, QXXRW signatures (Fig 1).

Major differences among TaCESAs resulted from the deletion of up to 45 AAs in hypervariable regions. The N-terminal of the PCW TaCESAs possessed more highly conserved motifs and fewer deletions in comparison to the SCW TaCESAs. ZnF consisted of 46 AAs in the predicted TaCESAs, with the exception of an 8 AAs deletion in TaCESA7 and its homoeologs, resulting in the following domain: CX2-CX6-ACX2-CX4-CX2-CX7-GX3-CX2-C as compared to the known domain (CX2-CX12-FXACX2-CX2PXCX2-CXEX5-GX3-CX2C), where X is any amino acid (Cosgrove 2005). Four of the TaCESAs out of 22 were missing the ZnF as did TaCESA9 because they were incomplete on the N-terminal end.

3.4.5 New motifs distinguishing PCW CESAs from SCW CESAs

A new motif distinguishing the PCW CESAs from the SCW CESAs was found within the ZnF. The motif, CQIC, was identified within the small motif, CXXC, reported earlier for differentiating CESAs from the CSL genes [8]. This motif was present in all the PCW TaCESAs. Although SCW TaCESAs also possessed a "CXXC" motif, the two middle amino acids in these proteins were variable. In the SCW TaCESA4, the polar amino acid glutamine was replaced by the negatively charged amino acid, glutamate; in TaCESA7, both the amino acids were replaced by the marginally hydrophobic amino acid alanine; and in TaCESA8, glutamine was replaced by a highly basic (positively charged) amino acid, arginine, and isoleucine was replaced by a relatively conservative substitution of alanine (Fig 4). Another conserved motif, SVICEXWFA, was located

within the second transmembrane domain in all the PCW CESAs. In the SCW-specific CESAs, TaCESA4, 7, and 8 this motif was variable but all the amino acid replacements were conservative. For example, isoleucine, a hydrophobic amino acid next to glutamate was replaced by an iso-amino acid, leucine, in CESA4; alanine was replaced by glycine, both somewhat hydrophobic, in CESA7; and valine and isoleucine, both hydrophobic amino acids, switched places in CESA8.

3.4.6 Conservation of motifs in monocots and dicots

The two motifs, CQIC and SVICEXWFA, distinguished the PCW from the SCW CESAs (Fig 4). That these motifs were conserved was confirmed by analysing the CESA proteins in the PCW and SCW groups from dicot (Arabidopsis) and monocot (barley, maize, rice, and wheat) species. Alignment results demonstrated that the CQIC and SVICEXWFA motifs were completely conserved only in the PCW-specific CESAs in all the plant species studied. The completely conserved amino acid residues in each motif across all the CESA proteins were CXXC and SXXCEXWF (Fig 1).

3.4.7 Unique motifs conserved among the CESA orthologs from different species

Motif analysis was performed by aligning CESA proteins from Arabidopsis, barley, maize, rice and wheat. Arabidopsis CESA4 and its orthologs from wheat, barley, maize, and rice exhibited 73-74% sequence similarity. In the case of SCW, nine motifs ranging from 2-15 amino acid residues in length provided ortholog-specific identity to the SCW CESAs from different species (Fig 5). These motifs were highly conserved among the orthologs from the five species analysed in this study. Only one gene from each species, with the exception of maize which had two closely related copies for one of the three SCW genes (CESA12 and 13), shared these motifs including a

dicot, Arabidopsis. This suggests that the genes for SCW had already duplicated before the separation of monocots and dicots. The number of amino acid residues among most of these motifs was also conserved among different species (Fig 5). CESA7 and 8 from wheat showed 71-75 % and 77-79 % sequence similarity with the corresponding orthologs from different species, respectively. Although the motifs were unique for CESA4, 7 and 8, they were highly conserved among the orthologs from different species (Fig 5).

Two PCW CESAs, AtCESA1, 3 and their orthologs from other species differed from AtCESA6 and its orthologs in structural features. AtCESA1 and 3 were highly similar (77-79%) to the corresponding orthologs from barley, maize, rice and wheat. Four motifs in TaCESA6 and three in TaCESA1 orthologs differentiated them from each other and all other CESAs (Fig 6).

3.4.8 Motifs differentiating CESAs from monocots and dicots

Arabidopsis CESA6 and its orthologs from other species in this study exhibited 68-70% sequence similarity but lacked any specific patterns that could differentiate them from the other CESAs. However, this group possessed motifs that were only conserved in the orthologs from monocots (grasses) but not in Arabidopsis. To confirm the specificity of these motifs for grasses, we retrieved the sequences of TaCESA2 orthologs from seven dicot species: *Arabidopsis thaliana* (AtCESA6), *Beta vulgaris* (BvCESA2), *Eucalyptus grandis* (EgCESA5), *Glycine max* (GmCESA2), *Gossypium hirsutum* (GhCESA5), *Populus trichocarpa* (PtCESA6) and *Solanum tuberosum* (StCESA3). The CESA2 and its orthologs from grasses were compared with its orthologs from dicot species. For this particular gene, nine motifs were highly conserved in the orthologs from grasses (Fig 7). But in dicots, these motifs were replaced by variable amino acid residues.

3.4.9 Phylogenetic analysis

The evolutionary history of the CESAs was inferred from the analysis involving 70 CESA protein sequences from different species. An unrooted phylogenetic tree revealed that the orthologs from Arabidopsis, barley, beet, cotton, maize, poplar, potato, rice, rose gum, soybean and wheat were grouped together. Branch lengths, which are indicative of the evolutionary distances were used to interpret the phylogenetic tree (Fig 8). The paralogs from various species were grouped in different clades from those of the orthologs. This suggests, again, that divergence of the *CesA* genes had occurred prior to the separation of monocots and dicots.

3.4.10 RNA-seq analysis of *TaCesA* genes

Gene expression of 21 of the 22 *TaCesA* genes was studied in five organs at three development stages. We left out the *TaCesA9* gene because it was represented by a highly truncated cDNA. A heat map displaying transcript abundance of the *CesA* genes from different wheat tissues is shown in Fig 9.

Transcript abundance data revealed the presence of two distinct groups. Group, I consisted of *TaCesA4A, B, D, TaCesA7B, D* and *TaCesA8B, D* genes, all involved in SCW synthesis. These genes were highly expressed in the mature tissues, for example, stem collected soon after anthesis, and at very low levels in the PCW formation (Fig 9). For example, *TaCesA7B, D* and *TaCesA8B, D* genes were expressed at extremely low levels in the spike and grain tissues (Fig 9).

Group II comprised the PCW *TaCesA* genes: *TaCesA1*, *2*, *3*, *5* and *6* along with their homoeologs from A, B and D genomes. These genes were expressed at lower levels in the mature tissues and at relatively high levels in the PCW forming cells (Fig 9). For example, all three

homoeologous copies of the *TaCesA3* gene were expressed in the grain and the leaf tissues. These genes were expressed moderately in the developing grain, which agrees with grain having a relatively low cell wall fraction. The expression of the *TaCesA5A* and *B* genes was highest in the grain tissues from 14 and 30 DAAs, whereas the *TaCesA5D* was moderately expressed in these tissues. The expression of *TaCesA5D* homoeolog was dramatically lower in the leaf tissues at 2 days after anthesis (DAA), whereas *TaCesA1D* was expressed at higher level. The transcript abundance of *TaCesA1A* was highest in the grain tissues at 2DAAs whereas *TaCesA6B* homoeolog was moderately expressed. The expression level of *TaCesA1B* was moderate in the root and grain tissues.

3.5 Discussion

Cellulose consists of paracrystalline microfibrils of multiple, unbranched β-1, 4-glucan chains, which are synthesised by the individual CESA polypeptides in the plasma membrane-localized rosette. *CesA* is a multigene family consisting of more than eight members in higher plants (Suzuki et al. 2006). Structure and function of the *CesA* genes in wheat remain undocumented. Most studies about structural and functional characterization of *CesAs* have been performed in Arabidopsis (Arioli et al. 1998; Richmond and Somerville 2000; Taylor et al. 2003), maize (Holland et al. 2000; Appenzeller et al. 2004), and rice (Tanaka et al. 2003; Wang et al. 2012a). Bread wheat, an allohexaploid, has a complex genome, ~17 Gb in size, ~80-90% of which consists of repetitive DNA (Mayer et al. 2014). The availability of large-scale genomic sequence information and conserved synteny between barley and wheat is valuable in exploring wheat gene function and structure (Mochida and Shinozaki 2013). In barley, the *CesA* gene family consists of nine genes (*HvCesA1* to *HvCesA9.* Three genes, *HvCesA1*, *HvCesA2*, and *HvCesA6*, are expressed during

primary wall formation, and another three, *HvCesA4*, *HvCesA7*, and *HvCesA8*, during secondary wall formation (Burton et al. 2004). In this report, we document 22 *CesA* genes from wheat, which we identified using a comparative genomics approach using barley sequences as anchors. As expected, most of the *TaCesA* genes each have three paralogs in the homoeologous genomes A, B and D. Four of the 22 genes deviated from this pattern: only one paralog was identified for *TaCesA9*, and two each for *TaCesA6*, *7*, and *8*. One of the genes, *TaCesA2*, had two paralogous copies on chromosomes 5B and 5D but the third on chromosome 4A, which was most likely because of a translocation between chromosomes 5A and 4A (Table 1) (Ma et al. 2013).

All the CESAs possess domains known to be highly conserved among all the plant species studied thus far (Richmond and Somerville 2000). Sequences in the non-conserved domains, however, are useful for the identification of the orthologs of individual *CesA* genes (Table 3). In the case of gene families, it is often difficult to determine true orthology among different species solely based on sequence similarity. Many previous studies reported *CesA* orthologs based on phylogenetic analyses (Burton et al. 2004; Wang et al. 2010a). We supplemented the phylogenetic analysis as a tool for the identification of the *CesA* orthologs by searching for the conserved motifs in addition to the ones already known (Ma et al. 2013).

Knowledge about the conserved structural motifs that can distinguish *CesA* genes involved in PCW and SCW formation as well as *CesAs* between monocots and dicots is limited. Distinct patterns of intron placement, removal, and the phases of insertion in *TaCesA* genes suggested that the phases of intron insertion remained conserved during the evolution of these genes (Trapp and Croteau 2001). Deviation of phase distribution from the expected 33% suggested a bias in intron insertions towards the 0 phase, that is, between the codons rather than within the codons (Csuros et al. 2011).

The motif CQIC in ZnF distinguishes the PCW and SCW CESAs from both the monocots and dicots. Distinct CSCs for the synthesis of primary and secondary cell walls have been reported (Arioli et al. 1998; Tanaka et al. 2003; Taylor et al. 2003). The high level of conservation of the CQIC motif suggests that it is possibly related to cellulose synthesis. This concurs with the observation in other major gene families, where domains and motifs were conserved during the evolution (Arioli et al. 1998; Taylor et al. 2003).

A similar trend of intron phase distribution and motif conservation was observed when we compared CESA1 of *Arabidopsis thaliana* with its orthologs from angiosperms (*Arabidopsis lyrata, Aquilegia coerulea, Brachypodium distachyon, Carica papaya, Citrus clementina, Citrus sinensis, Cucumis sativus, Eucalyptus grandis, Glycine max, Manihot esculenta, Medicago truncatula, Mimulus guttatus, Oryza sativa, Populus trichocarpa, Physcomitrella patens, Prunus persica, Ricinus communis, Setaria italica, Sorghum bicolor, Vitis vinifera, Zea mays*), Chlorophytes (*Chlamydomonas reinhardtii, Volvox carteri)***,** and pteridophyte (*Selaginella moellendorffii*).

We also identified new, highly conserved motifs among the CESA orthologs of five species (Arabidopsis, barley, maize, rice and wheat). Despite the variable protein sequence of each member of the CESA family among the orthologs from various species, the organisation of the motifs remained conserved.

RNA-seq expression profiling revealed that the three SCW genes (*TaCesA4, 7, 8*) and their homoeologs were co-expressed in the mature stem tissues (Fig 9). This observation provided support for these genes being functionally orthologous to the secondary wall-forming genes from other species, for example, Arabidopsis (Arioli et al. 1998; Richmond and Somerville 2000; Taylor

et al. 2003), barley (Burton et al. 2004), maize (Holland et al. 2000; Appenzeller et al. 2004), and rice (Tanaka et al. 2003; Wang et al. 2012a). Five genes (*TaCesA1, 2, 3, 5, and 6*) and their homoeologs from the A, B and D genomes of wheat constituted a second group involved in PCW synthesis.

Most of the *TaCesA* genes were differentially expressed among three different genomes of bread wheat, which is a common phenomenon in hexaploid wheat (Mochida et al. 2004). This differential expression pattern is attributable to the genetic divergence of paralogous genes during the evolution (Takata and Taniguchi 2015). *TaCesA* genes are distributed across the wheat genome (Fig 10). Similar distribution patterns were observed in Arabidopsis, barley and maize (Holland et al. 2000; Burton et al. 2004).

Our study compiles a list of the *CesA* genes in bread wheat, classified them into PCW and SCW formation, and maps them to the chromosomes. This information will be useful in breeding wheat for culm strength and biofuel-related traits.

3.6 Conclusion

We have identified 22 *CesA* genes from bread wheat and compared them with their orthologs from Arabidopsis, barley, maize, and rice. New structural motifs were identified, which allowed differentiation of the CESA proteins for their roles in primary or secondary wall (PCW or SCW) formation in higher plants. Further characterization of the motifs would be needed, however, to establish their respective biological roles. Several new motifs identified in this study would be useful as signatures for the identification of orthologs of the *CesA* genes from various species. The compilation of the *CesA* gene family in bread wheat along with the expression patterns and

genomic map positions of individual members will be helpful in improving culm strength for reduced lodging as well as improving the straw for biofuels.

Fig 3.1 Predicted protein features of wheat cellulose synthase genes. The numbers 1 to 8 in the purple rectangles refers to the transmembrane domains (TMDs). Black triangles localise the conserved motifs. The newly identified motifs CXXC and SXXCEXWF are highlighted in blue and previously reported motifs in black.

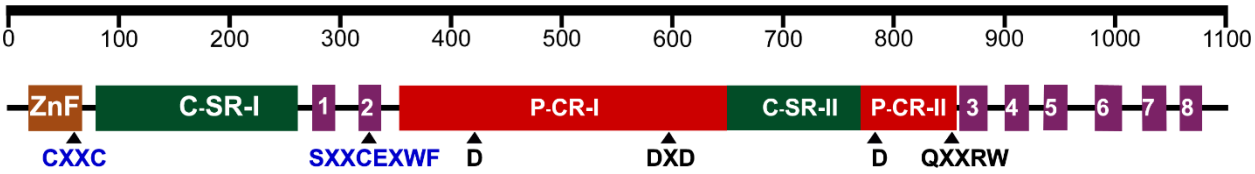

Fig 3.2 Structural features of the *TaCesA* genes. Drawn to scale, exons are represented by black boxes and introns by grey lines. Intron lengths are presented on top of each intron. PCW and SCW *CesA* genes are shown in blue and red colours, respectively.

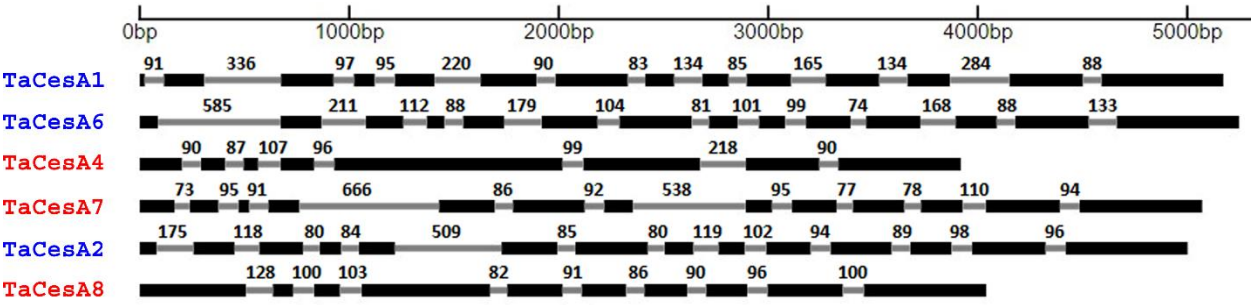

Fig 3.3 Amino acid sequence alignment of wheat CESA proteins. Drawn to scale with solid lines representing conserved amino acid sequences and the gaps representing the mismatches and deletions. Corresponding phases of intron evolution (0, 1, and 2) for the CESA proteins are shown on the top of the solid lines. Primary and secondary cell wall CESAs are shown in blue and red colour, respectively.

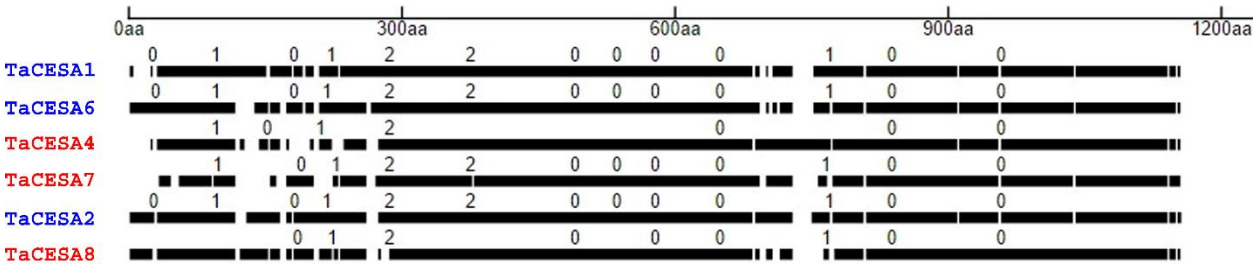

Fig 3.4 Motifs differentiating PCW and SCW CESA orthologs from different species. Conserved and non-conserved amino acids residues are highlighted in red and green respectively. Amino acid changes in the motifs are shown in blue.

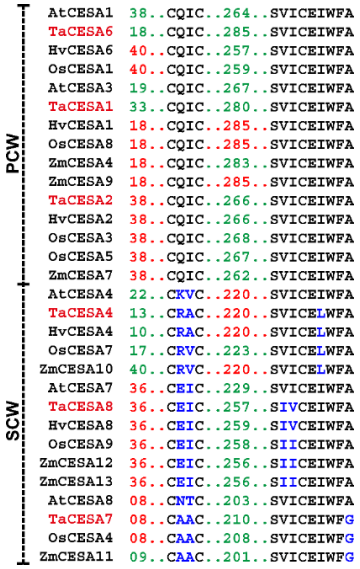

Fig 3.5 Conserved motifs differentiating the orthologs of SCW CESAs from *Triticum aestivum* (TaCESA), *Arabidopsis thaliana* (AtCESA), *Hordeum vulgare* (HvCESA), *Oryza sativa* (OsCESA), and *Zea mays* (ZmCESA). Conserved and non-conserved amino acids residues are highlighted in red and green respectively. Amino acid changes in the motifs are shown in blue.

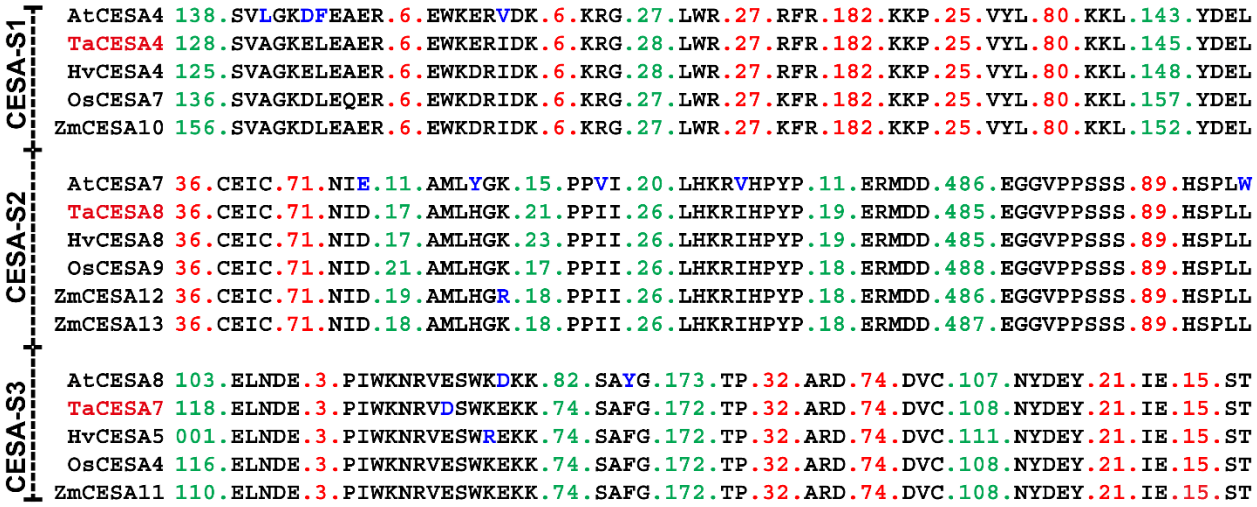

Fig 3.6 Conserved motifs differentiating the orthologs of PCW CESAs from *Triticum aestivum* (TaCESA), *Arabidopsis thaliana* (AtCESA), *Hordeum vulgare* (HvCESA), *Oryza sativa* (OsCESA), and *Zea mays* (ZmCESA). Conserved and non-conserved amino acids residues are highlighted in red and green respectively. Amino acid changes in the motifs are shown in blue.

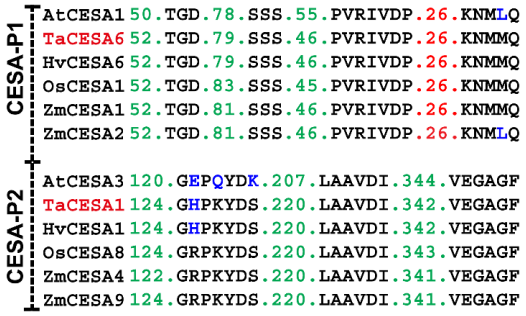

Fig 3.7 Monocots and dicots specific motifs of CESA orthologs from *Triticum aestivum* (TaCESA), *Arabidopsis thaliana* (AtCESA), *Beta vulgaris* (BvCESA), *Eucalyptus grandis* (EgCESA), *Glycine max* (GmCESA), *Gossypium hirsutum* (GhCESA), *Hordeum vulgare* (HvCESA), *Oryza sativa* (OsCESA), *Populus trichocarpa* (PtCESA), *Solanum tuberosum* (StCESA) and *Zea mays* (ZmCESA). Conserved and non-conserved amino acids residues are highlighted in red and green respectively. Amino acid changes in the motifs are highlighted in blue.

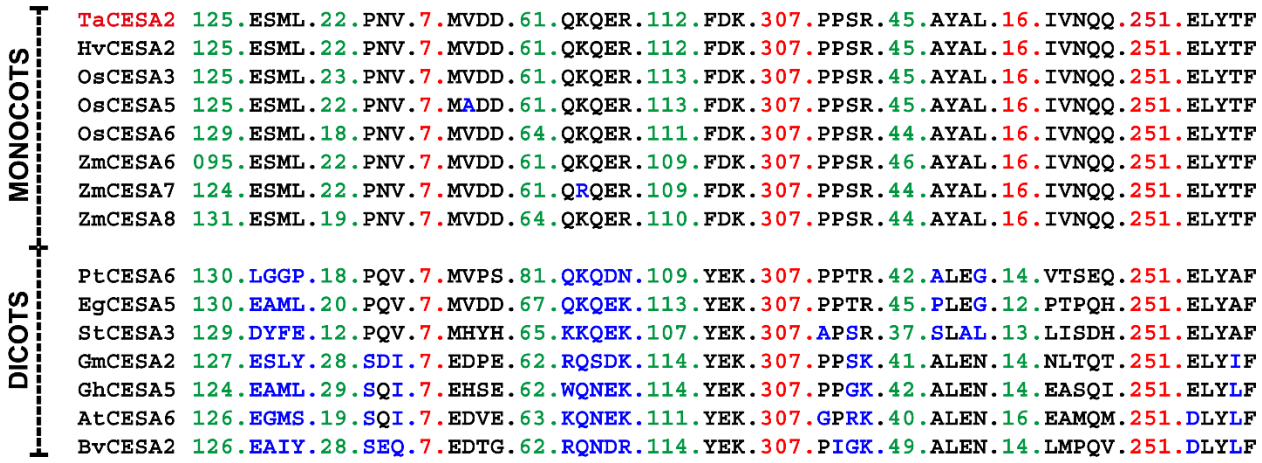

Fig 3.8 Unrooted phylogenetic tree of the CESAs from *Triticum aestivum* (TaCESA), *Arabidopsis thaliana* (AtCESA), *Beta vulgaris* (BvCESA), *Eucalyptus grandis* (EgCESA), *Glycine max* (GmCESA), *Gossypium hirsutum* (GhCESA), *Hordeum vulgare* (HvCESA), *Oryza sativa* (OsCESA), *Populus trichocarpa* (PtCESA), *Solanum tuberosum* (StCESA) and *Zea mays* (ZmCESA). The bar provides a scale for the branch length in the horizontal dimension. The line segment with the number '0.1' means that an equal length of the branch between the CESA proteins represents a change of 0.1 AA. Color codes for different species: Red - TaCESA, blue – AtCESA, purple - HvCESA, yellow - ZmCESA, green - OsCESA, and grey – BvCESA, EgCESA, GmCESA, GhCESA, PtCESA, StCESA.

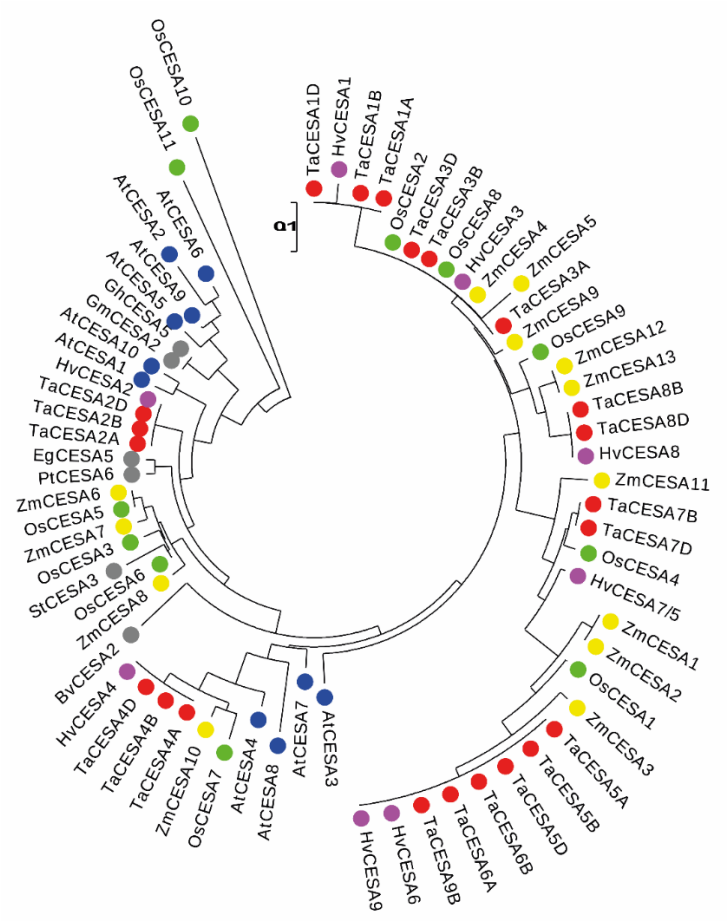

Fig 3.9 Heat map of 21 *CesA* transcripts by log2 counts per million (CPM) standard deviation in hexaploid wheat.

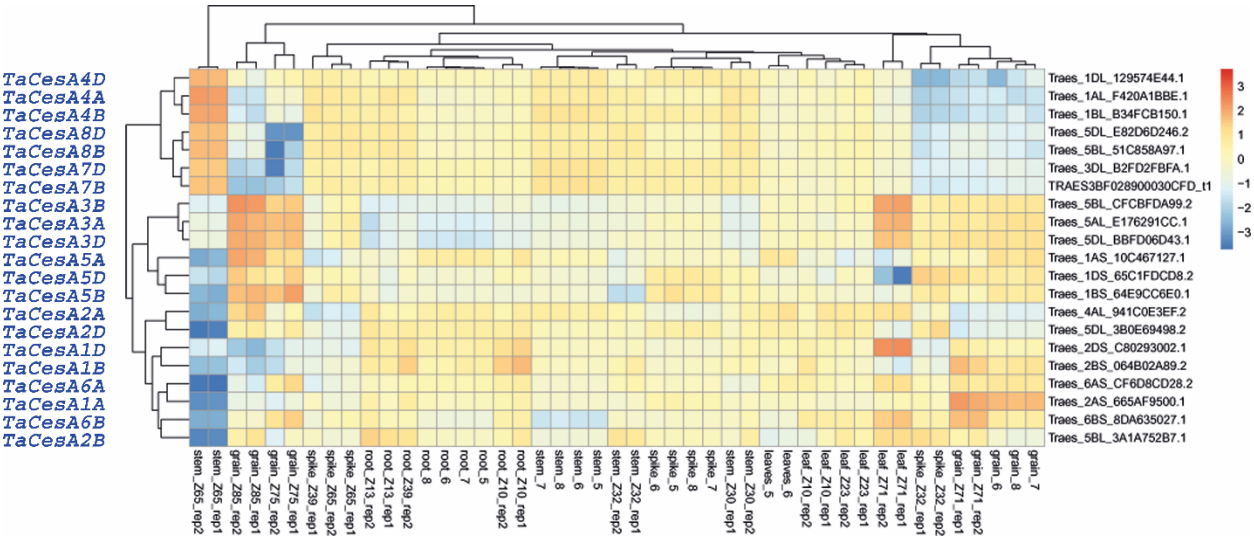

Fig 3.10 Map positions of *TaCesA* genes in the wheat genome. The exact locations are shown in Table 1.

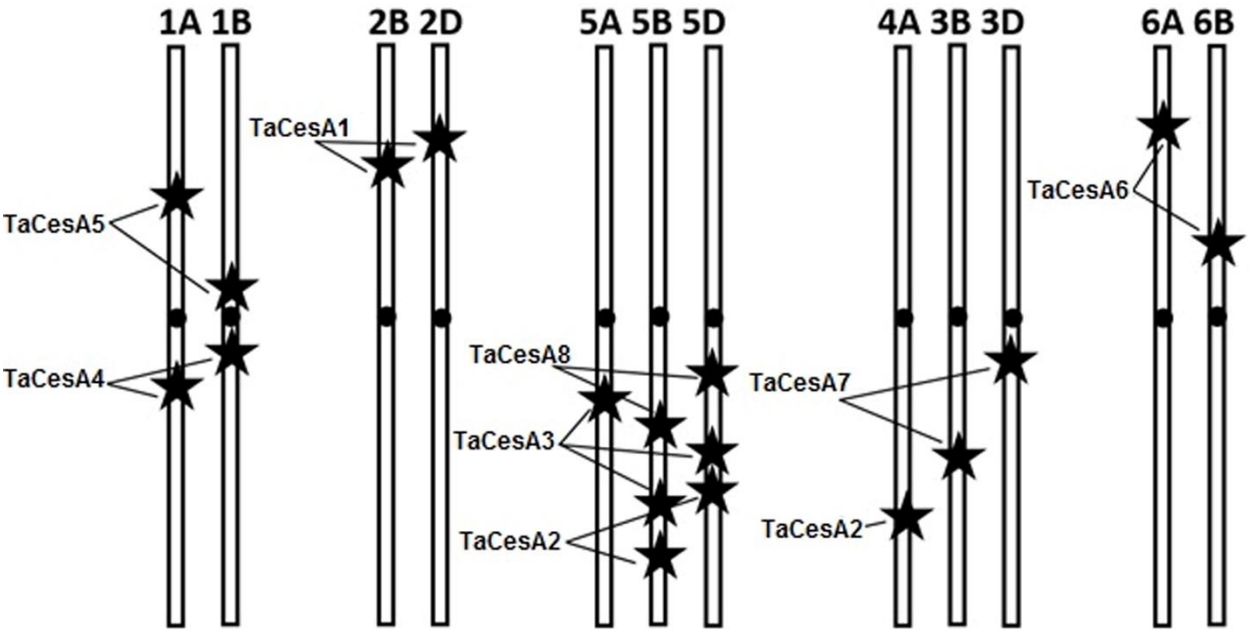

Table 3.1 *CesA* genes and their chromosomal locations in hexaploid wheat.

***CesA* gene**	**Map position (MB)**	**Ensembl ID**
TaCesA1A	NA	Traes_2AS_665AF9500.1
TaCesA1B	23.50	Traes_2BS_064B02A89.3
TaCesA1D	18.70	Traes_2DS_C80293002.1
TaCesA2A	176.7	Traes_4AL_941C0E3EF.2
TaCesA2B	262.70	Traes_5BL_3A1A752B7.1
TaCesA2D	151.78	Traes_5DL_3B0E69498.2
TaCesA3A	125.80	Traes_5AL_E176291CC.1
TaCesA3B	247.07	Traes_5BL_CFCBFDA99.2
TaCesA3D	144.92	Traes_5DL_BBFD06D43.1
TaCesA4A	93.16	Traes_1AL_F420A1BBE.1
TaCesA4B	48.70	Traes_1BL_B34FCB150.1
TaCesA4D	NA	Traes_1DL_129574E44.1/ EMT11949
TaCesA5A	29.31	Traes_1AS_10C467127.1
TaCesA5B	103.00	Traes_1BS_64E9CC6E0.1
TaCesA5D	NA	Traes_1DS_65C1FDCD8.2
TaCesA6A	9.23	Traes_6AS_CF6D8CD28.2
TaCesA6B	25.84	Traes_6BS_8DA635027.1
TaCesA7B	514.04	TRAES3BF028900030CFD_t1
TaCesA7D	42.80	Traes_3DL_B2FD2FBFA
TaCesA8B	163.48	Traes_5BL_51C858A97.1
TaCesA8D	60.35	Traes_5DL_E82D6D246.2
TaCesA9B	NA	Traes_2BS_9B34A7A43.2

NA- Precise location of these genes on the respective chromosomes is not known because of the incomplete assembly of the wheat genome.

Table 3.2 Structures of the *TaCesA* genes for PCW and SCW synthesis.

***CesA* gene**	**PCW or SCW**	**Gene length (nt)**	**Introns (#)**	**ORF length (AA)**	**Map to Chromosome**
TaCesA1	PCW	5175	13	1080	2
TaCesA2	PCW	5005	13	1091	5
TaCesA3	PCW	5127	13	1105	5
TaCesA4	SCW	3923	7	1044	1
TaCesA5	PCW	4,085	14	1078	1
TaCesA6	PCW	5251	13	1075	6
TaCesA7	SCW	5072	12	991	3
TaCesA8	SCW	4044	9	1055	5
TaCesA9	PCW	2184	5	537	2

Table 3.3 *TaCesA* genes and their orthologs from Arabidopsis, barley, maize, and rice involved in the formation of the primary cell wall (PCW) or secondary cell wall (SCW).

Gene Function	Wheat	Barley	Maize	Rice	Arabidopsis
PCW	*CesA5, 6* and *9*	*CesA6* and *9*	*CesA1, 2* and *3*	*CesA1*	*CesA1*and *10*
	*CesA1*and *3*	*CesA1*and *3*	*CesA4, 5*, and *9*	*CesA2, 8,10* and *11*	*CesA3*
	CesA2	*CesA2*	*CesA6, 7*, and *8*	*CesA3, 5*, and *6*	*CesA2, 5, 6*, and *9*
SCW	*CesA4*	*CesA4*	*CesA10*	*CesA7*	*CesA4*
	CesA8	*CesA8*	*CesA12* and *13*	*CesA9*	*CesA7*
	CesA7	*CesA5* and *7*	*CesA11*	*CesA4*	*CesA8*

CONNECTING STATEMENT FOR CHAPTER IV

Chapter IV, entitled "Functional characterization of secondary cell wall specific *CesA4* gene in bread wheat using Virus-Induced Gene Silencing (VIGS)" authored by Simerjeet Kaur, Kanwarpal S. Dhugga, Raj Duggavathi, Kulvinder S. Gill, and Jaswinder Singh has been submitted to "*Cellulose*".

As discussed in chapter III, Cellulose Synthase Complexes (CSCs) in secondary cell walls of wheat plants are composed of three genes *TaCesA4*, *TaCesA7*, and *TaCesA8*. These three genes co-expressed in the mature stem tissues of bread wheat. But the relative transcript abundance was found to be higher for *TaCesA4* genes, which indicates its major role in the secondary cell wall cellulose synthesis. However, the function of this gene requires further attention which could provide further understanding of cellulose synthesis in secondary cell walls. In study IV, the biological role of *TaCesA4* gene has been functionally evaluated using Virus-induced gene silencing (VIGS) approach. Three homoeologs (*TaCesA4A*, *TaCesA4B*, and *TaCesA4D*) were silenced collectively in bread wheat using the *TaCesA4* specific oligo designed from the conserved region of these homoeologs. Silenced plants showed a significant reduction in transcript abundance and cellulose content in the stem tissues. However, the anatomy of stem cross sections of silenced plants did not show any evidence of abrupt changes in the secondary cell wall of stems at the booting stage.

Chapter IV. Functional characterization of secondary cell wall specific *CesA4* gene in bread wheat using virus-induced gene silencing (VIGS).

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4.1 Abstract

Plant cell walls produce a bulk of renewable biomass vital for food, feed and biofuels. Cell wall consists of three layers including middle lamella, secondary cell wall and primary cell wall. The secondary cell wall is a thicker layer developed inside the primary cell wall. Cellulose is the main carbohydrate of the primary and secondary cell walls and involved in the shape, structure and strength of the plant. Recently, three major genes (TaCesA4, TaCesA7 and TaCesA8) have been identified in wheat which are appeared to play important roles in the synthesis of the secondary cell wall. In this study, efforts have been made to functionally characterize TaCesA4 using Virus-induced gene silencing (VIGS) approach. Inoculations were performed at the booting stage of the bread wheat (Chinese spring) plants grown under the temperature regime of 220C days and 180C nights with 23-50% relative humidity and 16 hrs of light. Quantification of the transcript at 21 dpi (Days post inoculation) revealed 87.17% decrease of TaCesA4 transcripts in the first internode of

silenced plants. Around 30% decline in the cellulose content was observed in silenced plants as compared to controls. However, negligible anatomical differences in the shape of cells and the arrangement of vascular bundles were observed between the stem cross-sections of silenced and control plants.

4.2 Introduction

In plants, cellulose microfibrils are known to be synthesized by a heteromeric rosette complex known as cellulose synthase complex (CSC). Each subunit of CSC has six cellulose synthase (CESA) isoforms that bound to the plasma membrane and catalyze the polymerization of β-1, 4-glucans using UDP-glucose as a substrate. CESA isoforms have been encoded by different *cellulose synthase A* (*CesA)* genes that play an important role in the synthesis of cellulose in primary and secondary cell wall (Endler and Persson 2011). In the case of *Arabidopsis thaliana*, *CesA1*, *CesA3*, and *CesA6* are involved in primary wall and *CesA4*, *CesA7* and *CesA8* appear to be required for cellulose synthesis in the secondary wall (Endler and Persson 2011). Cellulose in the primary cell wall determines the shape of cells, which is laid down during plant growth (Wasteneys 2004). The cellulose in secondary cell walls is deposited after the cell stop growing, because of its greater thickness, constitutes the bulk of terrestrial biomass (Joshi and Mansfield 2007). Moreover the higher degree of polymerization increase microfibril crystallinity of cellulose in secondary cell wall which determines the physical strength of the plant (Saxena and Brown 2005). Genes involved in secondary cell wall thickening are important candidates to study the genetic variability between diverse genotypes (Tian et al. 2014). Based on the structural, evolutionary and expression analysis of *CesA* genes in wheat, *TaCesA4*, *TaCesA7* and *TaCesA8* genes are appeared to play distinctive roles in the synthesis of the secondary cell wall (Taylor et al. 2003). Among secondary cell wall forming *CesAs*, higher transcript abundance of *TaCesA4*

has been observed in mature stem tissues of wheat (Kaur et al. 2016). Genetic evidence for the role of these genes in secondary cell wall formation came from the Arabidopsis irregular xylem mutants, *irx1* (*AtCesA8*), *irx3* (*AtCesA7*), and *irx5* (*AtCesA4)*, showing collapsed mature xylem cells due to lowered content of secondary cell wall cellulose (Taylor et al. 2003). Several *brittle culm* retrotransposons and EMS mutants for secondary cell wall *CesAs* in rice and a spontaneous *brittle stalk-2* mutant in maize showed a significant reduction in cellulose content as compared to wild-type plants (Ching et al. 2006; Zhang et al. 2009; Kotake et al. 2011; Wang et al. 2012a; Kaur et al. 2016). Gene expression studies coupled with reverse genetic approaches is a preferred method to functionally and rapidly annotate a particular gene (Held et al. 2008). In the current study, efforts have been made to functionally validate the role of *CesA4* gene in the wheat tissues using Virus-Induced Gene Silencing (VIGS) approach. VIGS is one of the powerful plant functional genomics tools (Singh et al. 2006; Bennypaul et al. 2012a; Singh et al. 2013) that exploits an RNA-mediated antiviral defence mechanism and triggers targeted gene silencing. VIGS is a fast and cost effective alternative to examining the function of uncharacterized genes, especially in polyploid crops, where stable transformation through RNAi is difficult to perform (Senthil-Kumar and Mysore 2011; Bhullar et al. 2014). Infection of plants by a virus engineered with fragments of a gene of interest activates post-transcriptional gene silencing as an innate defence response. Barley stripe mosaic virus (BSMV) is a single-stranded RNA virus consisting of tripartite α, β and γ genome. DNA plasmids carrying full-length cDNA clones of these three RNAs were constructed from BSMV strain ND18 (Petty et al. 1989). The insertion of a 178-bp fragment of the barley phytoene desaturase (*PDS*) gene into γ construct of BSMV resulted in the silencing of *PDS* with obvious phenotype after infection (Holzberg et al. 2002). The BSMV-based VIGS system was previously shown to silence the three homoeologous copies of a gene in wheat

efficiently (Bennypaul et al. 2012b). A similar approach has been utilized here to characterize *CesA4* genes in wheat.

4.2.1 Hypothesis *Cellulose synthase A 4* (*CesA4*) gene in wheat is required for the deposition of cellulose in mature stem tissues.

4.2.2 Objective I. Generation of appropriate constructs for VIGS

4.2.3 Objective II. Functional validation of *CesA4* gene in wheat using the VIGS system

4.3 Materials and methods

4.3.1 *TaCesA4* gene structure analysis

Full gene sequences of three homoeologs of *TaCesA4* were downloaded from Ensemblplants (http://plants.ensembl.org/Triticum_aestivum) (Kaur et al. 2016). Multiple sequence alignments were performed using Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al. 2011). The sequence manipulation suite: Color align conservation (http://www.bioinformatics.org/sms2/color_align_cons.html) was used to highlight the conserved regions of *TaCesA4* used to synthesize VIGS construct (Stothard 2000). Gene structure was predicted using the gene structure display server 2.0 (http://gsds.cbi.pku.edu.cn/) via the genomic and cDNA sequences of *TaCesA4* homoeologs.

4.3.2 *In silico* expression analysis of *TaCesA homoeologs*

Publicly available RNA-seq data generated from hexaploid bread wheat (var. *Chinese spring*) was used to predict the expression of *TaCesA4* homoeologs. The data was compiled from five different wheat tissues (Spike, root, leaf, grain and stem) collected at three stages of wheat development. The developmental stages with respect to each organ were reported in Zodoks scale; spike_z32

(two nodes), spike_z39 (meiosis), spike_z65 (anthesis), root_z10 (seedling), root_z13 (three leaves), root_z39 (meiosis), leaf_z10 (seedling), leaf_z23 (three tillers), leaf_z71 (2 days after anthesis), grain_z71 (2 days after anthesis), grain_z75 (14 days after anthesis), grain_z85 (30 days after anthesis), stem_z30 (spike at 1 cm), stem_z32 (two nodes), stem_z65 (anthesis). The relative expression of each *TaCesA4* homoeolog was presented as fragments per kilo base of transcript per million mapped reads (FPKM) (Choulet et al. 2014a).

4.3.3 Preparation of VIGS-construct

For the transient gene silencing experiment, 110 bp fragment of wheat *cellulose synthase A* (*TaCesA4*) gene was selected from a region conserved among homoeologous genes but unique to all other genes in wheat. The fragment was scanned for specificity to avoid off target genes using a BLAST search against GenBank database and with siRNA Scan tool (http://bioinfo2.noble.org/RNAiScan.htm). The fragment was cloned into pUC57 vector (GenScript, NJ, USA) and the sequence was confirmed. The Plasmid was then digested using the restriction enzymes *NotI* and *PacI* (New England Biolabs, MA, USA) to generate *NotI* and *PacI* ends in the cloned DNA fragment. The cDNA fragment was subsequently ligated to the pSL038-1 vector of BSMV γ genome (Scofield et al. 2005). The plasmids were linearized using *Mlu1* restriction enzyme for BSMV α, pγSL038-1 whereas, *Spe1* enzyme for BSMV β.

4.3.4 *In vitro* transcription of VIGS plasmids and rub inoculation

Infectious BSMV RNA was prepared from the linearized plasmids by *in vitro* transcription using a T7 DNA-dependent RNA polymerase (Thermo Fisher Scientific Inc., CA, USA) according to manufacturer's instructions. Three *in vitro* transcripts, BSMV α, β and γ (BSMV: 00/BSMV:

TaCesA4/ BSMV: *TaPDS*) in ratio 1:1:1 (2.5 µl each) were mixed with 22.5 µl of abrasive FES buffer to facilitate the viral entry (Bennypaul et al. 2012a). Ten plants were separately inoculated with each of test (BSMV: *TaCesA4*), positive control (BSMV: *TaPDS*) and negative control (BSMV: 00). FES buffer (1% sodium pyrophosphate, 1% bentonite, 1% celite in 0.1 M glycine, 0.06 M dipotassium phosphate) was used as abrasive buffer for rub inoculation. Plants were infected by rub-inoculating the flag leaf of each plant 2 to 3 at times at booting stage. Inoculated plants were grown at 22^0C days and 18^0C nights with 23-50% relative humidity and 16hrs light for 2-3 weeks, considered optimal for VIGS experiments.

4.3.5 RNA Isolation and cDNA synthesis

Stem tissues (internodes below the peduncle) of 21dpi (days post inoculation) plants, was collected and immediately placed in liquid nitrogen. For RNA extraction, samples were homogenised using a TissueLizer and then incubated in 1 ml of TRIzol reagent (Invitrogen, USA). Total RNA was extracted following the manufacturer's recommendations. Samples were treated with DNase1 (Promega Corp., WI, USA) and incubated in a 37^0C water bath for 30 minutes. cDNA was synthesized from 2 µg of RNA using an iScript cDNA synthesis kit (Bio-Rad, ON, Canada).

4.3.6 Real-time PCR

Primers for semi-qPCR and qRT-PCR were designed from the region unique to *TaCesA4* and outside the region which was used for making VIGS construct using clone manager suite software 6.0 (Table 1). The semi-qPCR and qRT-PCR were performed with three biological and three technical replicates as described previously (Bregitzer et al. 2007; Singh et al. 2013). Amplification was performed using a reaction volume of 25 µl containing 2 µl of cDNA template

and SYBR Green II master mix (Stratagene, Cedar Creek, USA) following the manufacturer's recommendations. The cycling conditions are as follows: five minutes of activation at 95^{o}C, followed by 30 cycles of 95^{o}C for 20 sec, 52^{o}C for 40 sec, and 72^{o}C for 40 sec, followed by a dissociation curve cycle of 95^{o}C for 1 min, 52^{o}C for 40 sec, and 95^{o}C for 40 sec using an Mx3005p PCR machine (Stratagene, Cedar Creek, USA). Gene silencing was expressed as a ratio of *TaCesA4* mRNA (normalized to *TaActin* mRNA) in BSMV: *TaCesA4* (test/silenced) inoculated plants to that inBSMV:00 (control/non-silenced) plants. qRT-PCR generated data was examined with Realtime PCR Miner (http://www.miner.ewindup.info/version2) and JMP software (version 3.2.2, SAS Institute Inc., Cary, NC, USA).

4.3.7 Estimation of cellulose content

Cellulose content was estimated as described by (Kaur et. al. unpublished) for three plants each from BSMV:00 and BSMV: *TaCesA4* inoculated plants. The first internode of the main tiller of each mature plant was taken and dried at 80°C. Dry sample (45-55 mg) was filled into a pre-weighted 2 ml Eppendorf tubes with a screw cap. 1.5 ml of a mixture of acetic acid: water: nitric acid (8:2:1) was added to each tube and vortexed (Appenzeller et al. 2004). All tubes were transferred to a steel rack and placed in a boiling water bath for four hours. Tubes were removed from the water bath and allowed to cool to room temperature. After the tubes reached room temperature they were placed in a swing-out rotor and centrifuged at 10,000 rpm for 10 minutes. The supernatant was aspirated off, washed with distilled water four times and finally washed with 90% ethanol. After each wash, the tubes were vortexed and centrifuged at 10,000 rpm for 10 minutes to aid in the formation of solid pellets. The caps were removed after the final wash and

the tubes were placed in the oven for drying at 80°C. The final weight of the tubes was used to calculate the percentage cellulose content on a dry matter basis.

% cellulose = Cellulose weight (final pellet dry weight)/Initial sample dry weight X100

4.3.8 Microscopic analysis of stem sections

Five stem samples (second internode from the top) per treatment (BSMV:00 and BSMV: *TaCesA4*) were taken at 21 dpi to analyse the anatomical features. The stem tissues were embedded in cryomolds containing Shandon CRYOMATRIX (Richard-Allan Scientific, Kalamazoo, USA) for cryosectioning. Stem cross sections (15 μm) were prepared using a cryotome (Leica, CM1850, Canada) machine at -10 °C. Cross-sections were stained with 5% toluidine blue (Sigma-Aldrich, Canada) for 5 min and washed with distilled water three times before mounting on glass slides (O'brien et al. 1964). Stained samples were observed under a fluorescence microscope (Nikon, Eclipse E800, USA).

4.3.9 Statistical analysis

Data from qPCR and cellulose content study was analyzed statistically using one-way analysis of variance (ANOVA) followed by student t-test (Cohen 1992).

4.4 Results

4.4.1 *TaCesA4* gene structure and construct designing

Three paralogs of *TaCesA4* gene were obtained corresponding to the three homoeolog genomes of hexaploid wheat (A, B and D). The genomic copies of three *TaCesA4* homoeologs were variable

in size; *TaCesA4A* (4614bp), *TaCesA4B* (3181bp), *TaCesA4D* (3063bp), however their coding DNA sequences (CDS) shared 98% identity. Multiple sequence alignment of genomic copies with their corresponding CDS revealed the intron-exon boundaries and translation start and stop sites. There were nine exons in *TaCesA4A*, whereas other two homoeologs (*TaCesA4B* and *TaCesA4D*) were found to possess only four exons. Although the exon-intron boundaries were highly conserved among the homoeologs, *TaCesA4B* and *TaCesA4D* were missing their first five exons from 5' end (Fig 1).

To confirm the functional role of *TaCesA4* gene, we designed VIGS construct to target all three homoeologous copies (*TaCesA4A, TaCesA4B* and *TaCesA4D*). A VIGS construct was designed such that it possess at least 95% nucleotide similarity among three homoeologs. It also contained at least one stretch of 21 nucleotides showing 100% nucleotide identity towards the target gene and this criterion did not meet by any non-target gene. Such unique region was selected from the C-SR-II (Class-Specific Region-II), upstream of DXD motif of *TaCesA4* gene (Kaur et al. 2016). This region is highly variable among different *CesA* genes (Fig S1a), however, this is highly conserved among the homoeologous copies of *CesA4* genes in bread wheat (Fig S1b). The C-SR-II for *TaCesA4* gene is approximately 400bp long, which comprises exon 7 of *TaCesA4A* (2600 to 3000), and exon 2 of *TaCesA4B* (1241 to1646 bp) and *TaCesA4D* (1073 to 1478 bp) respectively. A 110 bp fragment from this region was cloned into the γ vector of BSMV genome.

4.4.2 Homoeolog specific expression of *TaCesA4*

In silico gene expression of three *TaCesA4* homoeologous genes was examined in five organs at three development stages (Choulet et al. 2014a). A bar graph displaying transcript abundance of the *TaCesA4* homoeologs from different wheat tissues (spike, root, leaf, grain and stem) at three

stages of wheat development is shown in Fig 2. Transcript abundance data revealed relatively higher expression of *TaCesA4A* as compared to that of *TaCesA4B* and *TaCesA4D* in all 5 tissue samples. These genes were highly expressed in the mature stem tissues collected soon after anthesis. However, significantly lower expression levels were observed in grain, leaf, root and spike tissues during different developmental stages (Fig 2).

4.4.3 Optimization of VIGS in *Chinese spring* (*CS*) wheat cultivar

The silencing of *PDS* (*phytoene desaturase*) gene triggered the photobleaching of leaves due to loss of chlorophyll pigments. This photobleaching effect was used as the visual marker to optimize the VIGS system in the *Chinese spring* variety of hexaploid wheat. An intense effect of *PDS* gene silencing (BSMV: *TaPDS*) was observed in wheat plants (booting stage) grown in the greenhouse under the temperature regimen 22°C day/18°C night. Ten plants were inoculated among which eight plants showed intense symptoms of photo-bleaching while others showed mild phenotypes at 21 dpi. There were no symptoms of photo-bleaching in the plants inoculated with BSMV:00. These plants were morphologically similar to the un-inoculated *Chinese spring* plants (Fig 3).

4.4.4 Silencing of *CesA4* gene in wheat

At 21 dpi, plants inoculated with the BSMV: *TaCesA4* were phenotypically similar to the control (BSMV:00) plants as well as to the plants that were not inoculated. RNA was extracted from three plants each of BSMV:00 and BSMV: *TaCesA4* inoculated plants to confirm the transient silencing via their relative transcript abundance. As per semi-qPCR (Fig 4) and qRT-PCR analysis, relative transcript expression of *TaCesA4* normalised to reference gene *TaActin* in silenced plants (BSMV: *TaCesA4*) showed significant *(P=0.0065)* reduction (87.17%) compared to non-silenced

plants (BSMV:00), confirming the successful silencing of the target gene in the wheat stem (Fig 5).

4.4.5 Analysis of cellulose content in VIGS treated plants

Cellulose content was measured for five plants each of control (BSMV:00) and silenced plants (BSMV: *TaCesA4*). The percentage content of cellulose was significantly lower (29.27%) in the *TaCesA4* silenced plants as compared to the control plants at $P=0.0041$. An average percent cellulose content of control (BSMV:00)plants was 45.1% whereas the silenced plants (BSMV: *TaCesA4*) showed 31.9% cellulose in their stem tissue (Fig 6).

4.4.6 Histological analysis of stem tissues

To analyse the morphological characteristics of stem tissues of control (BSMV: 00) and silenced plants (BSMV: *TaCesA4*) plants, 15 μm transverse sections of second internode of were stained with toluidine blue to visualize the tissue architecture. In the wheat stem, vascular bundles consisting of xylem (tracheids) and phloem (sieve tube elements) were clearly observed. A hollow cavity inside the stem called internodal cavity was lined by the parenchyma cells. Xylem cells were large and thick-walled as compared to small phloem cells (Fig 7). It was observed that the xylem and phloem cells of intermodal and nodal tissues of the stem were intact in the silenced plants. The organization and appearance of cells in silenced plants were also similar to that of control plants. This confirmed that the silencing of *TaCesA4* gene at booting stage has no effect on the shape and arrangement of cells.

4.5 Discussion

Plant cell wall polysaccharides are getting attention more recently due to their extensive use as dietary fibres, food additives, a raw material for biofuels, and fodder for livestock (Taylor-Teeples et al. 2015). In addition, to providing mechanical support and a barrier against pathogen invasion, secondary cell wall accounts for the bulk of renewable cellulosic biomass. Cellulose, hemicellulose and lignin are the major constituents of the secondary cell wall, among which cellulose is the main load bearing network. Cellulose in the secondary cell wall is synthesised by a complex containing three CESA subunits. The cells of dicots and most of the monocots possess Type I cell walls whereas commelinoid monocots possess type II cell walls (Carpita 1996). The major cereals such as barley, oat, wheat, maize, and rice, as well as the C4 grasses, comes under commelinoid monocots (Vogel 2008). More than 1500 genes have been reported for cell wall related function in Arabidopsis, rice and maize (http://cellwall.genomics.purdue.edu), and over 1000 unannotated genes are estimated for their probable role in cell wall biogenesis (Yong et al. 2005).

Presently, the major aim of cell wall research is to assign specific functions to this large collection of genes at different developmental stages of plant and to understand the regulatory networks responsible for cell wall biosynthesis. Although bioinformatics approaches have been remained quite supportive in providing tentative functions to these genes (Holland et al. 2000; Burton et al. 2004; Yin et al. 2009; Wang et al. 2010b; Liepman and Cavalier 2012; Liu et al. 2012; Schreiber et al. 2014b), only a few of them have been characterised for their specific functional role (Dhugga et al. 2004b; Burton et al. 2006a; Cocuron et al. 2007; Burton et al. 2011b; Taketa et al. 2012). To explore the function of genes in cell wall biosynthesis, a vast majority of mutant resources are available for Arabidopsis, however, there are very limited cell wall mutants in grass

species. For example *irregular xylem* (*irx*) mutants, *irx1* (*AtCesA8*), *irx3* (*AtCesA7*) and *irx5* (*AtCesA4*) of Arabidopsis unveiled a collapsed xylem phenotype indicating requirement of these genes for due to secondary cell wall formation (Hernández-Blanco et al. 2007)

In the case of bread wheat, the genes of this complex are named as *TaCesA4*, *TaCesA7*, and *TaCesA8* (Kaur et al. 2016). *In vitro* expression studies in wheat showed the highest transcript abundance of *TaCesA4* in mature stem tissues. These expression patterns are supported by their involvement in the formation of secondary cell wall formation, which is laid down in the mature cells (Taylor-Teeples et al. 2015). Wheat genome comprises three homoeologs of *CesA4* gene, which are structurally different; *TaCesA4A* homoeolog possesses 9 exons while other two homoeologs have 4 exons. Interestingly, an ortholog of wheat *CesA4* gene in rice (*OsCesA7*) also possesses 9 exons (Wang et al. 2010b; Kaur et al. 2016). Although first five exons from 5' end have been found to be missing in *TaCesA4B* and *D*, yet their expression has been observed in the mature stem tissues. Nonetheless, the expression of these two homoeologs was not as prominent as of *TaCesA4A* homoeolog. Despite an essential part of plant's basic structural unit, functional characterization of *TaCesA4* has not been reported in wheat.

In the current study, we have employed VIGS to understand the role of *TaCesA4* in the secondary cell wall of wheat. Transient gene knockdown through VIGS has been successfully described in wheat for assigning functions to different genes (Scofield et al. 2005; Tai et al. 2005; Bennypaul et al. 2012b). VIGS has also been employed in (*Nicotiana benthamiana*) for the functional analysis of *CesA* genes inserted in potato X virus vectors (Burton et al. 2000). A reduction in transcript levels and cellulose content was recorded for the infected plants. VIGS with BSMV has also been shown as an effective means of transient gene silencing in wheat and barley (Holzberg et al. 2002; Scofield et al. 2005; Bennypaul et al. 2012a). Silencing of *PDS* gene

encoding a phytoene desaturase through VIGS was used as a positive control which leads to visual photo-bleaching symptoms (Ruiz et al. 1998; Bennypaul et al. 2012a). The efficiency of transient knockdown through VIGS is dependent on cultivars and growth conditions (Bennypaul et al. 2012a). Inoculation of BSMV: *CesA4* at booting stage of *Chinese spring* plants successfully silenced *TaCesA4*. (Cakir and Tör 2010; Bennypaul et al. 2012a). We observed significant difference (87.17 %) in *TaCesA4* expression of control and silenced plants using qRT-PCR. Also, there was 29.27% decrease in the cellulose content of silenced plants as compared to control plants. The comparable impact of *CesA* gene silencing was observed on the gene expression and cellulose content in tobacco (*CesA1* and *CesA2*) (Burton et al. 2000), barley (*CesA6*) (Held et al. 2008), and flax (*CesA4* and *CesA8*) (Chantreau et al. 2015).

Reduction of cellulose content was also recorded for the brittle culm mutants of rice (Tanaka et al. 2003; Taylor et al. 2003; Kotake et al. 2011; Wang et al. 2012a). Cellulose synthesis was inhibited in *CesA4* (irx5), *CesA7* (irx3), and *CesA8* (irx1 mutants (Hernández-Blanco et al. 2007). Although phenotypes of mutant plants varied in different plant species, but the reduction in cellulose content was a common phenomenon in all these studies. The anatomical changes in the stem sections of flax plants were more pronounced for the VIGS of genes related to primary cell wall *CesAs* (*CesA1, 3*, and *6*) and as compared to secondary cell wall *CesAs* (*CesA4* and *8*) (Chantreau et al. 2015). Similar to these observations, no obvious anatomical changes were observed in the stem sections of wheat plants after the silencing of *TaCesA4* gene. A possible explanation of this may be the growth stage of cells and tissues at the time of viral infection which largely determine anatomical features of cross sections. If the cells have grown to their full size and have adequate primary and secondary cell wall before the inoculation, they will appear normal after viral infection. Viral infection, in this case, can minimise or stop the further deposition of

cellulose due to the gene knockdown, but the decrease in the cellulose levels may not necessarily impact the cell shape and integrity.

Fig 4.1 Schematic depicting the structure of *TaCesA4* gene and its homoeologs. Red bar indicates the 110 bp region of the *TaCesA4* gene cloned into the BSMV γ vector.

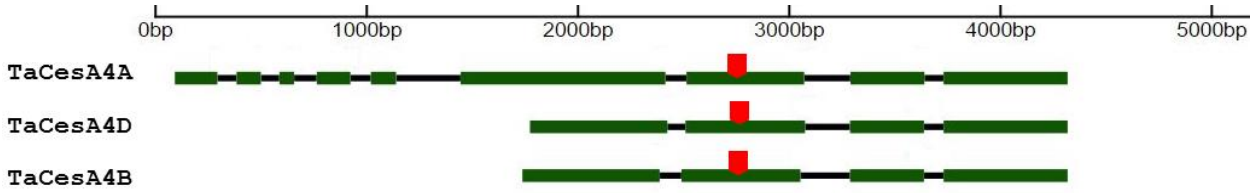

Fig 4.S1a Multiple sequence alignment of the fragment used for designing VIGS construct with other secondary cell wall related genes (*TaCesA4, TaCesA7* and *TaCesA8*) along with their homoeologs representing the non-conserved region.

```
TaCesA7_3DL     -AAGAAAAAGGTTGAAAAAACTGAGAAGGAAATGCACA---GAGA-------CTCCAGACGA----------------- 1773
TaCesA7_3B      -AAGAAAAAGGTTGAAAAAACTGAGAAAGAAATGCACA---GAGA-------CTCCAGAAGA----------------- 1770
TaCesA8_5BL     GAAGCGAAAGGGCGGCAAGGATGGG---------------------------CTGCCGGAGGGC---------------- 2010
TaCesA8_5DL     GAAGCGAAAGGGCGGCAAGGATGGG---------------------------CTGCCGGAAGGC---------------- 2013
TaCesA8_5AL     GAAGCGAAAGGGCGGCAAGGATGGG---------------------------CTGCCGGAGGGC---------------- 2013
TaCesA4_1DL     GCACCGCAAGTCGAGCAAGGACAAGAAGGGCGGCGGCGGCGGCGACGATGAGCCGCGCCGCGGCTCCTGGGGTTCTACA 907
TaCesA4_1BL     GCACCGCAAGTCAAACAAGGAGAAGAAGGGCGGCGGC---GGCGACGACGAGCCGCGGCGCGGCTCCTGGGGTTCTACA 904
TaCesA4_1AL     GCACCGCAAGTCGGACAAGGACAAGAAGGGCG---------GCGACGACGAGCCGCGCCGCGGCTCCTCGGGTTCTACA 1885
VIGS_Construct  ----------------------------------------GCGACGACGAGCCGCGCCGCGGCTCCTCGGGTTCTACA 39

TaCesA7_3DL     ----G--------------------------AGGACCTTGAATCTG-----------------------------CT 1791
TaCesA7_3B      ----G--------------------------AAGACCTTGAGTCTG-----------------------------CC 1788
TaCesA8_5BL     ----G--------------------------TGGCCGATG-------------------------------------- 2020
TaCesA8_5DL     ----G--------------------------TGGCCGATG-------------------------------------- 2023
TaCesA8_5AL     ----G--------------------------TGGCCGACG-------------------------------------- 2023
TaCesA4_1DL     AGAAGCGGGGCAAGAAGGATAAGCTCGGCGGCGGGCCGAAGAAGGGGTCGTACCGGAAGCAGCAGCGCGGGTACGAGCTG 987
TaCesA4_1BL     AGAAGCGGGGCAAGAAGGACAAGCTCGGCGGCGGGCCGAAGAAGGGGTCGTACAGGAAGCAGCAGCGCGGGTACGAGCTG 984
TaCesA4_1AL     AGAAGCGGGGCAAGAAGGACAAGCTCGGCGGCGGGCCGAAGAAGGGGTCGTACAGGAAGCAGCAGCGCGGGTACGAGCTG 1965
VIGS_Construct  AGAAGCGGGGCAAGAAGGACAAGCTCGGCGGCGGGCCGAAGAAGGGGTCGTACAGGAAGCAGCAGCGCGGG--------- 110

TaCesA7_3DL     ATTTTCAATCTACGGGAAATCGACAACTACGACGAGTATGAGCGGTCCATGCTTATCTCCCAGATGAGCTTTGAGAAGTC 1871
TaCesA7_3B      ATTTTCAATCTACGGGAAATCGACAACTACGACGAGTATGAGCGGTCCATGCTTATCTCCCAGATGAGCTTTGAGAAGTC 1868
TaCesA8_5BL     -------------GAGGAAT------------GGACGGCGACAAGGAGCAGATGATGTCCCAGATGAACTTTGAGAAGCG 2075
TaCesA8_5DL     -------------GAGGAAT------------GGACGGCGACAAGGAGCAGATGATGTCCCAAATGAACTTCGAGAAGCG 2078
TaCesA8_5AL     -------------GAGGAAT------------GGACGGCGACAAGGAGCAGATGATGTCCCAGATGAACTTCGAGAAGCG 2078
TaCesA4_1DL     GAGGAGATCGAGGAGGGGATAGAGGGGTACGACGAGCTGGAGCGCTCGTCGCTCATGTCGCAGAAGAGCTTCCAGAAGCG 1067
TaCesA4_1BL     GAGGAGATCGAGGAGGGCATCGAGGGGTACGACGAGCTGGAGCGCTCGTCGCTCATGTCGCAGAAGAGCTTCCAGAAGCG 1064
TaCesA4_1AL     GAGGAGATCGAGGAGGGCATCGAGGGGTACGACGAGCTGGAGCGCTCCTCGCTCATGTCGCAGAAGAGCTTCCAGAAGAG 2045
VIGS_Construct  ---------------------------------------------------------------------------- 110
```

Fig 4.S1b Multiple sequence alignment of the fragment of *TaCesA4* gene used for designing VIGS construct with its homoeologs representing the conserved region.

```
TaCesA4_1DL     GTGCGACTGCTGGCCCTCGTGGTGCTGCTGCTGCTGCTGCTTCGGCGGAGGGAAGCACCGCAAGTCGAGCAAGGACAAGA 853
TaCesA4_1BL     GTGCGACTGCTGGCCGTCGTGGTGCTGCTGCTGCTGCTGCTTCGGCGGAGGGAAGCACCGCAAGTCAAACAAGGAGAAGA 853
TaCesA4_1AL     GTGCGACTGCTGGCCCTCGTGGTGCTGCTGCTGCTGCTGCTTCGGCGGCGGGGAAGCACCGCAAGTCGGACAAGGACAAGA 1840
VIGS_Construct ---------------------------------------------------------------------------------- 0

TaCesA4_1DL     AGGGCGGCGGCGGCGGCGACGATGAGCCGCGCCGCGGGCTCCTGGGGTTCTACAAGAAGCGGGGCAAGAAGGATAAGCTC 933
TaCesA4_1BL     AGGGCGGCGGC---GGCGACGACGAGCCGCGGCGCGGGCTCCTGGGGTTCTACAAGAAGCGGGGCAAGAAGGACAAGCTC 930
TaCesA4_1AL     AGGGCG---------GCGACGACGAGCCGCGCCGCGGGCTCCTCGGGTTCTACAAGAAGCGGGGCAAGAAGGACAAGCTC 1911
VIGS_Construct ---------------GCGACGACGAGCCGCGCCGCGGGCTCCTCGGGTTCTACAAGAAGCGGGGCAAGAAGGACAAGCTC 65

TaCesA4_1DL     GGCGGCGGGCCGAAGAAGGGGTCGTACCGGAAGCAGCAGCGCGGGTACGAGCTGGAGGAGATCGAGGAGGGGATAGAGGG 1013
TaCesA4_1BL     GGCGGCGGGCCGAAGAAGGGGTCGTACAGGAAGCAGCAGCGCGGGTACGAGCTGGAGGAGATCGAGGAGGGCATCGAGGG 1010
TaCesA4_1AL     GGCGGCGGGCCGAAGAAGGGGTCGTACAGGAAGCAGCAGCGCGGGTACGAGCTGGAGGAGATCGAGGAGGGCATCGAGGG 1991
VIGS_Construct GGCGGCGGGCCGAAGAAGGGGTCGTACAGGAAGCAGCAGCGCGGG---------------------------------- 110

TaCesA4_1DL     GTACGACGAGCTGGAGCGCTCGTCGCTCATGTCGCAGAAGAGCTTCCAGAAGCGGTTCGGGCAGTCGCCGGTGTTCATCG 1093
TaCesA4_1BL     GTACGACGAGCTGGAGCGCTCGTCGCTCATGTCGCAGAAGAGCTTCCAGAAGCGGTTCGGGCAGTCGCCGGTGTTCATCG 1090
TaCesA4_1AL     GTACGACGAGCTGGAGCGCTCCTCGCTCATGTCGCAGAAGAGCTTCCAGAAGAGGTTCGGGCAGTCGCCGGTGTTCATCG 2071
VIGS_Construct ---------------------------------------------------------------------------------- 110
```

Fig 4.2 *In silico* expression of *TaCesA4* homoeologs in different wheat tissues, expressed as reads per kilo base of transcripts per million mapped reads (FPKM) in hexaploid wheat. Blue color bar represent *TaCesA4A*, black and green bars denotes *TaCesA4B* and *TaCesA4D* respectively.

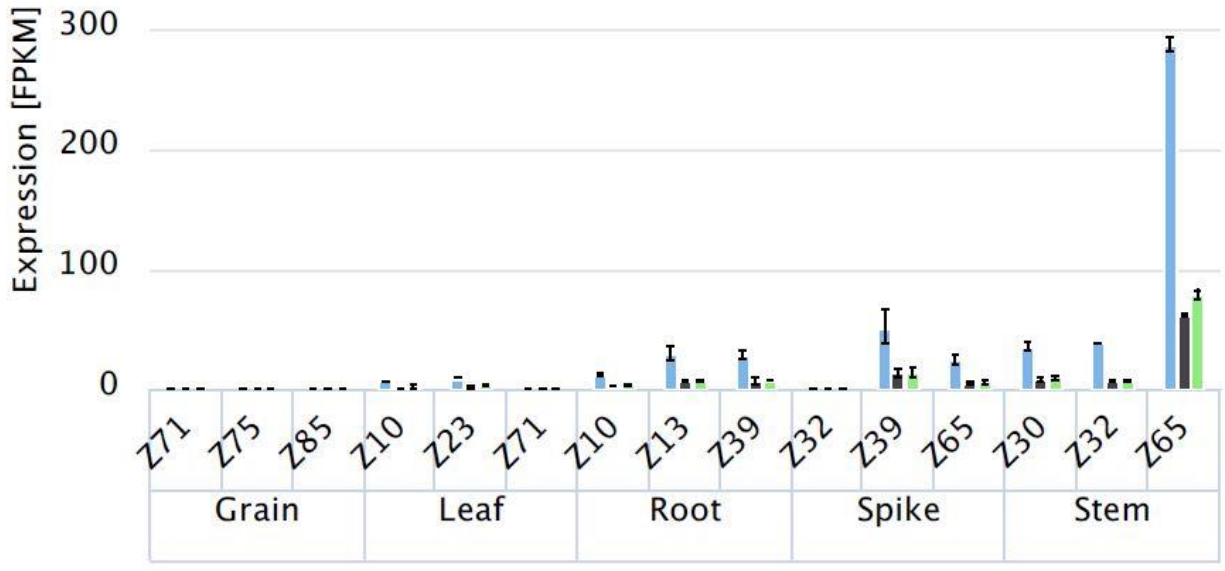

Fig 4.3 Silencing of the *phytoene desaturase* (*PDS*) gene. Leaf phenotypes of wheat plants inoculated with BSMV:00 and BSMV: *TaPDS* at 21 dpi.

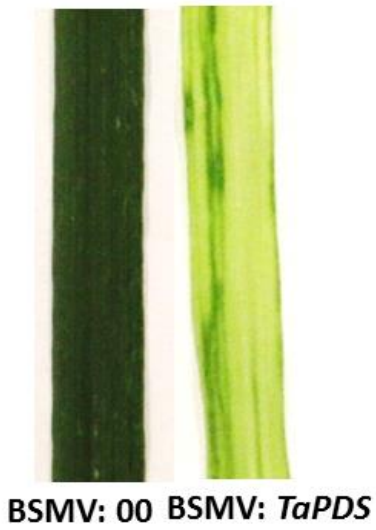

Fig 4.4 Semi-qPCR based expression of *TaCesA4* normalised to reference gene *TaActin* in silenced plants (BSMV: *TaCesA4*) and non-silenced plants (BSMV:00); Where L- marker, -ve- negative control.

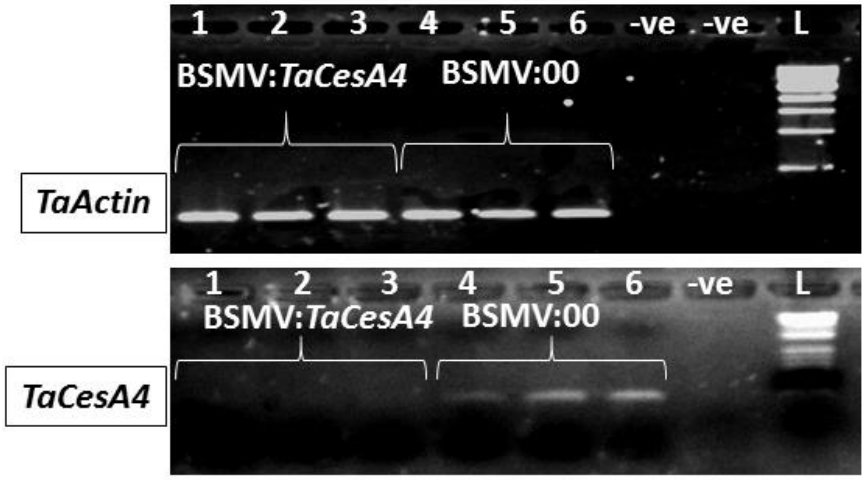

Fig 4.5 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses to confirm the gene knockdown as the relative transcript expression of *TaCesA4* normalized to *TaActin* mRNA in BSMV: *TaCesA4* inoculated plants as compared to control (BSMV:00) plants at 21 dpi.

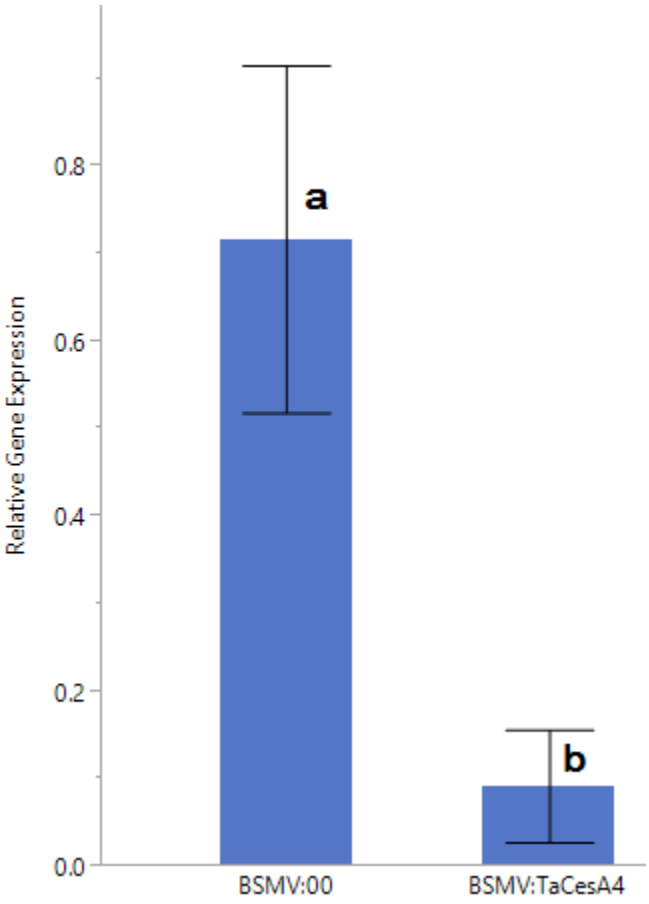

Fig 4.6 Cellulose content (% w/w) in *TaCesA4* silenced (BSMV: *TaCesA4*) plants as compared to control (BSMV:00) plants.

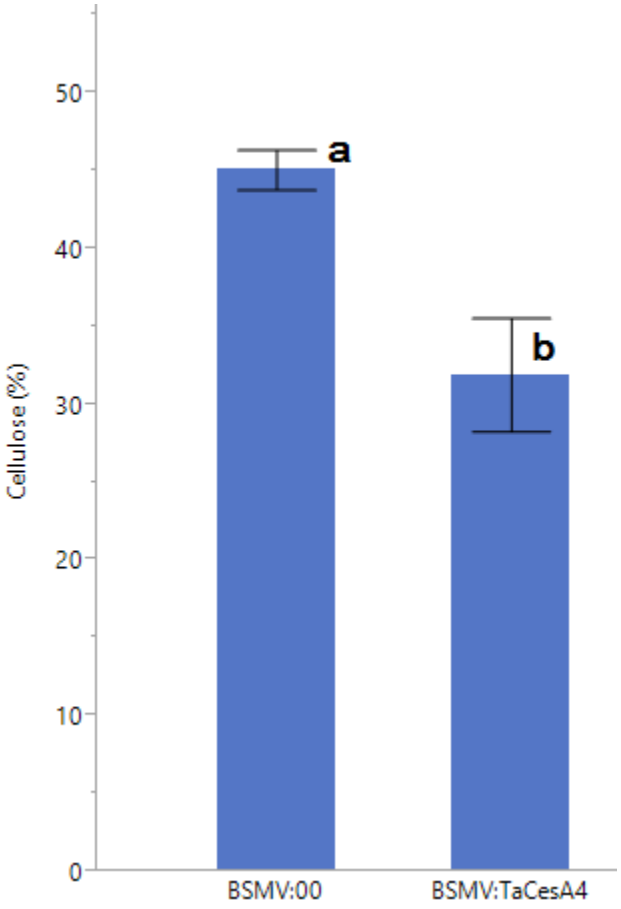

Fig 4.7 Transverse sections of stem tissues of control (BSMV: 00) and silenced (BSMV: *TaCesA4)* wheat plants at 20X and 4X magnification; where mx is meta xylem, px is protoxylem, ph is phloem.

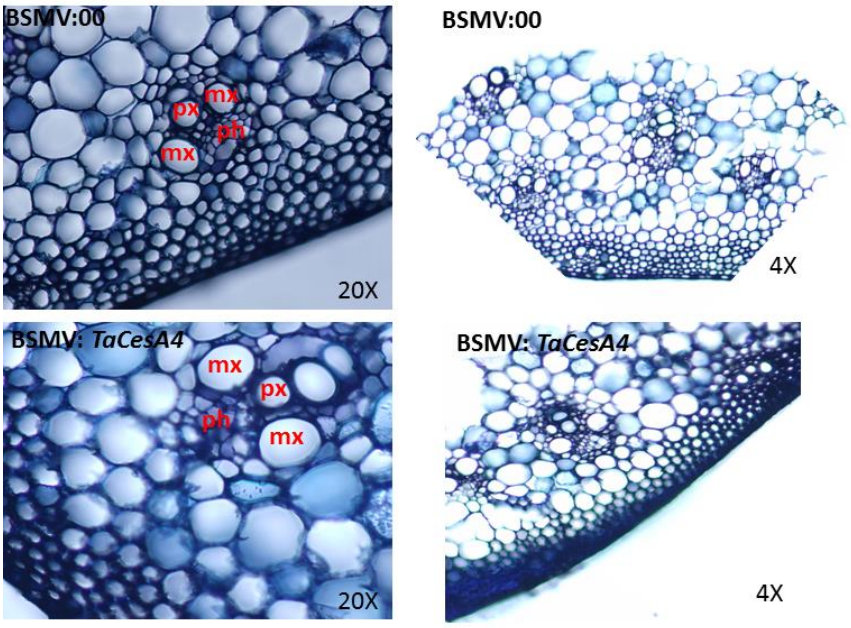

Table 4.1 Primers used for semi-qPCR, qRT-PCR and confirmation of VIGS construct.

		Primers	
Experiment	**Name**	**Forward**	**Reverse**
VIGS fragment amplification and sequencing	Gamma	TGATGATTCTTCTTCCGTTGC	TGGTTTCCAATTCAGGCATCG
VIGS gene expression	*TaActin*	TGTGCTTGATTCTGGTGATGGTGTG	CGATTTCCCGCTCAGCAGTTGT
VIGS gene expression	*TaCesA4*	CCGAAGAAGGGGTCGTACAG	CTCTTCTGCGACATGAGCGA

CONNECTING STATEMENT FOR CHAPTER V

Chapter V, entitled "Genome-Wide Association study (GWAS) revealed novel genes linked to natural variability of cellulose content in Bread Wheat (*Triticum aestivum*, L.)" authored by Simerjeet Kaur, Xu Zhang, Amita Mohan, Prashant Vikram, Sukhwinder Singh, Kanwarpal S. Dhugga, Zhiwu Zhang, Kulvinder Gill and Jaswinder Singh has been submitted to "*Frontiers in Plant Science*".

In chapter III and IV, we have explored the genes that are the major players for cellulose biosynthesis in wheat. These studies led to the identification of 22 *CesA* genes based on the comparative genomics approach. A gene (*TaCesA4*) expressing in the mature stems was validated for its contribution towards cellulose synthesis through VIGS. But our current knowledge is limited about the genetic associations of the existing natural variation in cellulose content. In chapter IV, we performed a comprehensive study about such genetic connections. We have evaluated 284 diverse wheat lines to estimate natural variation of cellulose content in the straw. This phenotypic variability was further linked to the SNP genotyping data generated by GBS (genotyping by sequencing). Genome-wide Association Studies (GWAS) led us to identify novel genetic association (β-tubulin and UDP-glycosyl transferase (UGT) family) linked to cellulose content in wheat straw. β-tubulin genes were previously reported to synthesise the microtubules that are associated with the delivery of CESA complexes to the plasma membrane (Gutierrez et al. 2009). The UGT family genes are known for the transfer of UDP-glucose to the catalytic sites for the synthesis of cellulose (Lairson et al. 2008). These novel associations will be valuable to devise marker-assisted/genomic selection strategies to monitor cellulose content in wheat breeding populations.

Chapter V. Genome- wide association study reveals novel genes linked to natural variation of cellulose content in bread wheat (*Triticum aestivum*, L.)

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5.1 Abstract

Plant cell wall provides dynamic structure and shape to the cells. Cell wall formation is a complex, coordinated and developmentally regulated process. Synthesis and remodelling of various cell wall components play a vital role in plant development and architecture. Cellulose is the most abundant biopolymer on earth and the most dominant constituent of plant cell walls. Because of its paracrystalline structure, cellulose is the main determinant of mechanical strength of plant tissues. As the most abundant polysaccharide on earth, it has been the main focus of cellulosic biofuel industry. It is important, thus, to explore the underlying mechanism of cellulose biosynthesis. This report presents results on the analysis of the stem cellulose content of 288 diverse wheat accessions and genome-wide association study (GWAS). The germplasm showed 6.56% coefficient of variation (CV) in cellulose content among diverse wheat accessions. Genotypic data comprising 21,073 SNPs was used to establish genome-wide marker-trait associations. The analysis led to the

identification of nine SNPs, which associated significantly (p<1E-05) with cellulose concentration. Four strongly associated (p<8.17E-05) SNP markers were linked to wheat unigenes. These unigenes were annotated using BLASTn search against various plant databases. Genes including *β-tubulin, Auxin-induced protein 5NG4* and a putative transmembrane protein of unknown function were found to be associated with cellulose content. Associated genes may be directly or indirectly involved in the synthesis of cellulose in wheat but further investigations are necessary to establish their respective involvements. GWAS results from this study have the potential for genetic manipulation of bread wheat and other small grain cereals to enhance culm strength.

5.2 Introduction

The increasing world population demands a sustainable increase in the production of food, feed and fuel crops (Scholey et al. 2016). Bread wheat (*Triticum aestivum*) occupies more agricultural area than any other food crop worldwide (http://www.wheatinitiative.org/). In addition to grain production, the annual worldwide production of wheat straw is around 350 million tons, which is used as cattle fodder in developing countries and is a potential feedstock for cellulosic ethanol production (Singhania et al. 2014). The use of grain for food and feed and straw residue for fuel could make wheat a dual purpose crop. Wheat straw, which is comprised of cellulose (~40%), hemicelluloses (~35%) and lignin (~25%), is one of the most abundant lignocellulosic raw materials in the world (Ruiz et al. 2013). Cellulose, a paracrystalline polysaccharide, is the main determinant of mechanical strength, which has implications in crop lodging, biotic and abiotic stresses. Cellulose amount in a unit length of the stem explains most of the variation for mechanical strength (Appenzeller et al. 2004). The proportion of cellulose in cell wall also affects the total sugar release during the process of enzymatic hydrolysis (FAN et al. 2012; Lindedam et al. 2012). An understanding of the natural variability of cellulose in plants and its association with

chromosomal regions could provide markers for enhancing grain and biomass yield (Ciesielski et al. 2014).

Cellulose consists of a linear chain of β (1→4) linked glucan (polyglucose) known to be synthesised by the members of superfamily *Glycosyltransferase 2* (*GT2*) called *Cellulose synthase A* (*CesA*) (Fujii et al. 2010; Kumar et al. 2016). Twenty-two *CesA* genes have been reported in hexaploid wheat (Kaur et al. 2016). In addition to the *CesA* genes, the *Glycosylhydrolase 9* (*GH9*) family genes are known to have an impact on the synthesis of cellulose in plants (Kotake et al. 2011). Based on the mutant analysis in Arabidopsis, a member of *GH9* family called *KORRIGAN1* (*KOR1*) has been reported to be involved in cellulose synthesis, cell expansion and intracellular trafficking of cellulose synthase complex (CSCs) (Szyjanowicz et al. 2004; Lei et al. 2014; Vain et al. 2014). Investigation of *brittle culm 1* mutants in rice and *brittle stalk 2* mutant in maize revealed the association of COBRA-like proteins with the cellulose microfibrils (Ching et al. 2006). Involvement of *Sucrose synthase* (*SuSy*) in channelizing substrate to cellulose synthase has also been reported (Fujii et al. 2010). Similarly several other proteins affect cellulose synthesis, including *chitinase-like 1* (*CSI1*) (Sánchez-Rodríguez et al. 2012), *companion of cellulose synthase* (*CC*) (Endler et al. 2015), *tracheary element differentiation-related* (*TED*) 6 and 7 (Rejab et al. 2015).

The involvement of several genes for cellulose synthesis highlights the complexity of the process, which needs further nvestigation to better comprehend the underlying mechanism (Kotake et al. 2011). Also, the variation for the proportion of cellulose in cell wall among wheat varieties is not been well understood. This study was planned to identify the genomic regions affecting the variability of cellulose content among diverse spring wheat genotypes through GWAS.

Genes associated with cell wall have been previously explored through GWAS in miscanthus (Slavov et al. 2014), Populus (Porth et al. 2013) maize (Li et al. 2016) and barley (Houston et al. 2015). In the case of barley genes of *Glycosyltransferase 2* and *Glycosylhydrolase* families were found to be associated with culm cellulose variation. However, none of the genes found in maize through GWAS of stalk cellulose content was specifically involved in the cellulose biosynthesis pathway. In the present study, the stem internodes of 288 spring wheat varieties were analysed for variation in cellulose content. Utilizing the 21,073 SNPs generated by DArT-seq GBS and cellulosic content, GWAS was performed by the fixed and random model circulating probability unification (FarmCPU) method (Liu et al. 2016). Genes, which were not reported previously for their role in cellulose formation, were identified as associated with the culm cellulose content. Gene-trait associations identified in this study might be useful in altering the lignocellulose composition of wheat and other grasses at a genetic level.

5.2.1 Hypothesis Variability of culm cellulose content in diverse wheat genotypes is linked to specific genomic regions.

5.2.2 Objective I. Analysis of cellulose content for diverse wheat lines

5.2.3 Objective II. GWA Study to identify novel genes linked to cellulose content in wheat

5.3 Materials and methods

5.3.1 Plant material

A worldwide collection of 288 diverse spring growth-habit wheat germplasm was used for the phenotypic and genotypic analysis. The collection included cultivars from different regions of United states, the International Maize and Wheat Improvement Centre (CIMMYT), Mexico, and historical lines dating back to 1871 (Mohan et al. 2013). The wide span of our collection was

intended to capture the maximum variation possible while maintaining a manageable population size. This worldwide collection also represents the various market classes of wheat based on color, hardiness and shape of the kernel: i.e. soft white spring (SWS), soft red spring (SRS), hard red spring (HRS), hard white spring (HWS), and club wheat cultivars (Mohan et al. 2013). The plants were grown in the greenhouse of the Plant Growth Facilities, Washington State University, Pullman at 22°C/18°C day/night temperature with 16 hours of light in 2014-15. Seeds were planted with randomised design to accommodate the effect of light.

5.3.2 Phenotypic analysis

The analysis on percentage cellulose was performed for 288 diverse spring wheat genotypes, with three replicates per genotype. The first internode (from the base) of the main tiller of each mature plant was taken and dried at 80°C. Measured amount of dried sample (45-55 mg) was put into a pre-weighted 2 ml Eppendorf tubes with a screw cap. A mixture of acetic acid: water: nitric acid (8:2:1) was added to each tube (1.5ml) and vortexed (Appenzeller et al. 2004). All tubes were transferred to a steel rack and placed in a boiling water bath for four hours. After four hours, tubes were removed from the water bath and allowed to cool at room temperature. After the tubes reached room temperature they were placed in a swing-out rotor and centrifuged at 10,000 rpm for 10 minutes. The supernatant was aspirated off, washed with distilled water four times and finally washed with 90% ethanol. After each wash, the tubes were vortexed and centrifuged at 10,000 rpm for 10 minutes to aid in the formation of solid pellets. The caps were removed after the final wash and the tubes were placed in the oven for drying at 80°C. The final weight of the tubes was used to calculate the percent cellulose content on a dry matter basis using the formula:

% cellulose = Cellulose weight (final pellet dry weight)/Initial sample dry weight x100

5.3.3 Population structure and GWAS analysis

The population structure was represented by the first Principal Components (PCs) calculated from all the SNPs. The three PCs were fitted as covariates in both the fixed effect model and the mixed linear model to eliminate the non-genetic effect confounded with population structure. The two models were iterated until converge on the estimated QTNs (Lipka et al. 2012; Ahmad et al. 2015; Tang et al. 2016).

A total of 21,073 SNP markers were obtained by analysing genomic DNA with the Genotyping-By-Sequencing (GBS) based approach (Mohan et al. unpublished). In brief, genotyping was carried out at DArT Pyt Ltd in Canberra-Australia, using a combination of HiSeq 2000 (Illumina) next-generation sequencing with DArT-seq GBS technology (called DArTseq ™). This method follows two-step complexity reductions by using two enzymes, PstI/HpaII and PstI/HhaI, along-with TaqI restriction enzyme to eliminate subsets of PstI -HpaII and PstI-HhaI fragments, respectively. The polled barcoaded samples were run in a single lane on an Illumina Hiseq 2000 instrument for sequencing. A proprietary analytical pipeline developed by DArT Pyt Ltd was used to obtain the DArT score and SNP tables (http://www.diversityarrays.com/). GWAS was conducted using a recently developed method, FarmCPU (Fixed and Random Model Circulating Probability Unification) (Liu et al. 2016) in R version 2.15.3. The model controls both non-genetic effects that confound with population structure, and genetic effects that confound with genetic loci having no genetic linkage with the test SNPs.

The confounded genetic effects controlled by Quantitative Traits Nucleotides (QTNs) were estimated using an algorithm named SUPER (Settlement of MLM under Progressively Exclusive Relationship). The whole genome was divided into bins. Each bin was represented by the most

significant SNP within each bin. The bin size and significant threshold were optimized by using the restricted maximum likelihood (REML) in a mixed linear model with kinship among individual lines calculated from the candidate bins. The set of bins with the optimum REML were used as the estimated QTNs. The estimated QTNs were directly fitted as covariates for testing SNPs in a fixed effect model to control the genetic effects confounded to the test SNPs. A Manhattan plot was generated using the –log10(*p*) values for each SNP with 1% Bonferroni test threshold (Team 2014). The significance of the genome-wide association between SNP marker and cellulose content was tested at FDR p <0.001. 5.3.4 Gene annotation.

The SNPs containing sequences were mapped against wheat unigenes downloaded from the NCBI database. The significant SNPs with associated unigese were annoted using BLASTn with the International Wheat Genome Sequencing Consortium (IWGSC) (Mayer et al. 2014) reference Sequence v1.0 (https://www.wheatgenome.org) posted on May 30, 2017. The functions to associated unigenes were also searched in orthologs found in another species.

5.4 Results

5.4.1 Cellulose content

A set of 288 diverse wheat lines was analysed for native variation in cellulose content (Appendix 5.1). Significant differences in percent cellulose content of wheat lines on a dry matter basis were identified. The coefficient of variation for cellulose content is 6.56% among the wheat lines. The cellulose content of germplasm ranged from 0.32 to 0.52 mg cellulose/mg of dry weight with an average of 0.45 mg cellulose/mg of dry weight). The wheat population showed a trend of a normal

distribution with respect to the cellulose variation and the density plot for the cellulose analysis is shown (Fig 1).

5.4.2 Principal component analysis and marker-trait associations

Principal component analysis (PCA) was performed to investigate the population structure. The first two PCs explained 8.13 and 4.90% variation in the population. The collection showed two distinct clusters, a minor and a major one. To simplify the population structure, the minor cluster containing 20 genotypes was removed from the final analysis and first PC was used as covariate while conducting GWAS (Fig 2).

A total of 21073 SNP markers with minor allele frequency (MAF) above 5% and the cellulose content data from 268 lines were used for GWAS analysis (Fig 3). Using the GWAS analysis, we found nine significant marker-trait associations with *p* values of less than 1E-05. The most significant correlation in our analysis corresponded to wheat chromosome 5AL with *p*-value 1.86E-07. The second most significant SNP being on chromosome 1AL with a *p*-value of 2.24E-07. In addition, we found significant SNPs corresponding to chromosome 1AL, 6BS, 1DL, 2DS, 4DL, 5BL, and 3B with *p* values <1E-05 respectively (Table 1). The quantile–quantile (QQ) plot drawn for calculated *p*-values was used to check spurious associations. The deviation of relatively a few markers from null expectations in the QQ plot is evidence for significant associations to be present (Fig 5).

5.4.3 Gene identification

Significant SNP markers resulting from GWAS were mapped to the wheat unigene database, their corresponding unigene identified. These unigenes were used to provide the most likely annotation through the NCBI BLAST and EnsemblPlant database. The searches resulted in the identification

of genes corresponding to these hits. The first SNP marker was found to be on the gene TRIAE_CS42_5AL_TGACv1_376159_AA1232950 and the second SNP marker corresponded to a genomic region containing unigene gnl|UG|Ta#S52545076. The third and fourth significant SNPs corresponded to the genes TRIAE_CS42_2DS_TGACv1_179544_AA0607850 and TRIAE_CS42_3B_TGACv1_224721_AA0800650.1 respectively. The gene TRIAE_CS42_5AL_TGACv1_376159_AA1232950 is uncharacterized in wheat as well as other plant species. The unigene gnl|UG|Ta#S52545076 showed 60% amino acid identity and 85% coverage with a gene in the *Tubulin* superfamily, *Tubulin β-1 chain* of *Triticum urartu*. TRIAE_CS42_2DS_TGACv1_179544_AA0607850 showed 82% identity and 97% coverage with the Auxin-induced protein *5NG4* of *Aegilops tauschii*, whereas TRIAE_CS42_3B_TGACv1_224721_AA0800650.1 was annotated based on 51% amino acid identity and 97% coverage with a putative transmembrane protein of *Medicago truncatula* (Table 1).

5.5 Discussion

From a larger set of 288 diverse bread wheat lines, we used 268 well-structured accessions to describe the genetic association of cellulose content variation. The most appropriate model was selected to obtain a higher level of confidence in our association results. We employed GBS for genome-wide SNP genotyping and conducted a comprehensive phenotypic analysis for multiple replications of 288 diverse wheat lines, to capture the variability in cellulose content. Phenotypic data was then combined with genotypic screening to implement Genome Wide Association Studies (GWAS) using Fixed and Random Model Circulating Probability Unification (FarmCPU); a new and more efficient method has been recently published that accounts for fixed and random effects to control false positives (Liu et al. 2016). The fixed effects include testing

SNPs and population structure represented by the first three principal components calculated from all the SNPs. The random effects were the genetic effect of individuals lines with variance and co-variance structure defined by the kinship calculated from the estimated Quantitative Traits nucleotides (QTNs). Most of the GWA mapping studies in wheat has been employed for the identification of genes or QTLs related to agronomic performance (Lopes et al. 2015; Jaiswal et al. 2016), grain yield (Sukumaran et al. 2015), disease resistance (Kollers et al. 2013; Gurung et al. 2014). To our knowledge, this is the first GWAS analysis related to the natural variation of cellulose content in wheat. Cellulose is a key component of plant cell walls and involved in mechanical strength in plants (Appenzeller et al. 2004). It is well documented that the *CesA* genes are involved in the synthesis of cellulose and recently a total of 22 *CesA* genes have been reported in wheat which differentially expresses in primary and secondary cell wall (Kaur et al. 2016). We have identified two significant associations for cellulose content in spring wheat. Although there were approximately 9 SNP markers that were associated [-log10(*p*)=7 to -log10(*p*)=5] with cellulose content (Table S1), we were able to map only four of these to the wheat unigene database. Greater marker density and population size used here provides higher confidence about these hits (Wang et al. 2012b).

The corresponding genomic regions for the SNP markers showing significant association with stem cellulose content were explored and the gene annotations were derived from the EnsemblPlant database. The fact that these genes showed significant association with cellulose content suggests that they may play a role in controlling the natural variation of cellulose in wheat lines. The involvement of many genes other than *CesAs* in controlling cellulose synthesis provides the evidence for the complexity of the process (Kotake et al. 2011). But there are still some missing links to completely understand the complex mechanism of cellulose synthesis.

Only a few studies have been performed to explore the additional genes involved in the cellulose biosynthesis pathway (Porth et al. 2013; Slavov et al. 2014; Houston et al. 2015; Li et al. 2016). Recently a GWA study in barley, a species syntenic to wheat, showed the involvement of genes co-expressing with *CesA* genes in culm cellulose content variation. Cellulose content was analysed for 288 two-rowed and 288 six-rowed spring type barley accessions genotyped with 3072 SNPs. GWAS results showed the significant hits involving genes mainly from *Glycosyltransferase* and *Glycosylhydrolases* (Houston et al. 2015). Similar to barley GWAS hits, our results also showed the involvement of *GT* gene family. However, we have encountered some unique hits that were probably missing in barley study because of the lower number of SNP markers used for the analysis. The present study has shown statistical evidence for marker-trait associations, which will add to our present knowledge of cell wall genetic architecture.

Our results pointed to the involvement of *β-tubulin* in the regulation of cellulose content. *β-tubulins* are proteins that form heterodimers with *α-tubulins* to form microtubules. These microtubules showed a closed association with cellulose microfibril deposition and formation of the secondary cell wall (Rao et al. 2016). There are many studies that have shown the functional association of cortical microtubules with cellulose synthase complexes, most of which were studied in Arabidopsis (Paredez et al. 2006; Chan et al. 2007; Wightman and Turner 2008; Crowell et al. 2009; Gutierrez et al. 2009; Chan et al. 2010).

Another important hit in our analysis is the Auxin-induced protein *5NG4*. This gene is a member of the plant drug/metabolite exporter (P-DME) (TC 2.A.7.4) family, also called WALLS ARE THIN1 (WAT1)-related proteins. Mutant studies in Arabidopsis have revealed its involvement in secondary cell wall formation in fibres. Comparative transcriptomics and metabolomics demonstrate the synchronised downregulation of the secondary cell wall *CesAs*

(*CesA8, CesA7 and CesA4*) and auxin metabolism genes (auxin-responsive genes and auxin influx transporter genes) in *wat1* mutants (Ranocha et al. 2010). The RNA-seq expression profiling of Chinese fir (*Cunninghamia lanceolate*) has also revealed the higher expression of PIN-like auxin efflux carrier and auxin-induced protein *5NG4* genes in relation to both cell division and cell expansion (Qiu et al. 2013). Our results also indicated the possible involvement of an uncharacterized gene (TRIAE_CS42_5AL_TGACv1_376159_AA1232950) in cellulose biosynthesis. This gene can be further explored for its specific role in the cell wall synthesis. The last hit in our analysis is a putative transmembrane protein of unknown function. Functional validation of these novel identified associations will further strengthen our understanding of their biological role in cellulose content variation found in the wheat stems. Though we have not yet drawn conclusions regarding the differences in cellulose content between different varieties of same species, our results indicate that additional genes are likely involved in the mechanisms responsible for the cellulose content variation in diverse wheat varieties.

5.6 Conclusion

Cellulose content in the culms of bread wheat varies from 0.32 to 0.52 mg /mg dry weight) in bread a diverse set of 288 genotypes. Genome-wide association analysis of 21073 SNPs with cellulose content variation helped identify 4 *de novo* genetic associations, which have the potential as molecular markers for manipulating cellulose content in wheat with the goal of improving culm strength.

Fig 5.1 Density plot showing the percentage cellulose content among 288 diverse spring wheat accessions.

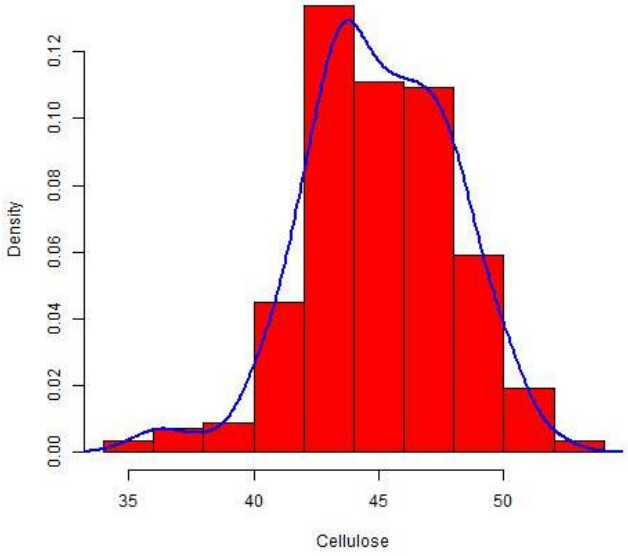

Fig 5.2 Principal component analysis of 288 diverse genotypes used for GWAS.

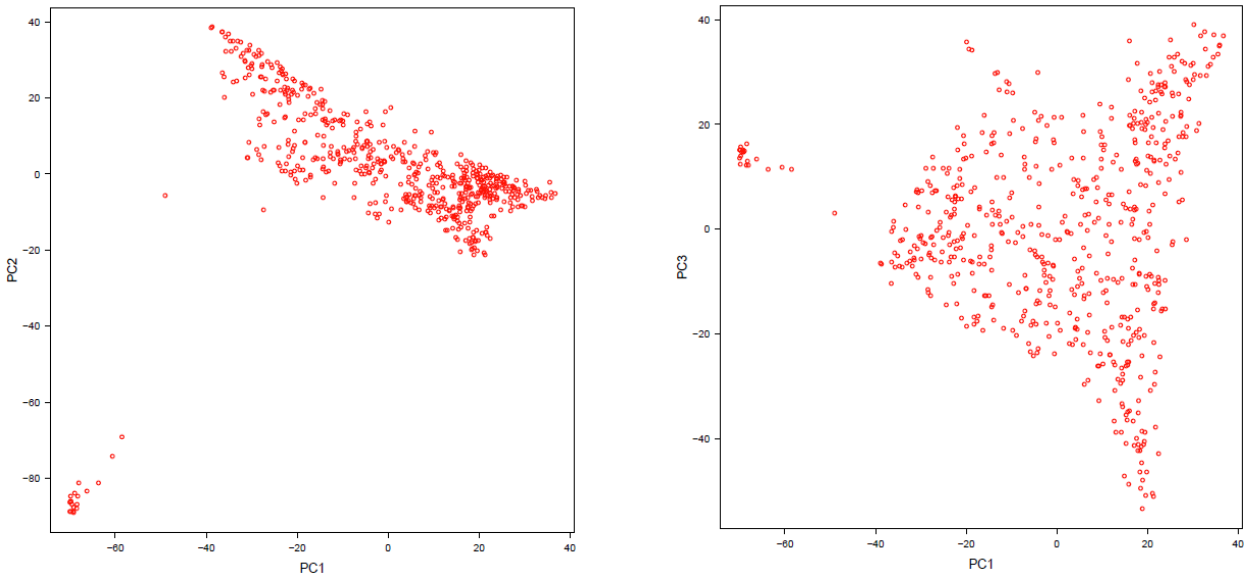

Fig 5.3 Minor allele frequency (MAF) patterns determined relative to allele calls for wheat genotypes based on 21073 SNPs.

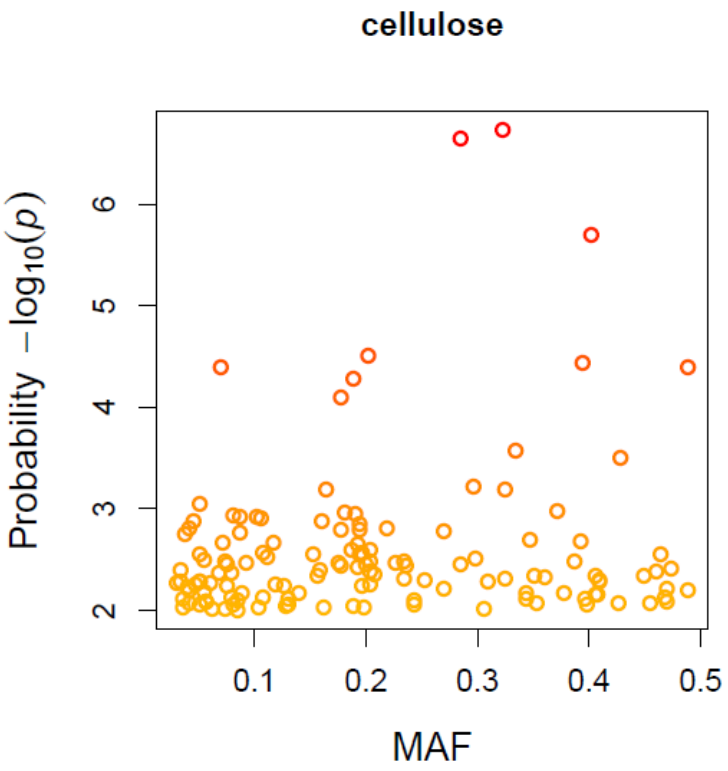

Fig 5.4 Manhattan plot of genome-wide association study (GWAS) on stem cellulose content (mg cellulose/mg dry weight) by using the FarmCPU. The -log10(*p*-values) from GWAS are plotted against the position on each of the 42 bread wheat chromosomes. U represents unassigned chromosome scaffolds. Two loci on chromosomes 1A and 5A were identified above the Bonferroni threshold correcting genome-wide multiple tests at type I error of 0.001 (green line).

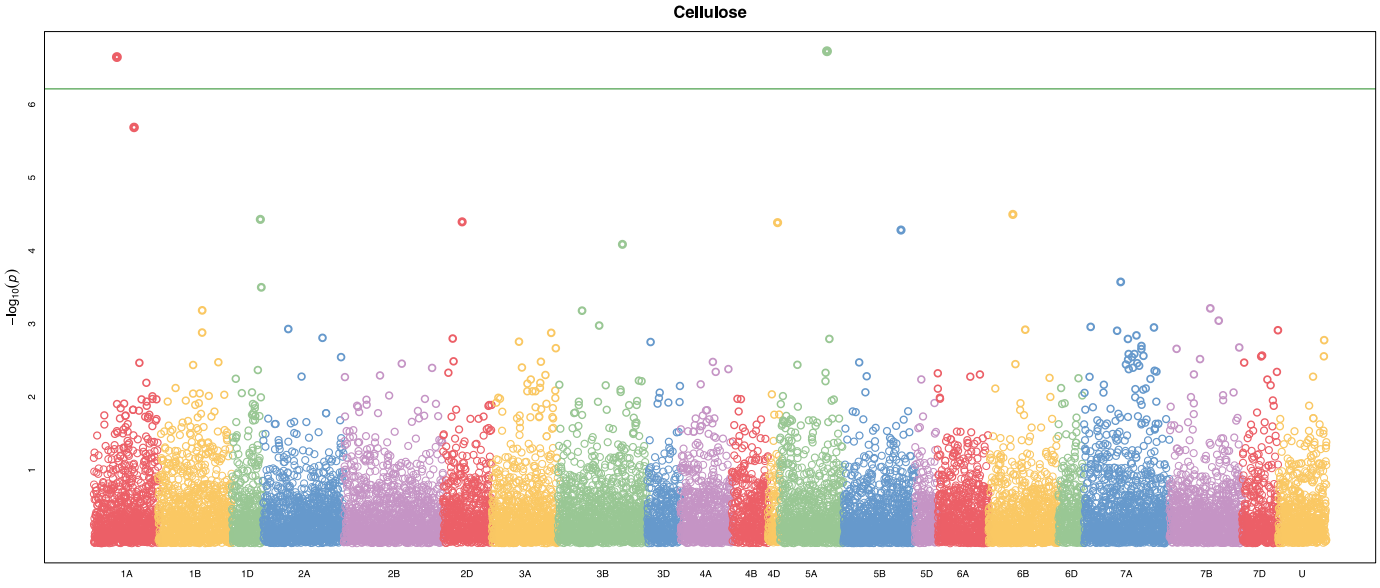

Fig 5.5 Quantile-quantile (QQ) plot showing the deviation from null hypothesis for associated SNP makers.

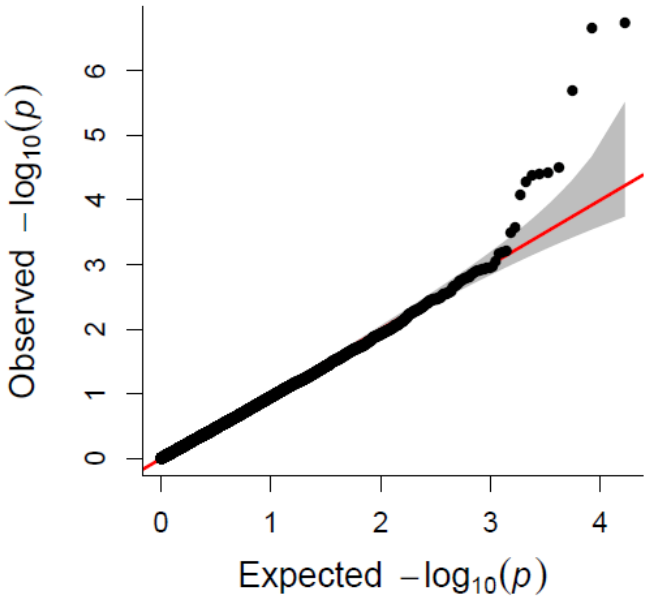

Table 5.1 Regions of wheat genome showing significant associations with stem cellulose content variation based on GWAS.

SNP ID	Allele	CHR	Scaffold:Position	P value	MAF	Unigene	Candidate annotation	Gene ID (Ensembl)
1096787|F|040	C>T	5AL	376159:25309	1.86E-07	0.323	gnl|UG|Ta#S13258805	Uncharacterized gene	TRIAE_CS42_5AL_TGACv1_376159_AA1232950S
1018641|F|062	T>C	1AL	138:45403	2.24E-07	0.285	N/A		
100315676|F|050	T>C	1AL	1074:43532	2.05E-06	0.402	gnl|UG|Ta#S52545076	*Tubulin β-1 chain*	TRIUR3_05395
1080815|F|044	T>C	6BS	514572:36113	3.18E-05	0.202	N/A		
3026141|F|05	A>C	1DL	63549:20036	3.72E-05	0.394	N/A		
1018617|F|035	C>T	2DS	179544:14866	4.02E-05	0.489	gnl|UG|Ta#S65598833	Auxin-induced protein *5NG4*	TRIAE_CS42_2DS_TGACv1_179544_AA0607850
1245047|F|039	C>T	4DL	344580:40916	4.12E-05	0.070	N/A		
1069330|F|06	T>A	5BL	406565:38744	5.21E-05	0.189	N/A		
2249069|F|014	G>A	3B	224721:15888	8.17E-05	0.177	gnl|UG|Ta#S61725485	Transmembrane protein, putative	TRIAE_CS42_3B_TGACv1_224721_AA0800650.1

Table 5.S1. Sequences of SNPs significantly associated with stem cellulose content variation.

SNP ID	Allele
1096787|F|040	CTTGCCACGACCGATTATCACCAACGACTGACAAGCCACGCCCCATTTTGGGCTGCCCTGCGCG
1018641|F|062	TCCAGCAACAAATGACTTGGTTGTATAGTCCGTAGGCACATCGGGAGTTGTTTCTTGTTGTAGT
100315676|F|050	CTCATGTCGTCTAGCACGTCGAACACCTCGGAGATGAGCCCCGTGTGGTCGGCGCTCGTCAGCT
1080815|F|044	CAGTTACACTAGAGAGTTGGATAAAAGCTTCTGCTATTTTCAAAGAAAATCGGTCACTTTGGAG
3026141|F|05	ACCGTGCGTGCCCGTGCACGTGTCCGTGCCGCCCGAGATCGGAAGAGCGGTTCAGCAGGAATGC
1018617|F|035	GATGCTCATGGTGATGGCTCCCCCCAGGCACAGAAGGGTCCCCACTATCTTGGCTCTTGTGTAC
1245047|F|039	GCAAGCTCTTGGGTTTCTTGGTTTCTAACAGAGGCATTGAAGCTAACCCGAGATCGGAAGAGCG
1069330|F|06	TTTTTCCAAAATTATGGTATTTTCTCTGCTTATAAAAAAGAACCCCCGACCTCTTTTTTAAAAC
2249069|F|014	CGTCCTCATGTGCGCGCTGCTCTACTTCCTCGACACCTCCGCGGACTACGCCAAGGGGATACAG

CONNECTING STATEMENT FOR CHAPTER VI

Chapter VI, entitled "Genome-wide analysis of the *Cellulose synthase-like* (*Csl*) gene family in bread wheat" authored by Simerjeet Kaur, Kanwarpal Dhugga, and Jaswinder Singh has been submitted to "*BMC Plant Biology*".

In chapters V, GWAS was performed for the identification novel genes controlling the cellulose content variation in diverse wheat genotypes. In addition to cellulose, hemicellulose is also an important component plant cell walls comprising roughly one-third of cell wall biomass. This is composed of several heteropolymers that interact with cellulose microfibrils through hydrogen bonds. Despite their major contribution towards the biomass and infrastructure cell walls, the synthesis of hemicelluloses is poorly understood in wheat. In this study, we have explored the *Cellulose synthase-like* (*Csl*) members, which have been known for the regulation/synthesis of hemicelluloses such as heteromannan, xyloglucan, heteroxylans, and mixed-linkage glucan. We have identified a total of 108 *Csl* genes using the gene family specific Pfam conserved domains. The classification of these genes based on phylogenetic analysis and tissue-specific expression has been discussed in chapter VI.

Chapter VI. Genome-wide analysis of the *Cellulose synthase-like (Csl)* gene family in bread wheat (*Triticum aestivum* L.)

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6.1 Abstract

Hemicelluloses are a diverse group of complex non-cellulosic polysaccharides, which constitute approximately one-third of the plant cell wall. Despite their extensive use as dietary fibres, food additives and raw materials for biofuels, genes involved in hemicellulose synthesis have not been extensively studied in small grain cereals. In this study, we have isolated the gene sequences for the *cellulose synthase-like* (*Csl*) family from wheat. A total of 108 genes (hereafter referred to as *TaCsl*) including two to three homoeologous copies for each were identified and named as *TaCslXY_ZA*, *TaCslXY_ZB*, or *TaCslXY_ZD*, where X denotes the subfamily, Y as the gene number and Z stands for chromosome number on the respective genomes of bread wheat. One-fourth of these genes had 2 to 3 splice variants, resulting in a total of 137 putative proteins. Close to 45% of *TaCsl* genes were found to be located on chromosomes 2 and 3. To gain insight into the potential functional role of this gene family, we performed *in silico* expression analysis in different tissues using a publically available dataset. Although most of the genes were expressed ubiquitously, some were tissue-specific. More than half of the genes had introns in phase 0, one-

third in phase 2, and a few in phase 1. This study provides new insights into the structure and function of the *Csl* gene family in hexaploid wheat.

6.2 Introduction

Non-cellulosic plant cell wall matrix polysaccharides generally referred to as hemicellulose, exhibit diverse linear or branched structures (Pauly and Keegstra 2008). These mainly encompass 1-4-β-glucan, 1,3;1,4-β-glucan, galactan, or glucomannan in grasses (Sorek et al. 2014). In addition, glucuronoarabinoxylan is a major grass well constituent. Because of the presence of heterogeneous substituents or other linkages in their polymer backbone, the structure of hemicellulose is non-crystalline and can be comparatively readily hydrolysed in comparison to cellulose. These polysaccharides can interact with cellulose chains through hydrogen bonds (Pauly et al. 2013).

Hemicellulosic polysaccharides in plants are made by *the cellulose synthase-like* (*Csl*) enzymes, which are members of a much larger superfamily of genes referred to as *glycosyltransferase 2* (*GT2*) (Richmond and Somerville 2000). These genes encoding these enzymes share sequence similarity with the *cellulose synthase A* (*CesA*) gene family known to form cellulose throughout the plant kingdom (Kaur et al. 2016). A variable number of *Csl* genes ranging from 30 to 50 have been identified from different plant species and are classified into nine subfamilies (*CslA–CslH* and *CslJ*) (Hazen et al. 2002). Cereals generally lack *CslB* and *CslG* families. Among the remaining families, *CslA*, *CslC*, and *CslD* are conserved in all land plants, whereas *CslF*, *CslH*, and *CslJ* are restricted to grasses (Farrokhi et al. 2006; Burton et al. 2011b). The subfamilies *CslB* and *CslG* were previously reported to be present only in dicots (Dhugga 2012). However, a recent study revealed the presence of *CslB* subfamily in monocots as well (Yin

et al. 2014). Diverse groups of *Csl* gene family have been reported to be involved in the biosynthesis of different cell wall polysaccharides. For example, subfamily *CslA* has been implicated in the biosynthesis of the β-1,4-mannan backbone of galactomannan and glucomannan (Dhugga et al. 2004a; Liepman et al. 2005). Similarly, *CslF* and *CslH* groups were reported to mediate the biosynthesis of 1-3;1-4-β-glucan in grasses (Burton et al. 2006b; Doblin et al. 2009) whereas *CslC* genes have been reported to be involved in the synthesis of the 1-4-β-glucan backbone of a xyloglucan and some other polysaccharides (Cocuron et al. 2007).

Wheat is a major cereal crop, which is grown on largest arable land in the world, is second only to maize in grain production, and feeds approximately 40% of the world population (Gupta et al. 2008). It has a large genome size (~17 Gb), of which ~80-90% is repetitive (Mayer et al. 2014). Because of its large genome size and hexaploid nature, *Csl* genes have not been well defined in wheat. Furthermore, the full genome sequence of bread wheat was not available until recently (Consortium 2014), which posed a challenge in exploring this complex gene family. Bread wheat possesses three homoeologous sets of seven chromosomes each distributed in three subgenomes (A, B and D). In general, homeologous copies of most of the genes are located on chromosomes of each genome. Moreover, *Csl* genes share a large sequence similarity with each other or within the subgroup, which makes it a challenging task to identify and characterise these genes in hexaploid wheat.

In the present study, we have explored the recently available resources to retrieve wheat genomic sequence. Comprehensive and large-scale data mining was performed using the Pfam domain models for the identification of *Csl* gene family in wheat. *TaCslD* has been studied in more detail for its gene structure and intron evolution, because of its evolutionary and structural

proximity to *CesA* genes and its probable role in cellulose or mannan synthesis (Verhertbruggen et al. 2011; Wang et al. 2011).

6.2.1 Hypothesis Orthologs of higher plants *Cellulose synthase-like* (*Csl*) genes are present one the A, B, D, homeolog genomes of bread wheat

6.2.2 Objective I. Identification of homeologus copies of *Csl* genes in wheat

6.2.3 Objective II. Phylogenetic and expression analysis of *Csl* genes

6.3 Materials and methods

6.3.1 Data sources and sequence retrieval

Wheat genome data was downloaded from the Ensembl Plants FTP server (ftp://ftp.ensemblgenomes.org/pub/current/plants/fasta/triticum_aestivum/), generated by the International Wheat Genome Sequencing Consortium (IWGSC) [29] and converted into a local BLAST database using the UNIX pipeline. BLAST analyses (BLASTN as well as BLASTP) were performed using the stand-alone command line version of NCBI (National Center for Biotechnology Information) blast 2.2.28+ (ftp://ftp.ncbi.nih.gov/blast/executables/LATEST/), released March 19, 2013. A query file was generated from Pfam domain models; PF00535 (*GT2*) domain and PF03552 (*Cellulose_synt*) downloaded from Pfam 30.0 June 2016 release (Finn et al. 2016). The sequences of splice variants were also retrieved from Ensembl Plants browser (http://plants.ensembl.org/Triticum_aestivum/Info/Index). Analysis of splice variants was conducted as described by Kim et al. (2007) (Kim et al. 2007b). Previously known *Csl* sequences from Arabidopsis, rice, and maize were downloaded from the Cell Wall Navigator database. For Brachypodium, sequences were retrieved from phytomine.

6.3.2 Blast searches for wheat homologs

All query files containing the two Pfam domain models (PF00535 and PF03552) were used to perform the BLASTn searches against the local blast database of bread wheat. All blast hits with E-value >1.0 were removed. Using cut-off E- value < 1.0, all previously known *CesA* genes were retrieved. After the compilation of all hits below the cut-off value, CD-hit program (Li and Godzik 2006) was used to get non-redundant sequences. The genes obtained were further filtered by confirming the presence of the conserved domains *Cellulose_synt/GT2* using a batch blast search at the CDD (conserved domain database) of NCBI. Homoeologous genes from each of the three genomes were named as *TaCslXA*, *TaCslXB* or *TaCslXD*, where *X* denotes the gene number and the last suffix stands for the respective genome. Alignment of the sequences of all newly identified wheat *Csl* genes is given in additional file 1.

6.3.3 Protein structure and motif/domain identification

Protein sequences were downloaded from the Ensembl Plants FTP server (ftp://ftp.ensemblgenomes.org/pub/current/plants/fasta/triticum_aestivum/), developed by the International Wheat Genome Sequencing Consortium (IWGSC) [29]. Multiple protein sequence alignments were performed using Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al. 2011). The resulting alignments were analysed for the presence of conserved catalytic motifs (DXD and D, D, QXXRW) of the *GT2* superfamily. The conserved patterns of aligned sequences were highlighted using the sequence manipulation suite: Color align conservation (http://www.bioinformatics.org/sms2/color_align_cons.html) (Stothard 2000). The conserved domains were predicted using CCD database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (Kaur et al. 2013; Marchler-Bauer et al.

2014). Due to the resemblance of *CslD* with *CesA* genes and its probable role in cellulose synthesis, we specifically focused on *TaCslD* subfamily. Gene structures and intron evolution of *TaCslD* were predicted using the gene structure display server 2.0 (http://gsds.cbi.pku.edu.cn/) via the genomic and cDNA sequences.

6.3.4 Evolutionary relationships of *Csl* genes

A total of 215 CSL proteins from Arabidopsis, Brachypodium, maize, rice and wheat were used to predict the phylogenetic history. The phylogeny of the *CslD* subfamily was also determined separately from these species. For phylogenetic analysis, the amino acid sequences of CSL proteins were aligned using the MUSCLE (Edgar 2004) and the evolutionary history was inferred using Neighbor-Joining methods (Saitou and Nei 1987). The tree was drawn to scale, with branch lengths being equivalent to the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed with a Poisson correction (Zuckerkandl and Pauling 1965) and are given as the number of amino acid substitutions per site. The rate of variation among sites was modelled with a gamma distribution (shape parameter = 1) and all positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). A file containing the FASTA sequences of 215 CSL proteins is provided as the text file S_1.

6.3.5 RNA-seq expression analysis

Publicly available RNA-seq data generated from hexaploid bread wheat (var. *Chinese spring*) was used to predict the expression of newly identified wheat *Csl* genes. The data was compiled from five different wheat tissues (leaf, spike, root, grain, and stem) collected at seedling, vegetative and

reproductive stages of development (Choulet et al. 2014a). The relative expression of each *TaCsl* subfamily was presented as a heat map generated from transcript per 10 million reads for each gene using wheat expression browser powered by expVIP (http://www.wheat-expression.com).

6.4. Results

6.4.1 Identification and classification of *Csl* gene family in bread wheat

Our search resulted in the identification of 108 cellulose synthase-like (*TaCsl*) genes from bread wheat using the conserved domain models PF00535 and PF03552, which are specific to the *GT2* superfamily. These genes include 2-3 homoeologous copies of each gene from the A, B and D genomes. To characterize the newly identified genes, a phylogenetic tree was constructed using multiple sequence alignments of full-length derived protein sequences from Arabidopsis, Brachypodium, maize, rice and wheat. An unrooted neighbor-joining (NJ) tree for the 215 *Csl* genes from these species is shown in Fig 1. *TaCsl* genes were grouped into seven subfamilies including *TaCslA* (32 genes*), TaCslC* (13 genes), *TaCslD* (12 genes), *TaCslE* (10 genes), *TaCslF* (29 genes), *TaCslH* (8 genes), and *TaCslJ* (4 genes) (Fig 2). The *TaCslA* and *TaCslC* sub-families were closely related as shown by their taxonomic distribution and phylogenies. These subfamilies were found to be highly conserved in all the land plants analysed in this study. A strong resemblance between *TaCslF* and *TaCslD* was observed, although *TaCslF* is specific only to grasses and *TaCslD* is present in all plants (Yin et al. 2014). Subfamilies *TaCslE*, *TaCslH*, and *TaCslJ* were phylogenetically diverse, however, *TaCslJ* was found to be closer to *TaCslA* and *TaCslC* subfamilies. The identified genes were named following the nomenclature of rice, which shares synteny with wheat. To avoid the complexity of the nomenclature, a suffix corresponding to the chromosome number and the specific wheat genome identifier (A, B, or D) have been used

for each gene name. For example, the first gene of subfamily *CslA*; *CslA1*, on the long arm of chromosome 1 of genomes A, B, and D is named as *TaCslA1_1AL*, *TaCslA1_1BL*, and *TaCslA1_1DL*, respectively.

6.4.2 Splice variants of *Csl* genes

Among *Csl* genes, 22 genes appeared to encode two or more proteins because of the presence of alternative splicing sites, as predicted by Ensembl database, which resulted in a total of 137 probable *Csl* protein products. Splice variants were discovered in all subfamilies of the *TaCsl* genes except *TaCslD* (Table 2). In subfamily *TaCslA*, 6 genes alternatively spliced to form 13 proteins whereas, in subfamily *TaCslC*, 5 genes were alternatively splicing resulting in 14 proteins. Similarly, for subfamilies *TaCslE* and *TaCslF*, alternate splicing resulted in 7 and 10 splice variants respectively. Similarly, alternative splicing of 1 and 2 genes respectively generated 3 and 4 proteins in the *CslH* and *CslJ* subfamilies (Fig 2). Of all the splice variants, 51% stemmed from the exon skipping, ~24% from the selection of alternative 5′ and 3′ splice sites and the rest, ~24%, from intron retention (Table 2).

6.4.3 Conserved motifs and domains

All predicted TaCSL proteins contain either the Pfam *glycosyltransferase family 2_3* (GT) domain (PF13641) or the *cellulose_synt* domain (PF03552). Subfamilies *TaCslA* and *TaCslC* contained the *GT 2_3* and *CslD, CslE, CslF, CslH, CslJ* subfamilies contained the *cellulose_synt* domain (Fig 2). All *TaCsl* genes possessed the motifs D, D, DXD and QXXRW except eight truncated genes that possessed either of these four motifs (*TaCslA7_2DS, TaCslD4_1BS, TaCslD4_5BS, TaCslF2_7BL, TaCslF6_7AL, TaCslF6_7DL, TaCslH3_3AS, TaCslH2_3B*). The motifs DXD and

QXXRW were diverse in different subfamilies of *Csl* genes, such as for *TaCslA* (DMD, QQH/FRW); *TaCslC* (DMD, QQHRW); *TaCslD* (DCD, QVLRW); *TaCslE* (DCD, QHKRW); *TaCslF* (DC/GD, QI/VL/VRW); *TaCslH* (DCD QF/YKRW); *TaCslJ* (DCD, QNKRW). These motifs are highlighted in alignment files in the Appendix 6.

6.4.4 Phylogenetic analysis of the *CslD* subfamily

The evolutionary history of the *CslD* subfamily from Arabidopsis, Brachypodium, rice, maize and wheat was inferred using the Neighbor-Joining method (Saitou and Nei 1987), in MEGA6 (Tamura et al. 2013) and the orthologs from various species were grouped into different clades (Fig 3). This was based on the rice *Csl* genes because complete nomenclature of rice genes is well documented. All the genes were divided into three clades. The first clade contained *CslD2* and *CslD1* genes from rice and their orthologs from different species. The tree homoeologous genes of wheat branched together with *OsCslD1*, wheat genes under this clade were named *TaCslD1_1AL*, *TaCslD1_1BL*, and *TaCslD1_1DL* from each of the 1AL, 1BL, and 1DL genomes. The second clade was branched into two subgroups containing the orthologs of rice genes *CslD3* and *CslD5* from different species. First subgroup of wheat genes were designated as *TaCslD3_2AS*, *TaCslD3_2BS*, and *TaCslD3_2DS*. The genes of the second subgroup were named *TaCslD5_7AL*, *TaCslD5_7BL*, and *TaCslD5_7DL*. The last clade was composed of the orthologs of the rice *CslD4* genes and wheat genes, named *TaCslD4_5BS*, *TaCslD4_1BS* and *TaCslD4_5DS*. Here we found only two homoeologs of *TaCslD4*, but a gene from the 1BS genome (*TaCslD4_1BS*) of wheat grouped together with *TaCslD4* genes (bootstrap = 100) (Table 1). This gene shared sequence identity of 85% with *TaCslD4_5BS* at amino acid level. *OsCslD* genes shared 73-86 % sequence identity with the corresponding wheat orthologs.

6.4.5 Gene structure and intron evolution of *TaCslD* subfamily

A total of 12 *TaCslD* genes were found in bread wheat. The length of *CslD* subfamily genes ranged from 1519-5864 bp. The *TaCslD4_1BS* gene was the shortest and *TaCslD1_1AL* was the longest. Homoeologous copies of all genes shared sequence identity ranging from 87-94% at the genomic scale. The variation in size among different genes was primarily due to the number and length of introns (Fig 4). Intron number in all the genes varied from 2 to 4. Two homoeologs: *TaCslD1_1AL* and *TaCslD1_1BL* each had three introns, however, a third homoeolog (*TaCslD1_1DL*) had four. Genes *TaCslD3*, *TaCslD4* and their homoeologs had three introns each, except *TaCslD4_1B*S with only two introns. *TaCslD5* and its homoeologs also had two introns each. Here we have predicted three different phases of intron evolution as 0, 1, or 2; referring to the insertion of an intron between two consecutive codons, between the first and the second base or second and the third base of a codon, respectively (Dhaliwal et al. 2014; Kaur et al. 2016). Genes from the *TaCslD* subfamily exhibited variable patterns of intron phase distribution. Introns 1, 2 and 3 of *TaCslD1_1AL*, *TaCslD1_1BL* and *TaCslD1_1DL* had 2, 0, and 0 phase distribution, the 4th intron of *TaCslD1_1DL* had a phase distribution of 0. Introns 1 and 2 of *TaCslD3_2AS*, *TaCslD3_2BS* and *TaCslD3_2DS* both had phase distribution of 0. The t*hird intron of* these genes was in phase 2, 1 and 2 respectively. Genes *TaCslD4_5BS*, *TaCslD4_5DS*, *TaCslD5_7AL*, *TaCslD5_7BL* and *TaCslD5_7DL* had introns 1 and 2 in phase 2 and 0 and the third intron of *TaCslD4_5BS* and *TaCslD4_5DS* were in phase 0 and 2, respectively. *TaCslD4_1BS* had introns 1 and 2 in phases 1 and 0. Among all the studied genes, the largest proportion of introns (60%) was found to be in phase 0, followed by phase 2 (33.3%) with very few in phase 1 (6%).

6.4.6 RNA-seq expression analysis of *TaCsl* genes from bread wheat

Publicly available RNA-Seq datasets were used to analyse the expression of *TaCsl* genes over three developmental stages different tissues of wheat including root, leaf, stem, spike, and grain. In the case of *TaCslA* genes, we have retrieved the expression of 32 *TaCslA* genes excluding splice variants. Two genes (*TaCslA1_6AS* and *TaCslA1_6BS*) were expressed in all the tissues except reproductive stem and leaves. Four genes (*TaCslA5_2BS*, *TaCslA5_2DS*, *TaCslA6_3B*, and *TaCslA6_3AL*) were expressed moderately. *TaCslA9* gene revealed exceptionally higher expression in reproductive leaf tissue while the transcript abundance of the remaining genes was very low (Fig 5A). The 13 genes of the *TaCslC* subfamily were expressed highly in root and spike tissues. Two genes, *TaCslC1* and *TaCslC7* and their homoeologs displayed moderate to higher expression in all the tissues at seeding and vegetative stage. One gene (*TaCslC10_5DL*) exhibited moderate to high expression levels in all the tissues studied except reproductive stem and grain tissues (Fig 5B). Most of the genes of *TaCslD* subfamily revealed moderate to a high expression level in spike and root tissues and their expression was very low in all other tissues (Fig 5C). Three of the ten *TaCslE* subfamily genes (*TaCslE2_6AL*, *TaCslE2_6BL* and *TaCslE3*) showed moderate to a high expression in all tissues.The remaining genes were expressed at a very low level in all tissues (Fig 5D). A mixed pattern of expression was observed in the large *TaCslF* subfamily. Three genes (*TaCslF6_7AL*, *TaCslF6_7BL* and *TaCslF6_7DL*) demonstrated higher expression in all the tissues except leaves at reproductive stage. Two genes (*TaCslF4_2BS* and *TaCslF4_2DS*) indicated higher expression in stem tissues, while low to moderate in all other tissues. All other genes revealed low to moderate expression in one or more tissues (Fig 5E). In the *TaCslH* subfamily, one out of eight genes (*TaCslH1_2BL*) showed moderate to high expression levels in leaves, stem and spike tissues. The remaining genes also unveiled low to moderate expression in

all the tissues (Fig 5F). Three out of four members of the subfamily *TaCslJ* possessed low to moderate expression levels in leaves and root tissues while one gene (*TaCslJ1_3DS*) was poorly expressed in all the tissues (Fig 5G).

6.5 Discussion

The grass cell walls are composed of about 20-40% non-cellulosic polysaccharides, while the amount and composition of these polysaccharides vary widely in different plant species (Saxena and Brown 1995). Several genes of the *Csl* family have been reported to encode the corresponding enzymatic proteins hemicellulose synthesis (Liepman et al. 2005; Burton et al. 2006a; Cocuron et al. 2007; Doblin et al. 2009; Goubet et al. 2009; Yin et al. 2009; Wang et al. 2010b). As a detailed understanding of the identity of *Csl* genes in wheat was lacking, thus we undertook this study to fill this gap in wheat cell wall formation.

We retrieved 108 *TaCsl* genes from wheat using two conserved domains, PF00535, and PF03552, which were previously shown to be present in the derived proteins of all the *Csl* genes (Yin et al. 2014). Around a quarter of the identified *Csl* genes were alternatively spliced, resulting in 29 splice variants. A recent study revealed that the alternative splicing is common in plants and accounts for about 20% of the loci transcribed in the leaf and spike tissues of *Aegilops tauschii* (Iehisa et al. 2017). This phenomenon is apparently meant to increase the diversity of gene products to increase the fitness of an organism (Zhou et al. 2003).

Physical mapping revealed the distribution of *TaCsl* genes on all wheat chromosomes except one, chromosome 4 (Fig S1). A similar trend of *Csl* gene distribution was observed in barley (Burton et al. 2008; Schreiber et al. 2014a; Schwerdt et al. 2015). More than half the *TaCsl* genes were located on chromosomes 2 (32%) and chromosome 3 (22%). These two chromosomes appear to be *TaCsl* hotspots and can be targeted in breeding efforts for altering cell wall composition. Five

of nine *CslF* genes in barley were located on chromosome 2H. A similar cluster of *CslF* genes was also detected in the conserved syntenic regions of Brachypodium, rice and sorghum on chromosomes 1, 7 and 2, respectively (Schwerdt et al. 2015).

In silico expression analyses across different tissues suggested that of the 32 genes from the subfamily *CslA* only half or so were expressed at varying levels. Moreover, there was no commonality seen between these genes based on their transcript abundance, as different genes of the same subfamily express differently in the root, leaf, stem, spike, and grain tissues during vegetative and reproductive growth stages. Reverse genetic and biochemical approaches in *Arabidopsis* have associated the *CslA* genes with glucomannan biosynthesis (Goubet et al. 2009).

In the case of subfamilies *TaCslC* and *TaCslD*, most of the genes showed relatively higher expression levels in root and spike tissues during the vegetative as well as reproductive phases. Heterologous expression studies in the case of *Pichia* revealed that the *CslC* genes are involved in the synthesis of the 1-4-β-glucan backbone of the xyloglucan and some other polysaccharides (Cocuron et al. 2007). Of all *Csl* genes, the *CslD* subfamily is conserved in all land plants and most closely related to the *CesA* gene family, between 40-50% amino acid sequence similarity (Doblin et al. 2001) Similar to *CesAs*, the *CslD* subfamily is ubiquitous in all plant genomes examined to date, unlike other, taxa-specific *Csl* subfamilies (Hunter et al. 2012). Previous reports also showed the involvement of certain members of the *CslD* subfamily in tip growth, development of root hairs (Kim et al. 2007a; Yuo et al. 2011), normal plant growth (Li et al. 2009; Hunter et al. 2012), pollen tube growth, and meristem morphology and architecture (Bernal et al. 2007; Li et al. 2009). More recently, their role in resistance against biotic stresses has been described (Douchkov et al. 2016). Adding to this discussion, our *in silico* expression analysis sheds light on the involvement of certain *TaCslD* genes during spike development. These results are relevant to the

reduction in the number and width of spikelets shown by mutant *slender leaf 1 (sle1)* that encodes the rice CSLD4 protein (Yoshikawa et al. 2013).

Two groups of *Csl* genes, *CslF* and *CslH,* have evolved independently in grasses (Burton et al. 2011a). A third group *CslJ* had been recently identified as grass specific (Farrokhi et al. 2006). Although *TaCslF6* gene showed higher expression in all the studied tissues except reproductive leaf tissue, it was the only member of the *TaCslF* subfamily which expressed highly in grain tissue. Several studies have demonstrated the functional role of *CslF6* and *CslH* in the synthesis of (1-3), (1-4) β-glucan (mixed-linked glucan or MLG) (Doblin et al. 2009; Nemeth et al. 2010; Taketa et al. 2012; Schreiber et al. 2014a). Of all the genes in these families, only *CslF6* was expressed in the grain, suggesting that it was responsible for MLG formation. MLG is a desirable polysaccharide as a dietary fiber but undesirable for the brewery industry. It should be possible to select natural variants for the expression of the *CslF6* gene to select for an increased or reduced MLG content depending upon the target market for the grain.

Differential expression patterns were observed among homoeologous copies from three different genomes of bread wheat, which agree with the studies reporting the unequal contributions of the three genomes towards the gene expression (Mochida et al. 2004; Hu et al. 2013; Tanaka et al. 2015). Interestingly, the homoeologous copies of *TaCslD* genes also differed from each other in terms of intron phase evolution; indicating structural and functional divergence of homoeologous gene copies (Fig 4). The three homoeologs of each gene were not observed for all genes identified here. This could be because of the incomplete sequencing information or because of the silencing of the genes during the evolution of allohexaploid wheat (Bottley et al. 2006; Aramrak et al. 2015; Jordan et al. 2015).

6.6 Conclusion

We have identified 108 *TaCsl* genes in bread wheat and classified them into seven subfamilies (*CslA, CslC, CslD, CslE, CslF, CslH, CslJ*). In most cases, two to three homoeologs of each gene were identified as was expected for a hexaploid crop like bread wheat. These genes were located on all the wheat chromosomes except chromosome 4, whereas chromosome 2 and 3 contained approximately half of all the *Csl* genes. Only of the homeoalleles of a single *CslF* gene, *CslF6* were expressed in the grain, suggesting its key role in mixed-linked glucan formation. Neither *CslJ* or *CslH* were expressed in the grain. Information in this report will be helpful in designing experiments to alter wall composition in wheat for various purposes.

Fig 6.1 An unrooted phylogenetic tree representing the *Cellulose synthase-like* gene family from Arabidopsis, maize, rice and wheat using MEGA6. Tree was constructed using Neighbour joining (NJ) method with 100 bootstrap value. Different colors represent the subfamilies with orthologous CSL proteins from different species. The bar provides a scale for the branch length in the horizontal dimension. The line segment with the number '0.5' means that an equal length of the branch between the CSL proteins represents a change of 0.5 AA.

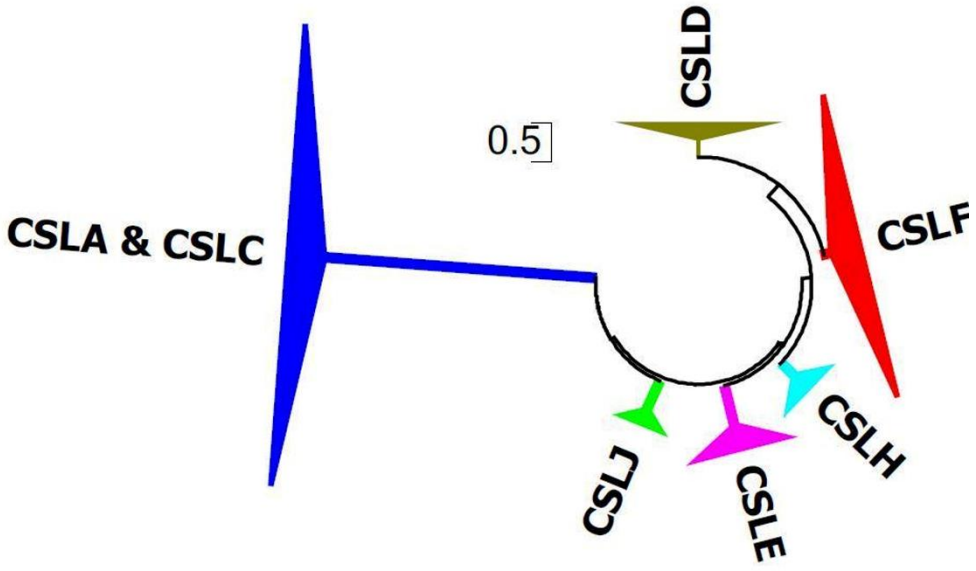


Fig 6.2 Distribution of *TaCsl* genes and their splice variants in seven subfamilies and their corresponding pfam domains used to identify *TaCsl* gene family.

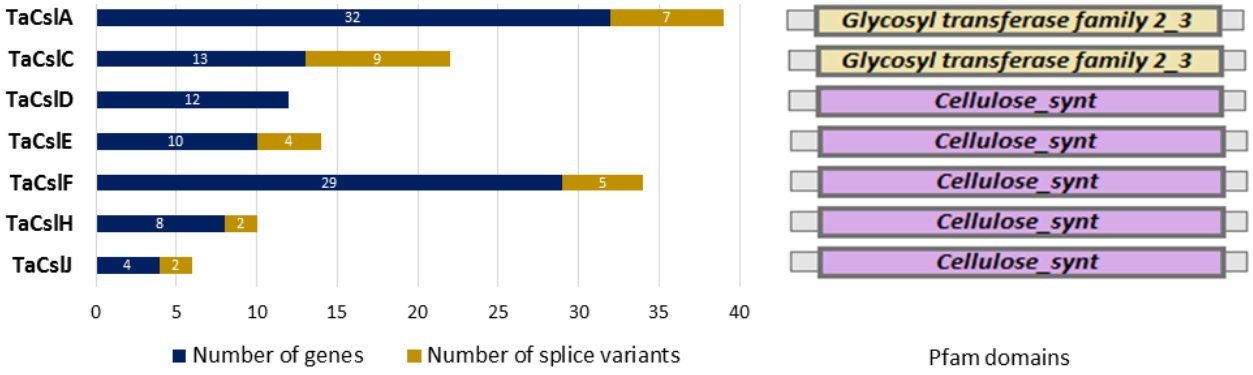


Fig 6.3 An unrooted phylogenetic tree representing the *CslD* subfamily from Arabidopsis, Brachypodium, maize, rice and wheat using MEGA6. Tree was constructed using Neighbour joining (NJ) method with 100 bootstrap value. Different colors represent orthologous *Csl* genes from different species. Arabidopsis-blue, Brachypodium-purple, maize-sky blue, rice-green, wheat-red.

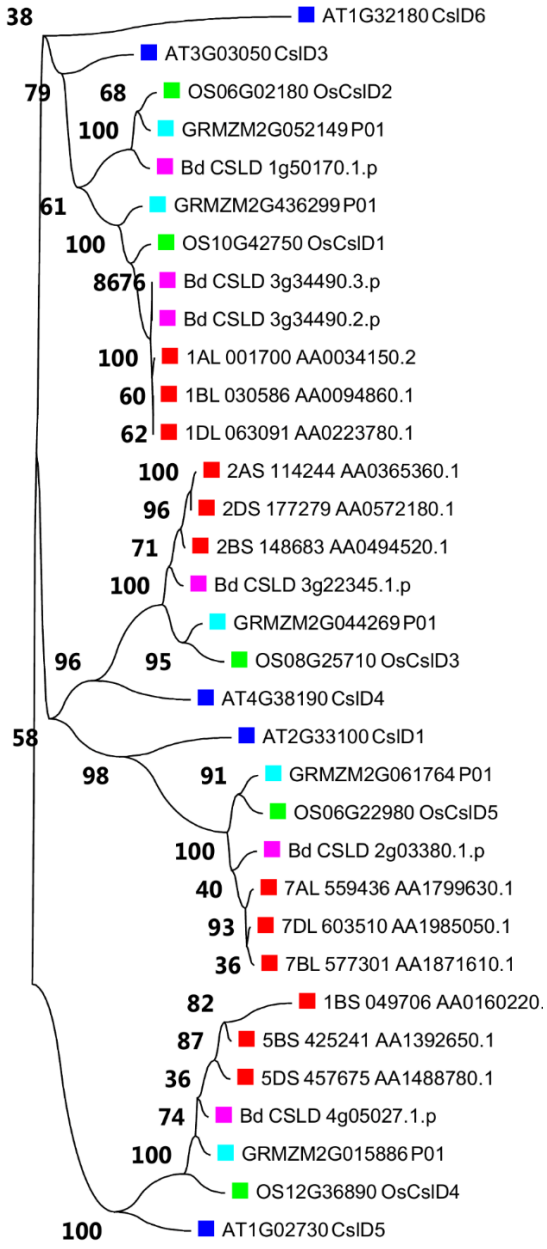


Fig 6.4 Structural features and phases of intron evolution of the *CslD* subfamily genes. Drawn to scale, exons are represented by red boxes and introns by back lines. Corresponding phases of intron evolution (0, 1, and 2) for the CslD genes are shown on the top of the black lines.

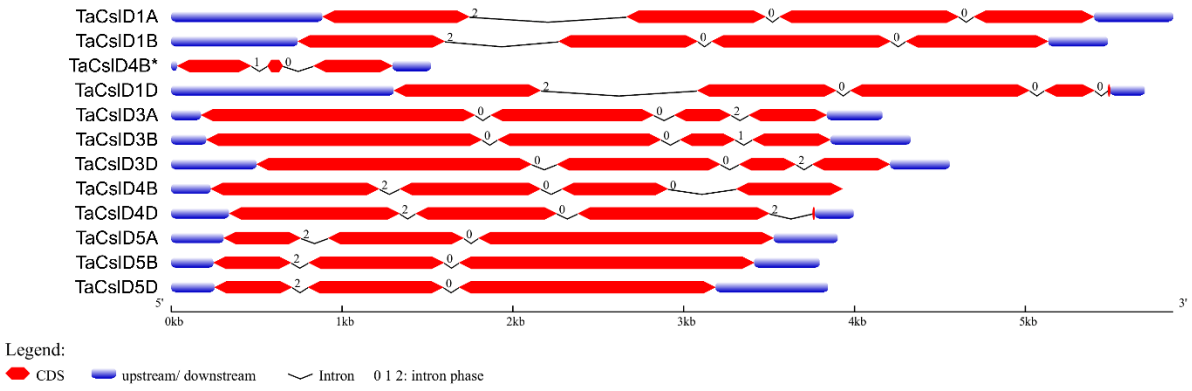
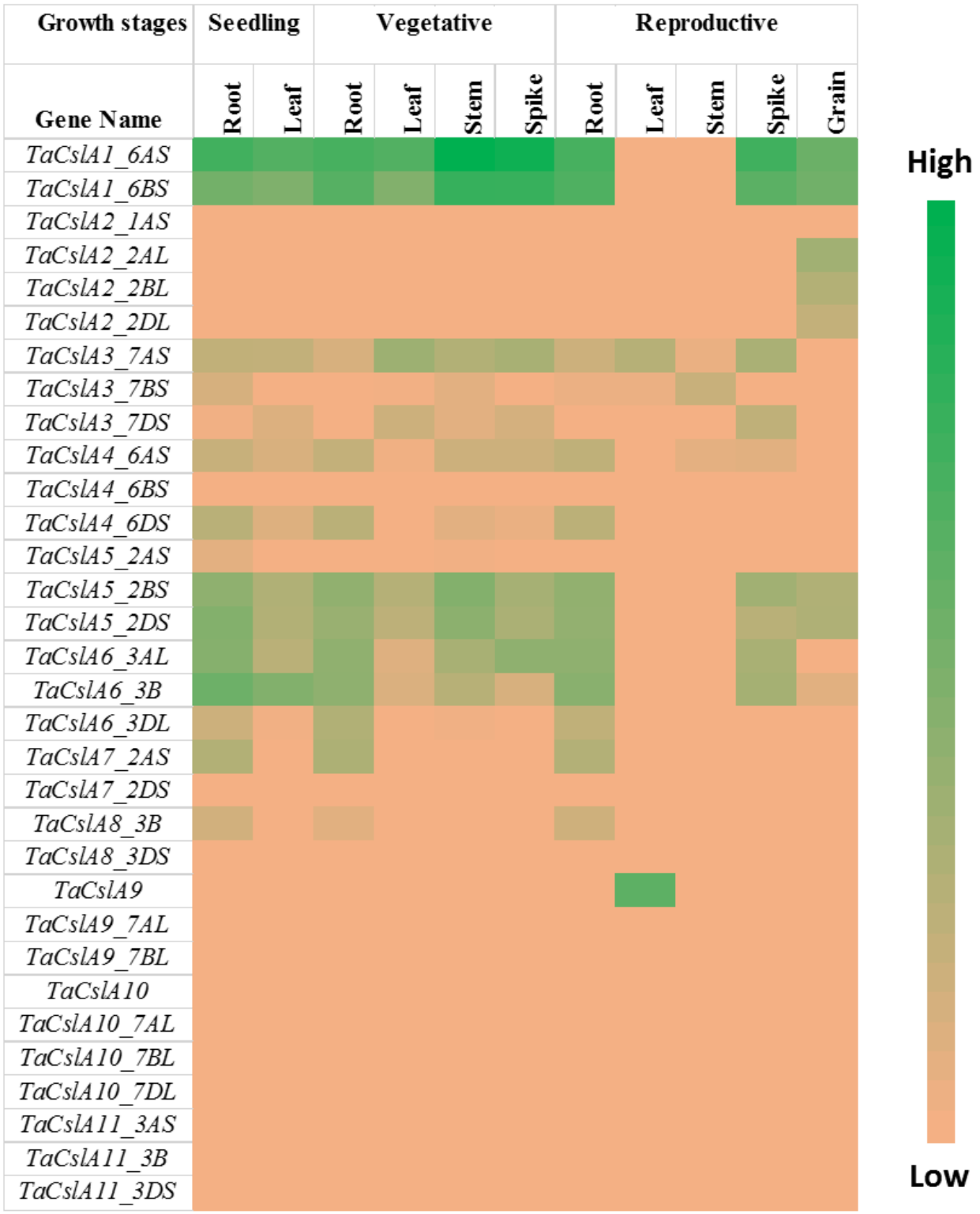


Fig 6.5 Heat map showing the expression profiling of wheat *Cellulose synthase-like* (*TaCsl*) genes at seedling, vegetative and reproductive stages. (A) *CslA* (B) *CslC* (C) *CslD* (D) *CslE* (E) *CslF* (F) *CslH* & *CslJ*. RNA-seq data from root, leaf, stem, spike and grain, of Chinese spring cultivar. The respective transcripts per 10 million values were used to construct heat map with scale bar showing expression of the genes.

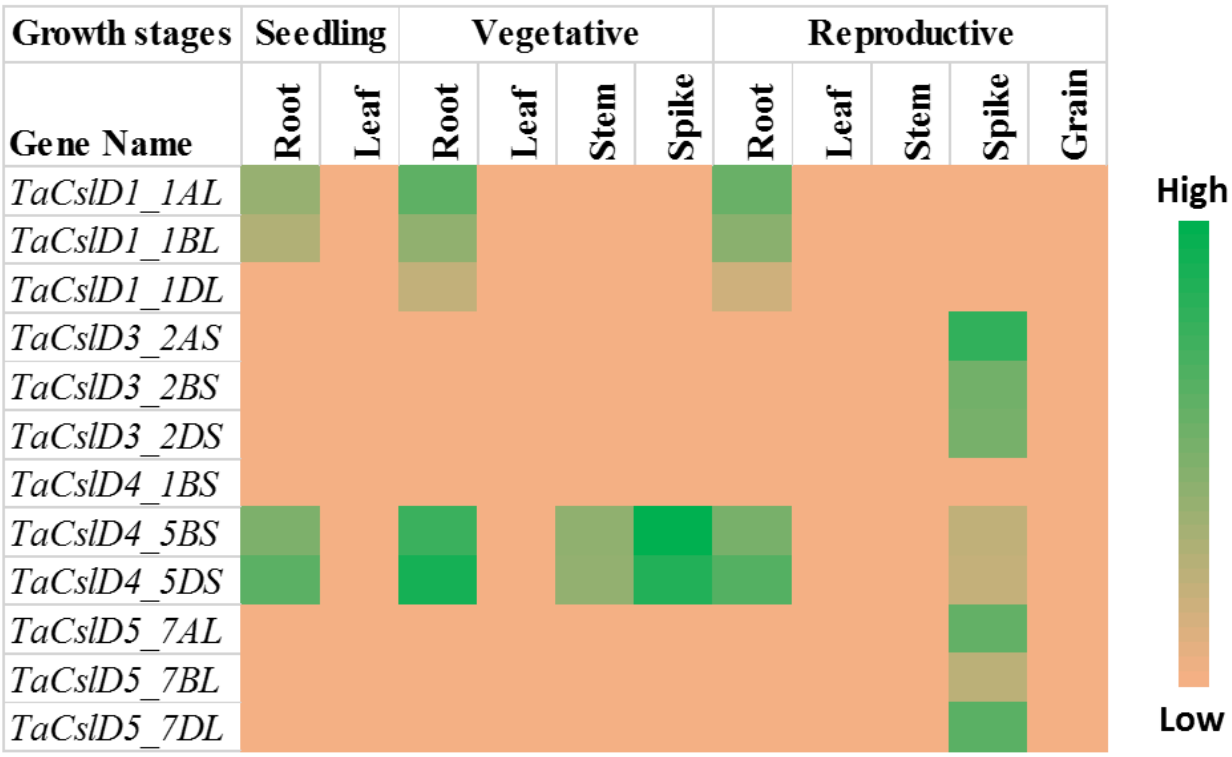
6.5A


Growth stages
Seedling
Vegetative
Reproductive
Gene Name
Root
Leaf
Root
Leaf
Stem
Spike
Root
Leaf
Stem
Spike
Grain
TaCslA1_6AS
TaCslA1_6BS
TaCslA2_1AS
TaCslA2_2AL
TaCslA2_2BL
TaCslA2_2DL
TaCslA3_7AS
TaCslA3_7BS
TaCslA3_7DS
TaCslA4_6AS
TaCslA4_6BS
TaCslA4_6DS
TaCslA5_2AS
TaCslA5_2BS
TaCslA5_2DS
TaCslA6_3AL
TaCslA6_3B
TaCslA6_3DL
TaCslA7_2AS
TaCslA7_2DS
TaCslA8_3B
TaCslA8_3DS
TaCslA9
TaCslA9_7AL
TaCslA9_7BL
TaCslA10
TaCslA10_7AL
TaCslA10_7BL
TaCslA10_7DL
TaCslA11_3AS
TaCslA11_3B
TaCslA11_3DS
High
Low

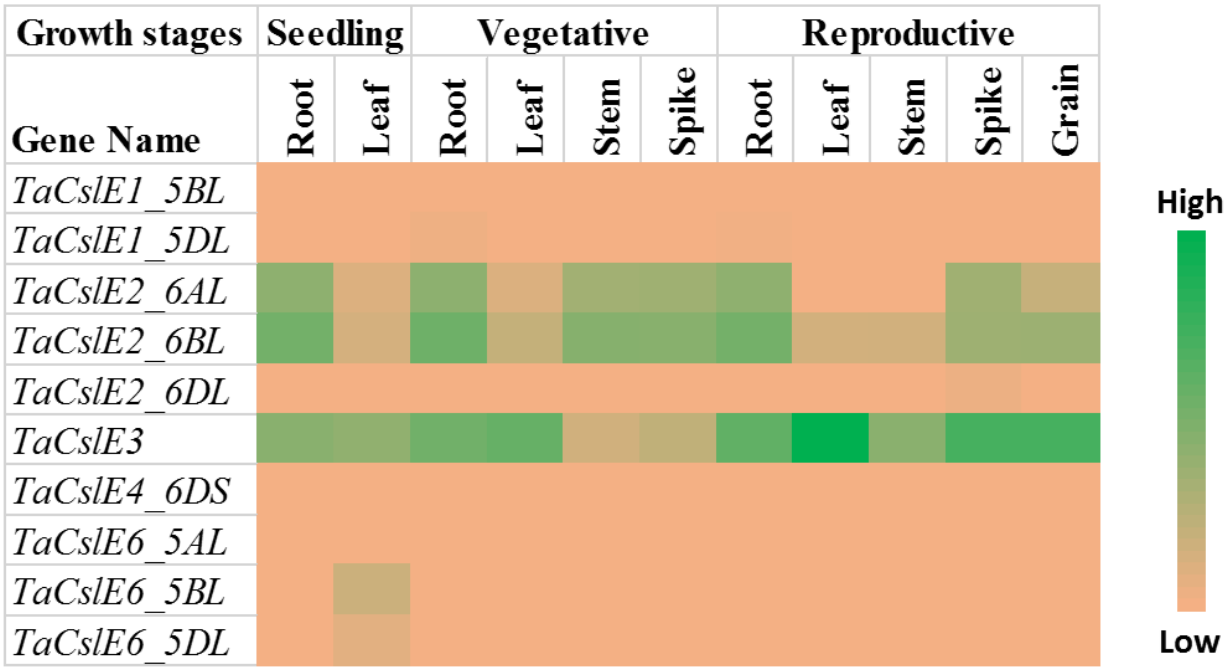
6.5B

Growth stages	Seedling		Vegetative				Reproductive				
Gene Name	Root	Leaf	Root	Leaf	Stem	Spike	Root	Leaf	Stem	Spike	Grain
TaCslC1_3AL											
TaCslC1_3DL											
TaCslC3_3AS											
TaCslC3_3B											
TaCslC3_3DS											
TaCslC7_1AL											
TaCslC7_1BL											
TaCslC7_1DL											
TaCslC9_1BL											
TaCslC9_1DL											
TaCslC10_5AL											
TaCslC10_5BL											
TaCslC10_5DL											

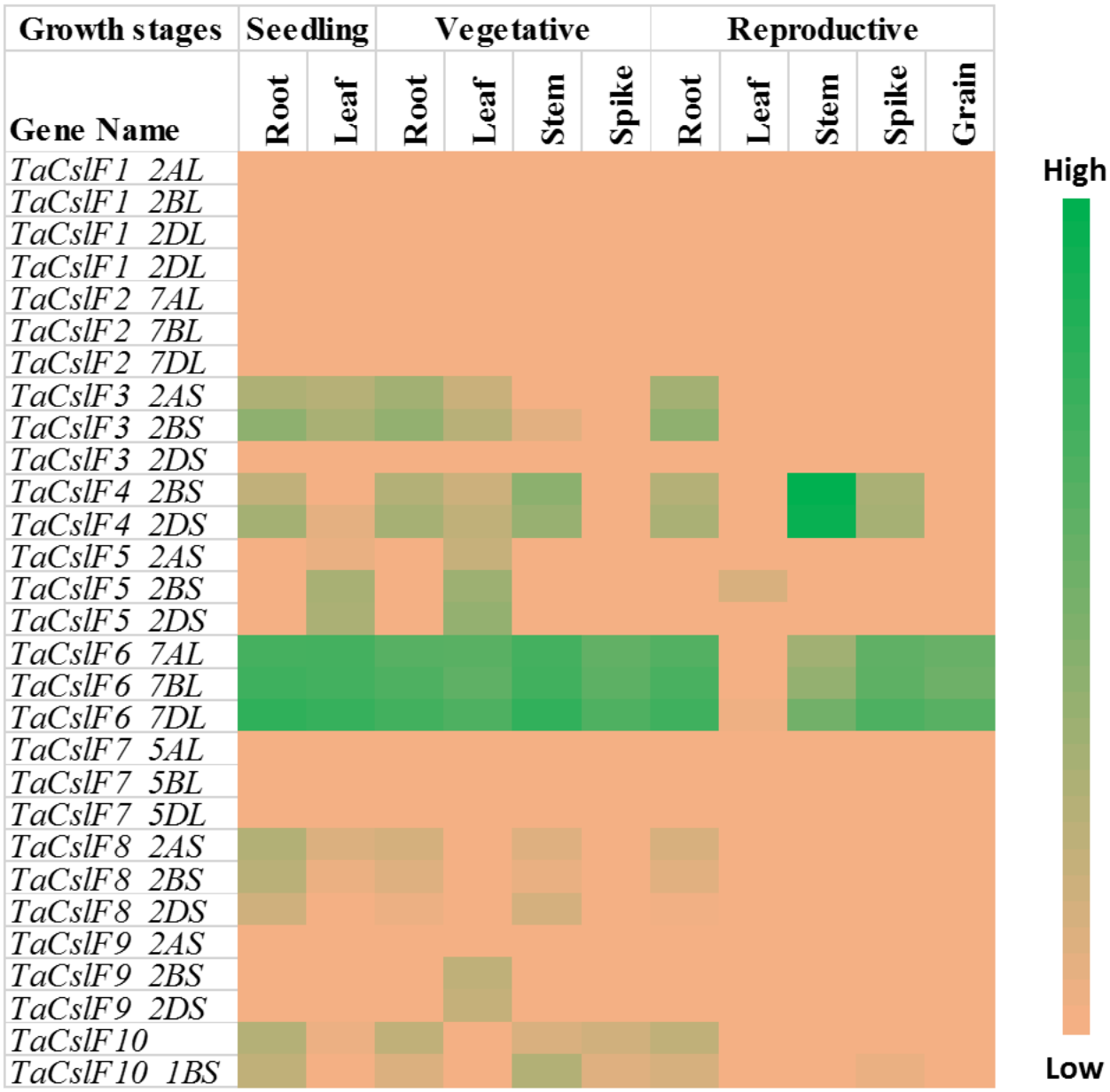
6.5C


Growth stages
Seedling
Vegetative
Reproductive
Gene Name
Root
Leaf
Root
Leaf
Stem
Spike
Root
Leaf
Stem
Spike
Grain
TaCslD1_1AL
TaCslD1_1BL
TaCslD1_1DL
TaCslD3_2AS
TaCslD3_2BS
TaCslD3_2DS
TaCslD4_1BS
TaCslD4_5BS
TaCslD4_5DS
TaCslD5_7AL
TaCslD5_7BL
TaCslD5_7DL
High
Low

6.5D


Growth stages
Seedling
Vegetative
Reproductive
Gene Name
Root
Leaf
Root
Leaf
Stem
Spike
Root
Leaf
Stem
Spike
Grain
TaCslE1_5BL
TaCslE1_5DL
TaCslE2_6AL
TaCslE2_6BL
TaCslE2_6DL
TaCslE3
TaCslE4_6DS
TaCslE6_5AL
TaCslE6_5BL
TaCslE6_5DL
High
Low

6.5E


Growth stages
Seedling
Vegetative
Reproductive
Gene Name
Root
Leaf
Root
Leaf
Stem
Spike
Root
Leaf
Stem
Spike
Grain
TaCslF1 2AL
TaCslF1 2BL
TaCslF1 2DL
TaCslF1 2DL
TaCslF2 7AL
TaCslF2 7BL
TaCslF2 7DL
TaCslF3 2AS
TaCslF3 2BS
TaCslF3 2DS
TaCslF4 2BS
TaCslF4 2DS
TaCslF5 2AS
TaCslF5 2BS
TaCslF5 2DS
TaCslF6 7AL
TaCslF6 7BL
TaCslF6 7DL
TaCslF7 5AL
TaCslF7 5BL
TaCslF7 5DL
TaCslF8 2AS
TaCslF8 2BS
TaCslF8 2DS
TaCslF9 2AS
TaCslF9 2BS
TaCslF9 2DS
TaCslF10
TaCslF10 1BS
High
Low

6.5F

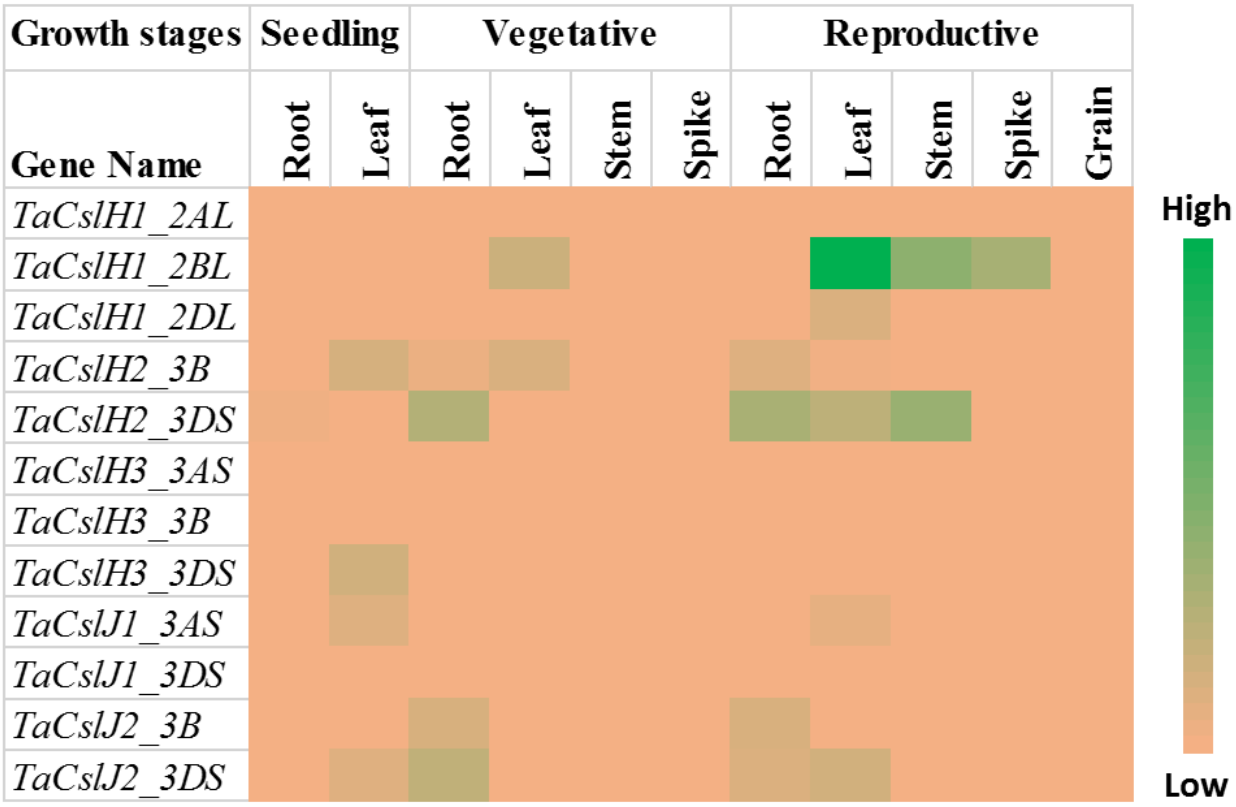


Fig 6.6 Pie chart showing the percentage of *TaCsl* genes on wheat chromosomes.

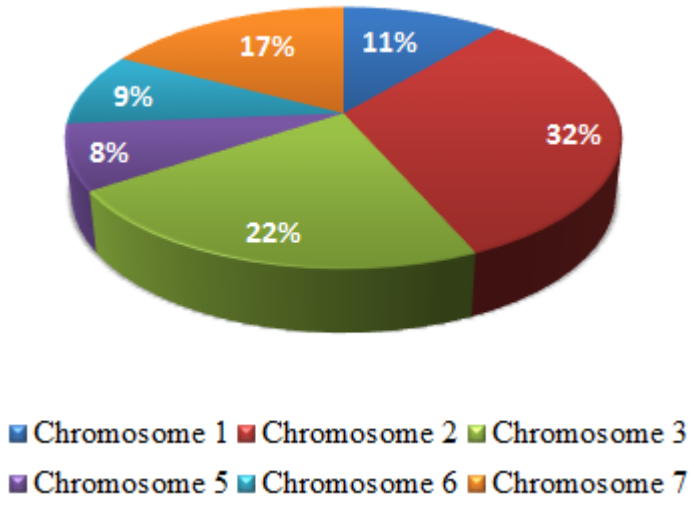


Table 6.1 Homoeologous copies of wheat *Csl* genes with their corresponding orthologs from rice.

No.	Ensembl ID	Gene Name	Corresponding gene in rice
1	TRIAE_CS42_6BS_TGACv1_513375_AA1639370.1	*TaCslA1_6BS*	*CslA1*
2	TRIAE_CS42_6AS_TGACv1_485966_AA1554960.1	*TaCslA1_6AS*	*CslA1*
3	TRIAE_CS42_2AL_TGACv1_093375_AA0278800.1	*TaCslA2_2AL*	*CslOS09G39920*
4	TRIAE_CS42_2BL_TGACv1_129747_AA0394630.1	*TaCslA2_2BL*	*CslOS09G39920*
5	TRIAE_CS42_2DL_TGACv1_160461_AA0550770.1	*TaCslA2_2DL*	*CslOS09G39920*
6	TRIAE_CS42_1AS_TGACv1_019142_AA0061550.1	*TaCslA2_1AS*	*CslOS09G39920*
7	TRIAE_CS42_7BS_TGACv1_592860_AA1945380.1	*TaCslA3_7BS*	*CslA3*
8	TRIAE_CS42_7DS_TGACv1_623146_AA2050070.1	*TaCslA3_7DS*	*CslA3*
9	TRIAE_CS42_7AS_TGACv1_569190_AA1809650.1	*TaCslA3_7AS*	*CslA3*
10	TRIAE_CS42_6DS_TGACv1_543811_AA1744360.1	*TaCslA4_6DS*	*CslA10/4/2*
11	TRIAE_CS42_6AS_TGACv1_487286_AA1569690.1	*TaCslA4_6AS*	*CslA10/4/2*
12	TRIAE_CS42_6BS_TGACv1_513376_AA1639390.1	*TaCslA4_6BS*	*CslA10/4/2*
13	TRIAE_CS42_2BS_TGACv1_146583_AA0468630.1	*TaCslA5_2BS*	*CslA5/7*
14	TRIAE_CS42_2AS_TGACv1_113418_AA0355820.1	*TaCslA5_2AS*	*CslA5/7*
15	TRIAE_CS42_2DS_TGACv1_177473_AA0578070.1	*TaCslA5_2DS*	*CslA5/7*
16	TRIAE_CS42_3DL_TGACv1_249033_AA0835410.1	*TaCslA6_3DL*	*CslA11*
17	TRIAE_CS42_3B_TGACv1_221079_AA0729630.1	*TaCslA6_3B*	*CslA11*
18	TRIAE_CS42_3AL_TGACv1_197519_AA0666560.1	*TaCslA6_3AL*	*CslA11*
19	TRIAE_CS42_2AS_TGACv1_113300_AA0354190.1	*TaCslA7_2AS*	*CslA5/7*
20	TRIAE_CS42_2DS_TGACv1_177798_AA0584795.1	*TaCslA7_2DS*	*CslA5/7*
21	TRIAE_CS42_3B_TGACv1_220828_AA0720500.1	*TaCslA8_3B*	*CslA11*
22	TRIAE_CS42_3DS_TGACv1_273022_AA0927600.1	*TaCslA8_3DS*	*CslA11*
23	TRIAE_CS42_U_TGACv1_642146_AA2112270.1	*TaCslA9*	*CslA9*
24	TRIAE_CS42_7BL_TGACv1_579090_AA1903960.1	*TaCslA9_7BL*	*CslA9*
25	TRIAE_CS42_7AL_TGACv1_558725_AA1795700.1	*TaCslA9_7AL*	*CslA9*
26	TRIAE_CS42_U_TGACv1_642146_AA2112290.1	*TaCslA10*	*CslA9*

27	TRIAE_CS42_7DL_TGACv1_602617_AA1962870.1	*TaCslA10_7DL*	*CslA9*
28	TRIAE_CS42_7AL_TGACv1_557254_AA1778850.1	*TaCslA10_7AL*	*CslA9*
29	TRIAE_CS42_7BL_TGACv1_578444_AA1895100.1	*TaCslA10_7BL*	*CslA9*
30	TRIAE_CS42_3AS_TGACv1_210508_AA0674280.1	*TaCslA11_3AS*	*CslA11*
31	TRIAE_CS42_3DS_TGACv1_272005_AA0912960.1	*TaCslA11_3DS*	*CslA11*
32	TRIAE_CS42_3B_TGACv1_223332_AA0780350.1	*TaCslA11_3B*	*CslA11*
33	TRIAE_CS42_3DL_TGACv1_251593_AA0882850.1	*TaCslC1_3DL*	*CslC1*
34	TRIAE_CS42_3AL_TGACv1_197197_AA0665370.1	*TaCslC1_3AL*	*CslC1*
35	TRIAE_CS42_3DS_TGACv1_271926_AA0910940.1	*TaCslC3_3DS*	*CslC3*
36	TRIAE_CS42_3B_TGACv1_220758_AA0718310.1	*TaCslC3_3B*	*CslC3*
37	TRIAE_CS42_3AS_TGACv1_211225_AA0686890.1	*TaCslC3_3AS*	*CslC3*
38	TRIAE_CS42_1DL_TGACv1_061928_AA0205730.1	*TaCslC7_1DL*	*CslC7*
39	TRIAE_CS42_1BL_TGACv1_030750_AA0099830.1	*TaCslC7_1BL*	*CslC7*
40	TRIAE_CS42_1AL_TGACv1_001272_AA0028090.1	*TaCslC7_1AL*	*CslC7*
41	TRIAE_CS42_1DL_TGACv1_062162_AA0209740.1	*TaCslC9_1DL*	*CslC10/9*
42	TRIAE_CS42_1BL_TGACv1_030501_AA0092480.1	*TaCslC9_1BL*	*CslC10/9*
43	TRIAE_CS42_5BL_TGACv1_404820_AA1311790.1	*TaCslC10_5BL*	*CslC10/9*
44	TRIAE_CS42_5DL_TGACv1_435778_AA1454840.1	*TaCslC10_5DL*	*CslC10/9*
45	TRIAE_CS42_5AL_TGACv1_374268_AA1195590.1	*TaCslC10_5AL*	*CslC10/9*
46	TRIAE_CS42_1BL_TGACv1_030586_AA0094860.1	*TaCslD1_1BL*	*CslD1*
47	TRIAE_CS42_1AL_TGACv1_001700_AA0034150.1	*TaCslD1_1AL*	*CslD1*
48	TRIAE_CS42_1DL_TGACv1_063091_AA0223780.1	*TaCslD1_1DL*	*CslD1*
49	TRIAE_CS42_2BS_TGACv1_148683_AA0494520.1	*TaCslD3_2BS*	*CslD3*
50	TRIAE_CS42_2DS_TGACv1_177279_AA0572180.1	*TaCslD3_2DS*	*CslD3*
51	TRIAE_CS42_2AS_TGACv1_114244_AA0365360.1	*TaCslD3_2AS*	*CslD3*
52	TRIAE_CS42_1BS_TGACv1_049706_AA0160220.1	*TaCslD4_1BS*	*CslD4*
53	TRIAE_CS42_5BS_TGACv1_425241_AA1392650.1	*TaCslD4_5BS*	*CslD4*
54	TRIAE_CS42_5DS_TGACv1_457675_AA1488780.1	*TaCslD4_5DS*	*CslD4*
55	TRIAE_CS42_7BL_TGACv1_577301_AA1871610.1	*TaCslD5_7BL*	*CslD5*
56	TRIAE_CS42_7AL_TGACv1_559436_AA1799630.1	*TaCslD5_7AL*	*CslD5*

57	TRIAE_CS42_7DL_TGACv1_603510_AA1985050.1	*TaCslD5_7DL*	*CslD5*
58	TRIAE_CS42_5DL_TGACv1_433536_AA1415830.1	*TaCslE1_5DL*	*CslE6/1*
59	TRIAE_CS42_5BL_TGACv1_406235_AA1342600.1	*TaCslE1_5BL*	*CslE6/1*
60	TRIAE_CS42_6DL_TGACv1_526558_AA1687090.1	*TaCslE2_6DL*	*CslE2*
61	TRIAE_CS42_6AL_TGACv1_471004_AA1500600.1	*TaCslE2_6AL*	*CslE2*
62	TRIAE_CS42_6BL_TGACv1_499967_AA1596110.1	*TaCslE2_6BL*	*CslE2*
63	TRIAE_CS42_U_TGACv1_683314_AA2158770.1	*TaCslE3*	*CslE6/1*
64	TRIAE_CS42_6DS_TGACv1_543277_AA1737920.1	*TaCslE4_6DS*	*CslE6/1*
65	TRIAE_CS42_5DL_TGACv1_433536_AA1415840.1	*TaCslE6_5DL*	*CslE6/1*
66	TRIAE_CS42_5BL_TGACv1_406235_AA1342610.1	*TaCslE6_5BL*	*CslE6/1*
67	TRIAE_CS42_5AL_TGACv1_376126_AA1232370.1	*TaCslE6_5AL*	*CslE6/1*
68	TRIAE_CS42_2DL_TGACv1_159781_AA0542640.1	*TaCslF1_2DL*	*CslF1/2/4*
69	TRIAE_CS42_2AL_TGACv1_094713_AA0301960.1	*TaCslF1_2AL*	*CslF1/2/4*
70	TRIAE_CS42_2DL_TGACv1_160109_AA0546890.1	*TaCslF1_2DL*	*CslF1/2/4*
71	TRIAE_CS42_2BL_TGACv1_130934_AA0420130.1	*TaCslF1_2BL*	*CslF1/2/4*
72	TRIAE_CS42_7BL_TGACv1_580651_AA1914920.1	*TaCslF2_7BL*	*CslF1/2/4*
73	TRIAE_CS42_7AL_TGACv1_557532_AA1782680.1	*TaCslF2_7AL*	*CslF1/2/4*
74	TRIAE_CS42_7DL_TGACv1_602590_AA1961740.1	*TaCslF2_7DL*	*CslF1/2/4*
75	TRIAE_CS42_2AS_TGACv1_113659_AA0359050.1	*TaCslF3_2AS*	*CslF3*
76	TRIAE_CS42_2DS_TGACv1_177641_AA0581710.1	*TaCslF3_2DS*	*CslF3*
77	TRIAE_CS42_2BS_TGACv1_148608_AA0494060.1	*TaCslF3_2BS*	*CslF3*
78	TRIAE_CS42_2BS_TGACv1_146146_AA0456710.1	*TaCslF4_2BS*	*CslF1/2/4*
79	TRIAE_CS42_2DS_TGACv1_179076_AA0604160.1	*TaCslF4_2DS*	*CslF1/2/4*
80	TRIAE_CS42_2DS_TGACv1_178985_AA0603230.1	*TaCslF5_2DS*	*CslF3*
81	TRIAE_CS42_2AS_TGACv1_112790_AA0345230.1	*TaCslF5_2AS*	*CslF3*
82	TRIAE_CS42_2BS_TGACv1_148027_AA0489970.1	*TaCslF5_2BS*	*CslF3*
83	TRIAE_CS42_7BL_TGACv1_577473_AA1876170.1	*TaCslF6_7BL*	*CslF6*
84	TRIAE_CS42_7AL_TGACv1_555973_AA1751470.1	*TaCslF6_7AL*	*CslF6*
85	TRIAE_CS42_7DL_TGACv1_607937_AA2011180.1	*TaCslF6_7DL*	*CslF6*
86	TRIAE_CS42_5BL_TGACv1_409916_AA1366600.1	*TaCslF7_5BL*	*CslF7*

87	TRIAE_CS42_5DL_TGACv1_433902_AA1424880.1	*TaCslF7_5DL*	*CslF7*
88	TRIAE_CS42_5AL_TGACv1_374191_AA1193100.1	*TaCslF7_5AL*	*CslF7*
89	TRIAE_CS42_2BS_TGACv1_148916_AA0495580.1	*TaCslF8_2BS*	*CslF8*
90	TRIAE_CS42_2DS_TGACv1_178471_AA0596060.1	*TaCslF8_2DS*	*CslF8*
91	TRIAE_CS42_2AS_TGACv1_112322_AA0335280.1	*TaCslF8_2AS*	*CslF8*
92	TRIAE_CS42_2AS_TGACv1_112322_AA0335290.1	*TaCslF9_2AS*	*CslF9*
93	TRIAE_CS42_2BS_TGACv1_147667_AA0486240.1	*TaCslF9_2BS*	*CslF9*
94	TRIAE_CS42_2DS_TGACv1_177329_AA0573830.1	*TaCslF9_2DS*	*CslF9*
95	TRIAE_CS42_U_TGACv1_641498_AA2096480.1	*TaCslF10*	*CslF9*
96	TRIAE_CS42_1BS_TGACv1_049866_AA0163180.1	*TaCslF10_1BS*	*CslF9*
97	TRIAE_CS42_2AL_TGACv1_094351_AA0296300.1	*TaCslH1_2AL*	*CslH1/2*
98	TRIAE_CS42_2DL_TGACv1_158387_AA0517170.1	*TaCslH1_2DL*	*CslH1/2*
99	TRIAE_CS42_2BL_TGACv1_129372_AA0380770.1	*TaCslH1_2BL*	*CslH1/2*
100	TRIAE_CS42_3B_TGACv1_221049_AA0728260.1	*TaCslH2_3B*	*Csl*
101	TRIAE_CS42_3DS_TGACv1_273502_AA0931770.1	*TaCslH2_3DS*	*Csl*
102	TRIAE_CS42_3DS_TGACv1_271739_AA0907200.1	*TaCslH3_3DS*	*Csl*
103	TRIAE_CS42_3AS_TGACv1_212952_AA0704280.1	*TaCslH3_3AS*	*CslH3*
104	TRIAE_CS42_3B_TGACv1_222234_AA0760340.1	*TaCslH3_3B*	*Csl*
105	TRIAE_CS42_3DS_TGACv1_272297_AA0918580.1	*TaCslJ1_3DS*	*Csl*
106	TRIAE_CS42_3AS_TGACv1_210908_AA0681280.1	*TaCslJ1_3AS*	*Csl*
107	TRIAE_CS42_3B_TGACv1_221705_AA0747940.1	*TaCslJ2_3B*	*Csl*
108	TRIAE_CS42_3DS_TGACv1_272756_AA0924850.1	*TaCslJ2_3DS*	*Csl*

Table 6.2 Status of splice variants of *Csl* genes in wheat genome.

Ensembl Gene ID	Gene name	Predicted amino acids	Splice site	Status
TRIAE_CS42_6BS_TGACv1_513375_AA1639370.1	TaCslA1_6BS	581	-	Wild type
TRIAE_CS42_6BS_TGACv1_513375_AA1639370.2		390	Exon 1 and 2	Exon skipping
TRIAE_CS42_6BS_TGACv1_513376_AA1639390.2	TaCslA4_6BS	528	-	Wild type
TRIAE_CS42_6BS_TGACv1_513376_AA1639390.1		393	Exon 1 and 2	Exon skipping
TRIAE_CS42_7AS_TGACv1_569190_AA1809650.1	TaCslA3_7AS	551	-	Wild type
TRIAE_CS42_7AS_TGACv1_569190_AA1809650.2		380	Exon 7, 8 and 9	Exon skipping
TRIAE_CS42_7AS_TGACv1_569190_AA1809650.3		503	Exon 9	Exon skipping
TRIAE_CS42_7DL_TGACv1_602617_AA1962870.2	TaCslA10_7DL	515	-	Wild type
TRIAE_CS42_7DL_TGACv1_602617_AA1962870.1		555	Intron 8	Intron retention
TRIAE_CS42_3DL_TGACv1_249033_AA0835410.2	TaCslA6_3DL	524	-	Wild type
TRIAE_CS42_3DL_TGACv1_249033_AA0835410.1		572	Intron 1	Intron retention
TRIAE_CS42_3B_TGACv1_221079_AA0729630.1	TaCslA6_3B	571	-	Wild type
TRIAE_CS42_3B_TGACv1_221079_AA0729630.2		538	Exon 2	Exon skipping
TRIAE_CS42_5BL_TGACv1_404820_AA1311790.1	TaCslC10_5BL	712	-	Wild type
TRIAE_CS42_5BL_TGACv1_404820_AA1311790.2		468	Exon 5	Alternative 5’ site
TRIAE_CS42_5BL_TGACv1_404820_AA1311790.3		504	Exon 1	Exon skipping
TRIAE_CS42_5DL_TGACv1_435778_AA1454840.1	TaCslC10_5DL	708	-	Wild type
TRIAE_CS42_5DL_TGACv1_435778_AA1454840.2		502	Exon1	Exon skipping
TRIAE_CS42_5AL_TGACv1_374268_AA1195590.3	TaCslC10_5AL	703	-	Wild type
TRIAE_CS42_5AL_TGACv1_374268_AA1195590.2		496	Exon 5	Alternative 5’ site
TRIAE_CS42_5AL_TGACv1_374268_AA1195590.1		501	Exon 5	Exon skipping
TRIAE_CS42_3DL_TGACv1_251593_AA0882850.1	TaCslC1_3DL	704	-	Wild type
TRIAE_CS42_3DL_TGACv1_251593_AA0882850.2		493	Exon 5	Exon skipping
TRIAE_CS42_3DL_TGACv1_251593_AA0882850.3		679	Exon 1	Alternative 3’ site
TRIAE_CS42_3AL_TGACv1_197197_AA0665370.1	TaCslC1_3AL	704	-	Wild type

TRIAE_CS42_3AL_TGACv1_197197_AA0665370.2		560	Exon 5	Alternative 3’ site
TRIAE_CS42_3AL_TGACv1_197197_AA0665370.3		679	Exon 5	Alternative 5’ site
TRIAE_CS42_6AL_TGACv1_471004_AA1500600.1	TaCslE2_6AL	667	-	Wild type
TRIAE_CS42_6AL_TGACv1_471004_AA1500600.2		737	Intron 8	Intron retention
TRIAE_CS42_6AL_TGACv1_471004_AA1500600.3		635	Exon 4	Alternative 5’ site
TRIAE_CS42_5DL_TGACv1_433536_AA1415830.1	TaCslE1_5DL	728	-	Wild type
TRIAE_CS42_5DL_TGACv1_433536_AA1415830.2		684	Exon 4	Exon skipping
TRIAE_CS42_5BL_TGACv1_406235_AA1342600.1	TaCslE1_5BL	734	-	Wild type
TRIAE_CS42_5BL_TGACv1_406235_AA1342600.2		728	Exon 1	Exon skipping
TRIAE_CS42_2DS_TGACv1_177641_AA0581710.1	TaCslF3_2DS	847	-	Wild type
TRIAE_CS42_2DS_TGACv1_177641_AA0581710.2		735	Exon 2	Alternative 3’ site
TRIAE_CS42_2DS_TGACv1_179076_AA0604160.1	TaCslF4_2DS	783	-	Wild type
TRIAE_CS42_2DS_TGACv1_179076_AA0604160.2		700	Exon 1	Exon skipping
TRIAE_CS42_2BS_TGACv1_147667_AA0486240.1	TaCslF9_2BS	877	-	Wild type
TRIAE_CS42_2BS_TGACv1_147667_AA0486240.2		796	Exon 1	Exon skipping
TRIAE_CS42_5BL_TGACv1_409916_AA1366600.1	TaCslF7_5BL	745	-	Wild type
TRIAE_CS42_5BL_TGACv1_409916_AA1366600.2		815	Intron 2	Intron retention
TRIAE_CS42_5AL_TGACv1_374191_AA1193100.1	TaCslF7_5AL	792	-	Wild type
TRIAE_CS42_5AL_TGACv1_374191_AA1193100.2		807	Intron 1	Intron retention
TRIAE_CS42_2AL_TGACv1_094351_AA0296300.1	TaCslH1_2AL	737	-	Wild type
TRIAE_CS42_2AL_TGACv1_094351_AA0296300.2		660	Exon 9	Exon skipping
TRIAE_CS42_2AL_TGACv1_094351_AA0296300.3		480	Exon 6,7,8 and 9	Exon skipping
TRIAE_CS42_3AS_TGACv1_210908_AA0681280.1	TaCslJ1_3AS	738	-	Wild type
TRIAE_CS42_3AS_TGACv1_210908_AA0681280.2		766	Intron 4	Intron retention
TRIAE_CS42_3DS_TGACv1_272756_AA0924850.2	TaCslJ2_3DS	609	-	Wild type
TRIAE_CS42_3DS_TGACv1_272756_AA0924850.1		734	Intron 1	Intron retention

CHAPTER VII. GENERAL DISCUSSION AND FUTURE STUDIES

7.1 General discussion

Plant cells exhibit special characteristics known as cell walls that provide basic infrastructure, mechanical support and a barrier against pathogen invasion throughout plant's lifecycle. Cell walls being the most abundant renewable biomass are getting attention for their use as dietary fibres, food additives, a raw material for biofuels, and fodder for livestock (Taylor-Teeples et al. 2015). These are the dynamic structures composed of complex polysaccharides such as celluloses, hemicelluloses, pectins and lignins along with highly glycosylated proteins (Doblin et al. 2010). These components vary greatly in their relative proportion and fine structure with the developmental stages and between different species (Fincher 2009; Hatfield et al. 2009).

Primary cell walls usually composed of cellulose, hemicellulose and pectins and provide shape and flexibility to young plant cells. Whereas secondary cell walls are composed of cellulose, hemicellulose and lignin and provide thickness and rigidity to mature plant cells. Secondary cell walls contribute more towards the total biomass production owing to their relatively higher thickness (Keegstra 2010). Considering their vital functions in plants, various medicinal and industrial uses, cell walls are getting much attention for research.

Biofuels from lignocellulosic biomass represent a potential source of energy with low carbon emissions. On the global scale, 3.7×10^{15} g of lignocellulosic biomass is produced per year from the residues of barley, maize, rice, soybean, sugar cane and wheat crops (Bentsen et al. 2014). This enormously abundant biomass can generate the energy equivalent to the 66 % of the energy required for transport worldwide (Baldwin et al. 2017). Among the various crop residues, wheat straw is one of the most practical biomass feedstocks used for the production of commercial

biofuels (Baldwin et al. 2017). Current varieties of wheat have not been designed for cellulosic biofuel production, however, great potential exists at the genetic level to alter lignocellulose composition of wheat and other grasses (Ong et al. 2014). Therefore, an efficient utilisation of lignocellulosic biomass as raw materials for biofuels or other bioproducts requires a thorough investigation of cell wall genetic architecture.

Celluloses and hemicelluloses present in the lignocellulose account for the bulk of renewable biomass. Cellulose is the major structural component of plants and composed of linear chains of β-1, 4-glucan units, synthesised at plasma membranes. A number of genes have been reported to be associated with cellulose synthesis in different plant species. A major class of these genes is known as *Cellulose synthase A* (*CesA*) (Suzuki et al. 2006). On the other hand, hemicelluloses are the group of heterogeneous polysaccharides synthesised on the Golgi membranes. These includes xyloglucans, xylans, mannans and glucomannans, and β-(1-3, 1-4)-glucans in the walls of terrestrial plants. However, *Cellulose synthase-like* genes (*Csl*) genes account for the synthesis of various hemicellulose components in diverse tissues at different developmental stages of plants. Typically, structural and functional characterization of *Csl* genes (Liepman et al. 2005; Burton et al. 2006a; Cocuron et al. 2007; Doblin et al. 2009; Goubet et al. 2009; Yin et al. 2009; Wang et al. 2010b) and *CesA* genes have been performed in Arabidopsis (Arioli et al. 1998; Richmond and Somerville 2000; Taylor et al. 2003), maize (Holland et al. 2000; Appenzeller et al. 2004), and rice (Tanaka et al. 2003; Wang et al. 2012a).

However, due to complex nature of wheat genome, *CesA*/*Csl* gene families have not been well defined in wheat. Furthermore, the full genome sequence of bread wheat has not been available until recently, which posed a major challenge in exploring these complex gene families. Being hexaploid wheat possess three genomes and corresponding homoeologous copies of each

gene are expected. Genes of *CesA*/*Csl* belongs to a highly conserved superfamily of genes known as *Glycosyltransferase 2*. These genes share a large sequence similarity among each other or within the subgroup, which makes it a difficult task to identify and characterise these genes in hexaploid wheat.

The results generated in chapter III, are the first report of identification and comprehensive structural analysis of *CesA* genes in bread wheat. Total 22 *CesA* genes including their paralogs from the homoeologous bread wheat genomes A, B and D, were identified using a comparative genomics approach. These genes were analysed for specific structural features such as domains, motifs and phases of intron evolution. Previous studies have shown the involvement of distinct CSCs for the synthesis of primary and secondary cell walls in plants (Arioli et al. 1998; Tanaka et al. 2003; Taylor et al. 2003). Following that, a novel motif "CQIC" was identified in the present study that structurally differentiates PCW and SCW CESAs from both the monocots and dicots. Additionally, several other motifs were identified that were highly conserved among the CESA orthologs from different species (Arabidopsis, barley, maize, rice and wheat). The newly identified motifs will enable researchers to easily extricate PCW or SCW related *CesAs* and to identify one to one orthologs of different *CesA* genes in various plant species. Comparable to the distribution patterns of *CesA* genes in Arabidopsis, barley and maize (Holland et al. 2000; Burton et al. 2004), *TaCesAs* were also found to be scattered all over the wheat genome, which reflect their significance in plants. *In vitro* expression analysis showed higher transcript abundance of three SCW *TaCesAs* (*TaCesA4, TaCesA7,* and *TaCesA8*) in mature stem tissues. Among these three essential components of SCW (Tanaka et al. 2003; Taylor et al. 2003; Kotake et al. 2011; Wang et al. 2012a), *TaCesA4* showed relatively higher expression in mature stem tissues.

Being an essential component of synthesis of cellulose in SCW, *TaCesA4* gene identified in chapter III was selected to further validate its function in bread wheat. Chapter IV explains the functional characterization of *TaCesA4* gene using BSMV-based VIGS, which has recently emerged as a rapid functional genomics tool in cereals (Bennypaul et al. 2012a). A significantly lower transcript abundance and cellulose content in the *TaCesA4* silenced plants correlates with the previous finding suggesting its role in the cellulose synthesis (Tanaka et al. 2003; Taylor et al. 2003; Kotake et al. 2011; Wang et al. 2012a). Conversely, the histological analysis revealed that the silencing of SCW *CesA* at booting stage does not pose much effect on the shape and arrangement of xylem, phloem and mesophyll cells in the stem tissues.

In addition to *CesA* genes, some other classes of genes including *Glycosyl Hydrolase 9* (*GH9*) and *Sucrose synthase* (*SuSy*) have been reported to affect the cellulose synthesis in plants (Fujii et al. 2010). The involvement of several genes in this process explains the complexity of underlying mechanism (Kotake et al. 2011). Chapter V was planned to explore the novel genomic regions affecting the variability of cellulose content among diverse spring wheat genotypes. The stem internodes of 265 spring wheat varieties were analysed in triplicate for cellulose content variation. The percentage cellulose data was associated with GBS generated 21073 SNP markers using genome-wide association studies (GWAS) using fixed and random model circulating probability unification (FarmCPU) (Liu et al. 2016). Novel genes (β-tubulin, and Auxin-induced protein, 5NG4 and UDP-glycosyl transferase 85A2) were discovered, that are linked to the differences in cellulose content among different wheat genotypes. The genes identified in this study were previously known for their association with cellulose microfibril deposition (Paredez et al. 2006; Chan et al. 2007; Wightman and Turner 2008; Crowell et al. 2009; Gutierrez et al. 2009; Chan et al. 2010; Rao et al. 2016), cell division and expansion (Qiu et al. 2013), transfer of UDP-

glucose to the catalytic sites (Lairson et al. 2008), but not for cellulose content variation. Further characterization of these genes can help us to better understand the genetic architecture of cellulose biosynthesis. Moreover, the analysis of cellulose content variability could be an important screening tool for selection of genotypes tolerant to crop lodging, which is a common problem in most cereal crops (Ching et al. 2006).

Chapter VI represents the first report of comprehensive and large-scale data mining for the identification of *Csl* genes in bread wheat. A total of 108 *TaCsl* genes were retrieved from available sequence databases using two conserved domains: PF00535, and PF03552 (Yin et al. 2014). The newly identified genes were categorized into different subfamilies (*CslA, CslC, CslD, CslE, CslF, CslH, CslJ)* based on the phylogenetic analysis (Yin et al. 2009; Yin et al. 2014). As expected, none of the wheat genes were clustered with so-called dicot specific *CslB* and *CslG* subfamilies (Schwerdt et al. 2015). A detailed analysis of gene structure and intron evolution was performed for *TaCslD* sub-family, as this family palys a major role in mannan synthesis (Verhertbruggen et al. 2011; Wang et al. 2011), tip growth, development of root hairs (Kim et al. 2007a; Yuo et al. 2011), normal plant growth (Li et al. 2009; Hunter et al. 2012), pollen tube growth, and meristem architecture (Bernal et al. 2007; Li et al. 2009), and resistance to biotic stresses (Douchkov et al. 2016). Tissue or developmental stage specific *in silico* expression of different *TaCsl* genes concurred with the variability of cell wall composition among different cells and tissues (Lin et al. 2016). In-depth analysis of gene structure, evolution, and expression of this family offers a valuable resource for breeding and genetic modifications to improve wheat varieties for desirable biomass with appropriate resistance against various stresses.

7.2 Future studies

- Functional characterization of novel motif (CQIC) using CRISPR-Cas9 will generate information for better understanding of cell wall structure and functions
- New molecular markers can be devised from functionally validated *TaCesA4* for marker-assisted breeding of wheat for the selection of lodging tolerance and culm strength
- Over expression of *TaCesA4*, β-tubulin, UDP-glycosyltransferase 85A2 genes may allow researchers to increase the cellulose content further
- Upon functional validation, SNPs associated with cellulose content could be used as molecular markers to identify and design appropriate bioenergy crops
- 265 diverse wheat varieties analysed for cellulose content could probably be used as a training set for genomic selection project to predict the breeding values of wheat genotypes
- *Csl* gene enrichment sequencing of EMS mutants could be performed to further validate the physiological roles of these genes.

VIII. APPENDIX

Appendix 5.1 Table showing the percent variation of cellulose content among 288 diverse wheat lines along with their countries of origin.

Line No.	Name	Country of origin	% Cellulose
KSG001	GABO 60	Mexico	46.70472619
KSG002	NACOZARI F 76	Mexico	43.06074918
KSG003	YECORA ROJO 76	Mexico	41.57629579
KSG004	ANNAPURNA 1	Nepal, India	47.03962704
KSG005	KLEIN DRAGON	Argentina	45.1394718
KSG006	MEXIPAK65	Pakistan	43.47821804
KSG007	BLUEBIRD 15	Mexico	43.01749685
KSG008	ABU GHRAIB#3	Iraq	45.2932595
KSG009	FAISLABAD 83	Pakistan	48.25707821
KSG010	PUNJAB 88	Pakistan	47.77705132
KSG011	SAN CAYETANO S 97	Mexico	40.35340966
KSG012	BR 18	Brazil	43.04271265
KSG013	KENYA KWALE	Kenya	44.91836948
KSG014	TEMPORALERA M87	Kansas	42.11726904
KSG015	ESTANZUELA PELON 90	Uruguay	43.80735931
KSG016	CHAM 6	Syria	44.95349446
KSG017	TINAMOUII	Mexico	42.12532419
KSG018	ARIVECHI M 92	Mexico	46.22849525
KSG019	YAQUI 50	Mexico	45.86505317
KSG020	NARINO 59	Colombia	47.53416518
KSG021	PENJAMO T 62	Mexico	44.08791132
KSG022	PITIC62	Mexico	43.39717696
KSG023	CRESPO	Colombia	43.44941278
KSG024	NADADORES M 63	Mexico	43.27110991
KSG025	SONORA 64	Mexico	43.45442644
KSG026	INIA F66	Mexico	42.62369488
KSG027	BAJIO	Mexico	47.57101559
KSG028	KALYANSONA	India	44.8328799
KSG029	SAFED LERMA	India	43.50507742

KSG030	SONALIKA	India	43.66591928
KSG031	CALIDAD	Argentina	43.06248997
KSG032	UP301	India	35.06884153
KSG033	POTAM S 70	Mexico	41.05781368
KSG034	MARCOS JUAREZ INTA	Argentina	46.21094668
KSG035	TANORI F 71	Mexico	45.44585477
KSG036	ARZ	lebanon	44.90792988
KSG037	JUPATECO F 73	Mexico	43.09056956
KSG038	MAYA 74	guatamala	44.13141554
KSG039	SALAMANCA 75	Spain	44.62962963
KSG040	LIESBECK	South Africa	43.34082318
KSG041	PAVON F 76	Mexico	42.54707117
KSG042	SAKHA 8	Egypt	43.99996933
KSG043	CHIVITO	Australia	39.87634078
KSG044	HERMOSILLO M77	Mexico	36.17321
KSG045	SERI M 82	Mexico	44.14783762
KSG046	UP262	Nepal, India	43.91580032
KSG047	BAHAWALPUR 79	Pakistan	43.74167182
KSG048	SAKHA 69	Egypt	44.43018497
KSG049	HARTOG	Australia	43.7402199
KSG050	PIRSABAK 85	Pakistan	44.79982167
KSG051	GONEN	Turkey	46.35639402
KSG052	RAYON F 89	Mexico	41.49339147
KSG053	NESSER	Jordan	40.50872523
KSG054	ICA YACUANQUER	Colambia	43.71542116
KSG055	TIA.1	Mexico	43.22401319
KSG056	BORLAUG M 95	Mexico	43.14365215
KSG057	PBW343	India	42.65420936
KSG058	INIFAP M 97	Chile	42.22214915
KSG059	TOBARITO M 97	Mexico	42.82890699
KSG060	GRANERO INTA	Argentina	42.19966414
KSG061	PROINTA OASIS	Argentina	45.83226527
KSG062	ITAPUA 40-OBLIGADO	Paraguay	44.86773775
KSG063	KLEIN DRAGON	Argentina	43.49546734
KSG064	BAW898	Bangladesh	41.6239607
KSG065	CUMHURIYET 75	Turkey	39.23444561
KSG066	MILLALEAU INIA	Chile	42.15707452

KSG067	IAN 8-PIRAPO	Turkey	42.46548654
KSG068	PAVON	Mexico	44.79717813
KSG069	POINTA FEDERAL	Argentina	44.1163193
KSG070	SONALIKA	Punjab, India	42.04477453
KSG071	ANDES-56	Colombia	44.15091988
KSG072	SARIAB-92	Pakistan	42.16081471
KSG073	OROFEN 60	Chile	40.16797882
KSG074	LERMA ROJO 64	Mexico	41.695595
KSG075	V-17	Mexico	45.88738332
KSG076	PJ62/GB55	Mexico	46.46232439
KSG077	ZAMINDAR 80	Pakistan	36.96560847
KSG078	PAKISTAN 81	Pakistan	42.62663038
KSG079	CORDILLERA 3	Paraguay	45.06010228
KSG080	IDAHO 61M3404	Idaho	46.96628522
KSG081	IDAHO 62M9-224	Idaho	43.44005421
KSG082	LEMHI 66	Idaho	47.83103307
KSG083	64AB9405	ID	43.04125263
KSG084	TWIN	Idaho	41.84246834
KSG085	OWENS	Idaho	44.5684991
KSG086	IDO190	Idaho	44.83899583
KSG087	IDO232	Idaho	42.35110827
KSG088	COPPER	Idaho	42.15872689
KSG089	VANDAL	Idaho	45.67624932
KSG090	IDAHO 266	Idaho	41.42568531
KSG091	WHITEBIRD	Idaho	44.69516279
KSG092	FREX	Indiana	41.80197902
KSG093	II-53-521	Minnesota	47.64675168
KSG096	II-55-1	Minnesota	39.91464209
KSG097	II-58-60	Minnesota	45.50838985
KSG098	II-62-78	Minnesota	40.84739058
KSG099	MN 6616M	Minnesota	41.96671847
KSG100	WHEATON	Minnesota	43.63580016
KSG101	II-64-20	Minnesota	40.51274456
KSG102	MN 6898	Minnesota	42.2826087
KSG103	VANCE	Minnesota	43.06664091
KSG104	NORM	Minnesota	47.16934327
KSG105	VERDE	Minnesota	38.19458938

KSG106	MCVEY	Nebraska	47.62559438
KSG107	JUSTIN	North Dakota	49.31623442
KSG108	ND 202-2	North Dakota	48.38509648
KSG109	ND 271	North Dakota	49.81917336
KSG110	ND 229-1	North Dakota	49.85835538
KSG111	ND 287	North Dakota	46.91647733
KSG112	FORTUNA	North Dakota	49.50539882
KSG113	LEEDS	North Dakota	45.79200901
KSG114	ND 59-120A	North Dakota	44.30834075
KSG115	ND 407	North Dakota	45.67017079
KSG116	WALDRON	North Dakota	40.83967449
KSG117	ND 22	North Dakota	47.78958387
KSG118	ND 66	North Dakota	42.4103521
KSG119	CI014952	North Dakota	47.55488531
KSG120	CI014953	North Dakota	44.38873116
KSG121	ROLETTE	North Dakota	45.65575577
KSG122	D 6647	North Dakota	45.28231895
KSG123	ND 467	North Dakota	48.8061043
KSG124	ND 476	North Dakota	39.67881485
KSG125	ELLAR	North Dakota	47.47339873
KSG126	EDMORE	North Dakota	47.66839378
KSG127	COTEAU	North Dakota	46.89117454
KSG128	D804	North Dakota	41.7127634
KSG129	MONROE	North Dakota	44.96333195
KSG130	D7925	North Dakota	46.3238966
KSG131	ND 13-137	North Dakota	46.88940781
KSG132	AMIDON	North Dakota	47.91221172
KSG133	MUNICH	North Dakota	43.57684523
KSG134	PIERCE	North Dakota	44.6623158
KSG135	ND 2710	North Dakota	43.36096219
KSG136	STW 598874	Oklahoma	46.21235205
KSG137	YSCA-1	Oklahoma	42.4899502
KSG139	SEL. 90	Washington	46.36374266
KSG140	WA 6101	Washington	40.0853117
KSG141	WA 7175	Washington	46.06972355
KSG142	SPILLMAN	Washington	43.58764394
KSG143	ARS95 451	Washington	45.09472781

KSG144	ARS95 457	Washington	48.02306331
KSG145	EDEN	washington	43.60548617
KSG146	ALPOWA	Washington	43.43949184
KSG147	ALTURAS	Idaho	43.75510767
KSG148	CHALLIS	Montana	44.410047
KSG149	EDWALL	washington	43.36811475
KSG151	JUBILEE	Idaho	47.37820634
KSG152	VANNA	ARIZONA	47.1130596
KSG153	TARA 2002 AKA TARA	Washington	41.09099118
KSG154	SCARLET	Washington	46.83861316
KSG155	JEFFERSON	Idaho	43.34845811
KSG156	HOLLIS	Washington	46.53028667
KSG157	CALORWA	Washington	45.42869581
KSG158	ZAK	washington	50.19169639
KSG159	WAWAWAI	Washington	46.65080457
KSG160	CENTENNIAL	idaho	45.10790766
KSG161	MACON	washington	49.06372049
KSG162	LOLO	Idaho	45.97618203
KSG163	KLASIC	Nebraska	48.77983321
KSG164	IDO377S	Washington	45.83206825
KSG165	YECORA ROJO	Mexico	47.67711192
KSG166	SAXON	Colorado	43.86641714
KSG167	NEWANA	Montana	45.92721642
KSG168	URQUIE	Washington	45.29367021
KSG169	RUSHMORE	South Dakota	52.13290804
KSG170	RAMONA	California	47.25687962
KSG171	HARD FEDERATION AKA PI041079	Australia	46.42912562
KSG172	REDCHAFF	Washington	48.07375876
KSG173	SELKIRK	Canada	47.15380762
KSG176	SAUNDERS	Canada	47.2226853
KSG177	LEE	Minnesota	50.1167154
KSG178	PEAK	Idaho	46.80380726
KSG179	AKA PROBRAND 751	Nebraska	46.80622613
KSG180	WADUAL	Washington	46.88229358
KSG181	WAKANZ	Washington	50.50736472
KSG182	CANTHATCH	Canada	47.40863478
KSG183	CONLEY	North Dakota	50.24719581

KSG184	PEAK 72	Idaho	50.30414443
KSG185	PROSPUR	Minnesota	49.3063974
KSG186	KITT AKA PI518818	Minnesota	40.42727527
KSG187	WAMPUM	Washington	46.32785815
KSG188	WALLADAY	Washington	43.49644857
KSG189	PONDERA	Montana	43.94650672
KSG190	STERLING	Idaho	42.85652588
KSG191	MCKAY	Idaho	47.18660804
KSG192	WAID	Washington	45.85480139
KSG194	NORANA	Montana	45.85363639
KSG195	OLAF	North Dakota	43.50033908
KSG196	BORAH	Idaho	47.90570783
KSG197	WAVERLY	Washington	42.24863107
KSG198	TREASURE	Idaho	43.74368596
KSG199	WESTBRED 906R	Arizona	43.59406286
KSG200	WESTBRED 911	Arizona	43.16655132
KSG201	BLISS	idaho	48.88787787
KSG202	WARD	North Dakota	44.00276206
KSG203	BOUNTY 208	Colorado	46.39909736
KSG204	ANZA	California	47.21021021
KSG205	MORAN	Idaho	48.76949155
KSG206	UNION	oregon	45.29289627
KSG207	UTAC	Utah	44.46124083
KSG208	WHITE FIFE AKA PI061345	Japan	46.52450797
KSG209	WHITE MARQUIS	Minnesota	43.85544415
KSG210	SEA ISLAND	Colorado	48.51260963
KSG211	RUBY	Canada	47.76026137
KSG212	RIVAL	North Dakota	50.55001294
KSG213	LEMHI	Idaho	46.40397413
KSG214	LITTLE CLUB	Oregon	48.76414788
KSG215	MARFED	Washington	49.90889593
KSG216	TOUSE	Utah	47.86276959
KSG217	THATCHER	Minnesota	48.18024363
KSG218	SUPREME	Canada	48.16087216
KSG219	SPINKCOTA	South Dakota	45.55200744
KSG220	SONORA	Mexico	42.30245184
KSG221	GALGALOS AKA PI009872	Armenia	50.41821948

KSG222	FEDERATION 67	Idaho	47.80453371
KSG223	FEDERATION AKA PI041080	Australia	43.81425027
KSG224	REWARD	Canada	48.19479594
KSG225	RESCUE	Canada	48.79979483
KSG226	RELIANCE	Oregon	52.01883133
KSG227	REGENT	canada	48.87994127
KSG228	RED BOBS	Canada	50.13675778
KSG229	RAMONA 50	California	42.14782993
KSG230	ORFED	Washington	43.56689822
KSG231	OREGON ZIMMERMAN	Oregon	48.73593185
KSG232	ONAS 53	California	48.07730773
KSG234	MIDA	North Dakota	50.03260797
KSG235	MARQUIS	Canada	47.73903971
KSG236	PACIFIC BLUESTEM	Oregon	48.54956975
KSG237	PACIFIC BLUESTEM 37	California	48.17517535
KSG238	PILOT	North Dakota	48.5406682
KSG239	PREMIER	North Dakota	48.31727123
KSG240	ALLEN	Washington	45.64510296
KSG241	AWNED ONAS	California	46.61107559
KSG242	BAART EARLY SELECTION	California	43.7966177
KSG243	CANADIAN RED	California	44.43374264
KSG244	CADET	North Dakota	43.73795884
KSG245	BLUECHAFF	Oregon	41.85275831
KSG246	BIG CLUB	Oregon,Calafornia	44.45097118
KSG247	HARD FEDERATION (-31)	Oregon	40.96056197
KSG248	HENRY	Wisconsin	45.20997332
KSG249	HOPE	South Dakota	48.83954145
KSG250	HYBRID 63	Washington	35.98484848
KSG252	KINNEY	Oregon	41.69922384
KSG253	KENHI	Canada	47.60807328
KSG254	CERES	North Dakota	48.79524715
KSG255	WESTBRED EXPRESS	Arizona	37.88456853
KSG256	LAGODA	Russian	45.88576706
KSG257	FLOMAR	Washington	49.08835286
KSG258	HYBRID 123	Washington	36.31063321
KSG259	DICKLOW	Utah	47.27318508
KSG260	GYPSUM	Colorado	46.77553779

KSG261	HYPER	Washington	46.60738832
KSG262	IDAED	Idaho	45.03714753
KSG263	INDIAN	Idaho	45.02423314
KSG264	BAART 46	California	51.13209342
KSG265	NEW ZEALAND	Nevada	41.82661343
KSG267	PILCRAW	California	44.72485318
KSG268	RINK	Oregon	47.30452914
KSG269	SURPRISE	Vermont	46.2047431
KSG270	WHITE FEDERATION	Australia	40.98373984
KSG271	BUNYIP	Australia	43.41207034
KSG272	CURRAWA	Australia	44.45410701
KSG273	WILBUR	Oregon	43.52105489
KSG274	EARLY BAART	California	45.34595338
KSG275	MAJOR	Australia	46.16155964
KSG276	LEMHI 53	Idaho	43.77935104
KSG277	SPRINGFIELD	Idaho	46.76870101
KSG278	FIELDER	Idaho	45.70008742
KSG279	FIELDWIN	Idaho	43.40397723
KSG282	SCHLANSTEDT	GermaNew York	44.55455946
KSG283	PRESTON	Canada	46.26326331
KSG284	CHINOOK	North Dakota	42.48168278
KSG285	MANITOU	Canada	44.92639949
KSG286	RED RIVER 68	California	43.1708695
KSG287	ERA	Minnesota	40.37224917
KSG288	BOUNTY 309	Colorado	42.14828517
KSG289	WINSOME	Oregon	42.22707263
KSG290	AIM	ARIZONA	44.8834374
KSG291	BRONZE CHIEF	USA	41.58139661
KSG292	KODIAK DWARF	USA	48.33561419
KSG293	KUBANKA	USA	43.58474159
KSG294	KAHLA	Algeria	48.80351813
KSG295	SENTRY	North Dakota	47.9702318
KSG297	WELLS	North Dakota	50.18567426
KSG298	WANDELL	Washington	49.59982381
KSG299	PRODURA	Minnesota	45.85905942
KSG301	WL 444	-	45.28841885
KSG302	POMERELLE	Idaho	47.0343288

Appendix 6.1 List of *CslA* subfamily genes, their protein length (amino acids) and multiple sequence alignment of amino acids showing the conserved motifs (D, D, DXD, QXXRW) specific to *Cellulose synthase like* (*Csl*) family (highlighted with red boxes).

S.No	Gene name with number of splice variants (CslA)	No. of amino acids (aa)
1	TRIAE_CS42_2BS_TGACv1_146583_AA0468630.1	581 aa
2	TRIAE_CS42_2AS_TGACv1_113418_AA0355820.2	580 aa
3	TRIAE_CS42_2DS_TGACv1_177473_AA0578070.1	581 aa
4	TRIAE_CS42_2AS_TGACv1_113300_AA0354190.1	579 aa
5	TRIAE_CS42_2DS_TGACv1_177798_AA0584795.1	881 aa
6	TRIAE_CS42_6BS_TGACv1_513375_AA1639370.1	518 aa
7	TRIAE_CS42_6AS_TGACv1_485966_AA1554960.1	518 aa
8	TRIAE_CS42_U_TGACv1_642146_AA2112270.1	522 aa
9	TRIAE_CS42_7BL_TGACv1_579090_AA1903960.1	375 aa
10	TRIAE_CS42_7AL_TGACv1_558725_AA1795700.1	518 aa
11	TRIAE_CS42_6DS_TGACv1_543811_AA1744360.1	531 aa
12	TRIAE_CS42_6AS_TGACv1_487286_AA1569690.1	528 aa
13	TRIAE_CS42_6BS_TGACv1_513376_AA1639390.2	528 aa
14	TRIAE_CS42_U_TGACv1_642146_AA2112290.1	512 aa
15	TRIAE_CS42_7BS_TGACv1_592860_AA1945380.1	547 aa
16	TRIAE_CS42_7DS_TGACv1_623146_AA2050070.1	545 aa
17	TRIAE_CS42_7AS_TGACv1_569190_AA1809650.1	551 aa
18	TRIAE_CS42_7DL_TGACv1_602617_AA1962870.1	555 aa
19	TRIAE_CS42_7AL_TGACv1_557254_AA1778850.1	515 aa
20	TRIAE_CS42_7BL_TGACv1_578444_AA1895100.1	515 aa
21	TRIAE_CS42_3DL_TGACv1_249033_AA0835410.1	572 aa
22	TRIAE_CS42_3B_TGACv1_221079_AA0729630.1	571 aa
23	TRIAE_CS42_3AL_TGACv1_197519_AA0666560.1	573 aa
24	TRIAE_CS42_3B_TGACv1_220828_AA0720500.1	570 aa
25	TRIAE_CS42_3DS_TGACv1_273022_AA0927600.1	568 aa
26	TRIAE_CS42_2AL_TGACv1_093375_AA0278800.1	527 aa
27	TRIAE_CS42_2BL_TGACv1_129747_AA0394630.1	528 aa
28	TRIAE_CS42_2DL_TGACv1_160461_AA0550770.1	548 aa
29	TRIAE_CS42_1AS_TGACv1_019142_AA0061550.1	515 aa
30	TRIAE_CS42_3AS_TGACv1_210508_AA0674280.1	566 aa
31	TRIAE_CS42_3DS_TGACv1_272005_AA0912960.1	570 aa
32	TRIAE_CS42_3B_TGACv1_223332_AA0780350.1	925 aa

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TRIAE_CS42_3AS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_3B_TGACv1 MLLLKIDIAKAFDTVSWEYILELLQRMNFPAHWRDRIALLLSSVSSAYLLKGDPGPAILHQRGLRQGDPLSAILFILVIV 80
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TRIAE_CS42_3DS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_3DS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_3DL_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_3B_TGACv1 ------------------------------------------------------------------------------- 0
TRIAE_CS42_3AL_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_6BS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_6AS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_7AL_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_U_TGACv1_ ------------------------------------------------------------------------------- 0
TRIAE_CS42_U_TGACv1_ ------------------------------------------------------------------------------- 0
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TRIAE_CS42_7AL_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_7BL_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_7DL_TGACv ------------------------------------------------------------------------------- 0
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TRIAE_CS42_1AS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_7DS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_7AS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_7BS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_6DS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_6BS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_6AS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_2BS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_2DS_TGACv ------------------------------------------------------------------------------- 0
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TRIAE_CS42_2AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2DS_TGACv ---------------------------------------------------------------------------------- 0

TRIAE_CS42_3AS_TGACv ---------------------------------------------------------------------------------- 0
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TRIAE_CS42_3B_TGACv1 ---------------------------------------------------------------------------------- 0
TRIAE_CS42_3DS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_3DS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_3DL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_3B_TGACv1 ---------------------------------------------------------------------------------- 0
TRIAE_CS42_3AL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_6BS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_6AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7AL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_U_TGACv1_ ---------------------------------------------------------------------------------- 0
TRIAE_CS42_U_TGACv1_ ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7BL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7AL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7BL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7DL_TGACv ---------------------------------------------------------------------------------- 0
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TRIAE_CS42_2BL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_1AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7DS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7BS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_6DS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_6BS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_6AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2BS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2DS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2AS_TGACv ---------------------------------------------------------------------------------- 0
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TRIAE_CS42_3AS_TGACv ---------------------------------------------------------------------------------- 0
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TRIAE_CS42_3B_TGACv1 ---------------------------------------------------------------------------------- 0
TRIAE_CS42_3AL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_6BS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_6AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7AL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_U_TGACv1_ ---------------------------------------------------------------------------------- 0
TRIAE_CS42_U_TGACv1_ ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7BL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7AL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7BL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7DL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2AL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2DL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2BL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_1AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7DS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7BS_TGACv ---------------------------------------------------------------------------------- 0
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TRIAE_CS42_6BS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_6AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2BS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2DS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2AS_TGACv ---------------------------------------------------------------------------------- 0
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TRIAE_CS42_3B_TGACv1 IPKRFYKDVDKARWRFLWVHDHEVTGGRCKVNWRLVTSPVDHGGLGIPSMERFARALCLRWLWLAWTDPARPWARMGTPC 320
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TRIAE_CS42_3DS_TGACv ---------------------------------------------------------------------------------- 0
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TRIAE_CS42_3DL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_3B_TGACv1 ---------------------------------------------------------------------------------- 0
TRIAE_CS42_3AL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_6BS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_6AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7AL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_U_TGACv1_ ---------------------------------------------------------------------------------- 0
TRIAE_CS42_U_TGACv1_ ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7BL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7AL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7BL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7DL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2AL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2DL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_2BL_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_1AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7DS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7AS_TGACv ---------------------------------------------------------------------------------- 0
TRIAE_CS42_7BS_TGACv ---------------------------------------------------------------------------------- 0
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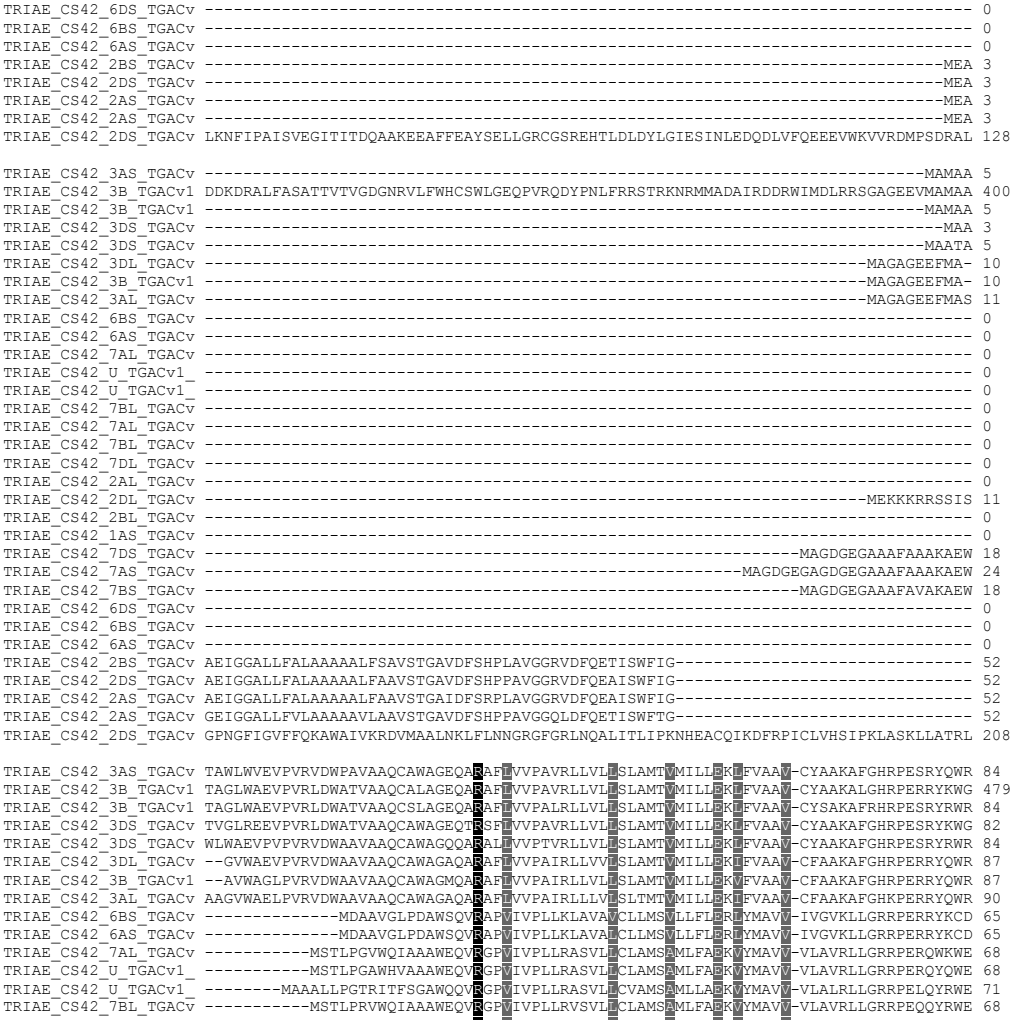

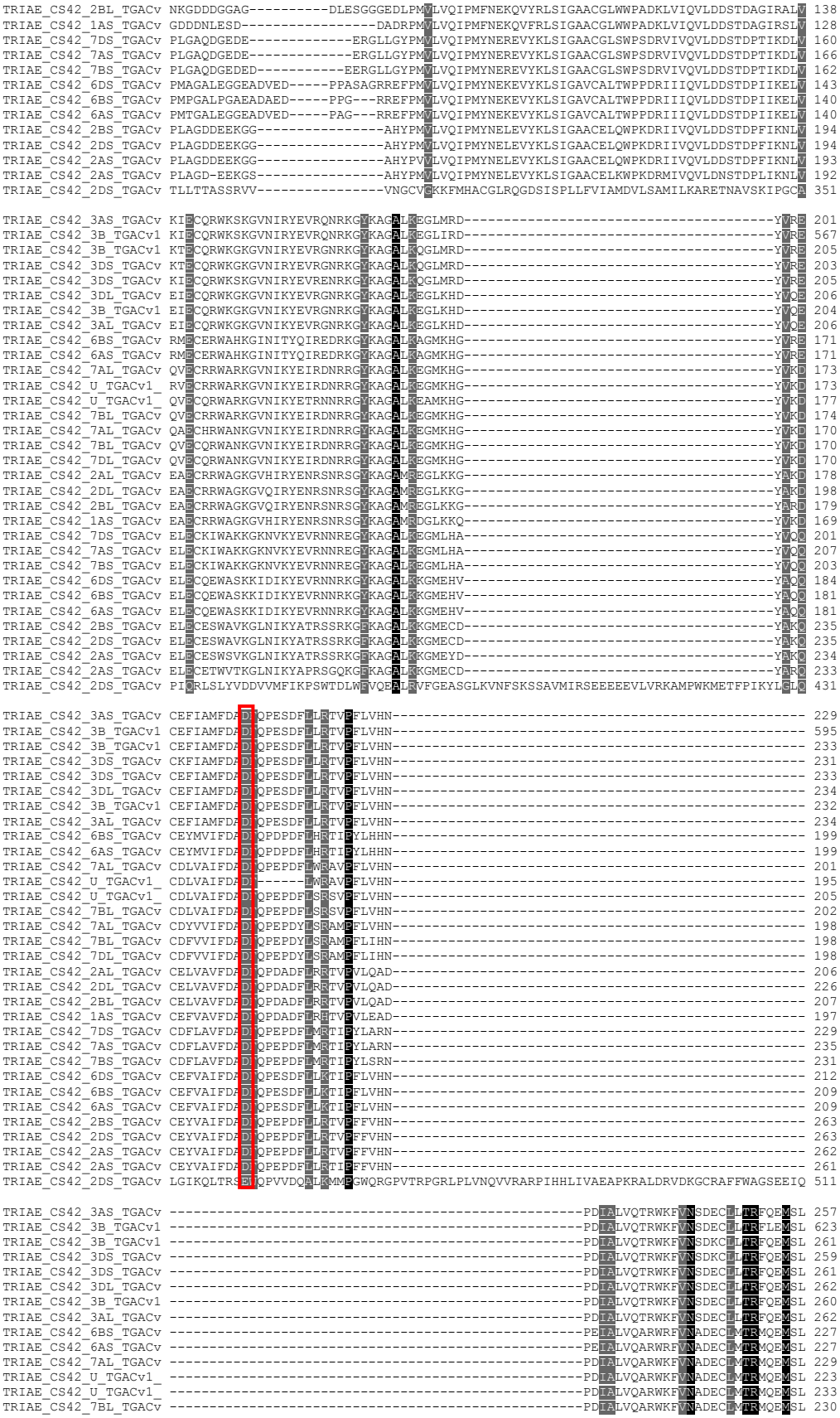

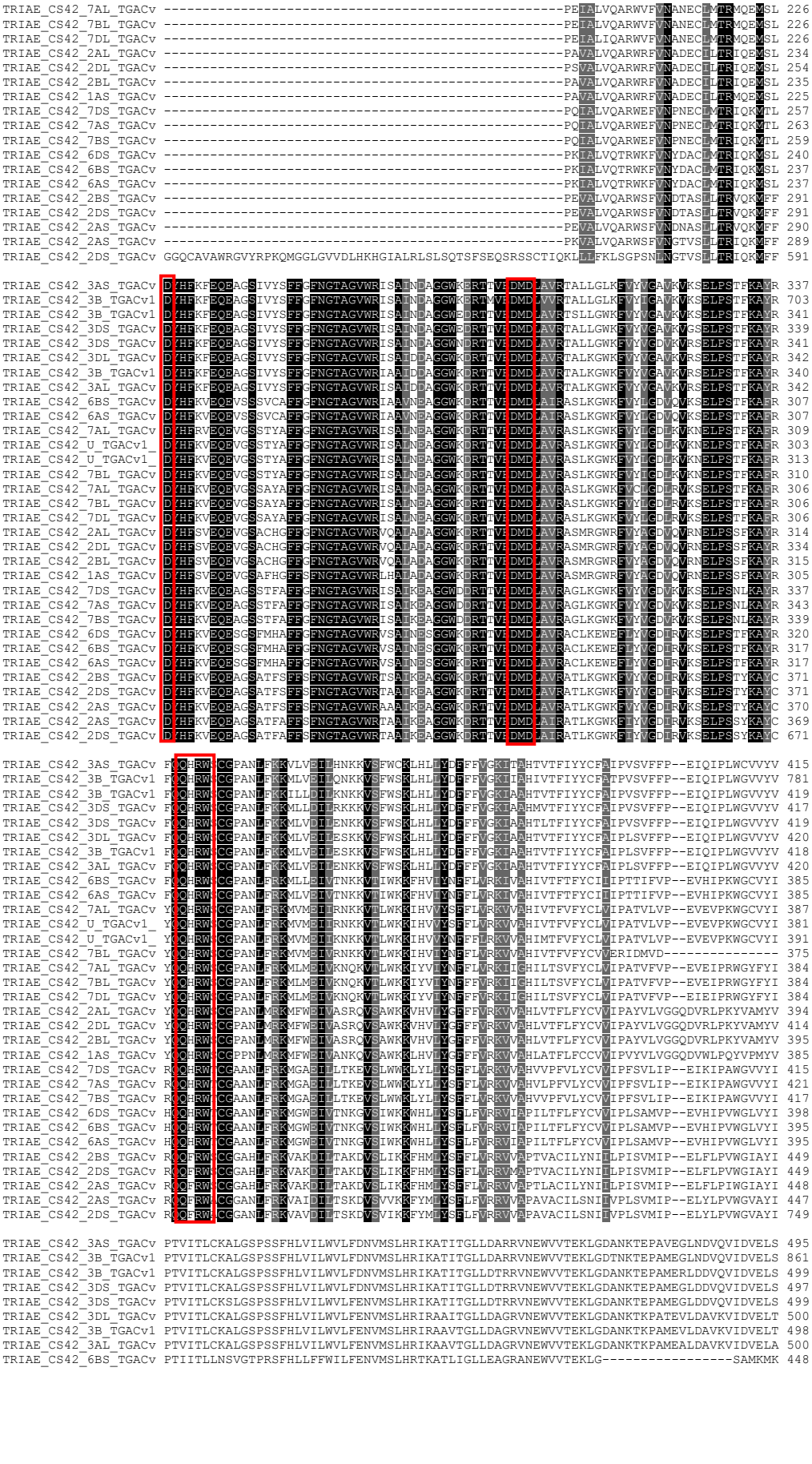

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TRIAE_CS42_7AL_TGACv PTIITLLNAVGTPRSVHLVVFWVLFENVMSLHRAKATFIGLLEAGTVNEWVVTEKLG-----------------DTLKAK 450
TRIAE_CS42_U_TGACv1_ PTIITLLNAVGTPRSVHLVVFWVLFENVMSLHRAKATFIGLLEVGTVNEWVVTEKLG-----------------DTLKAK 444
TRIAE_CS42_U_TGACv1_ PAIITLLSVVGTPRSVHLVIFWALFENVMSLHRTKATFIGLLEAHTVNEWVVTEKLG-----------------DTVKTK 454
TRIAE_CS42_7BL_TGACv ---------------------------------------------------------------------------- 375
TRIAE_CS42_7AL_TGACv PTVITLLNAVGTPRSFHLVIFWVLFENVMSLHRTKATFSGLLELGRVNEWVVTEKLG-----------------DILKMK 447
TRIAE_CS42_7BL_TGACv PTVITLLNAVGTPRSFHLVIFWVLFENVMSLHRTKATFSGLLELGRVNEWVVTEKLG-----------------DVLKMK 447
TRIAE_CS42_7DL_TGACv PTIITLLNAVGTPRSFHLVIFWVLFENVMSLHRTKATFSGLLELGRVNEWVVTEKLG-----------------DVLKMK 447
TRIAE_CS42_2AL_TGACv PAIITLLNAVCTPRSWHLLVFWILFENVMSMHRSKATIIGLVEASRANEWVVTEKLGSV------------TS-TPAATT 461
TRIAE_CS42_2DL_TGACv PAIITLLNAVCTPRSWHLLVFWILFENVMSMHRSKATIIGLVEASRANEWVVTEKLGSV------------TSSTPAATT 482
TRIAE_CS42_2BL_TGACv PAIITLLNAVCTPRSWHLLVFWILFENVMSMHRSKATIIGLVEASRANEWVVTEKLGSV------------TS-TPAAAT 462
TRIAE_CS42_1AS_TGACv AAVLTLLNAVCTPRSCHLLVFWILFENVMSIHRCKATIIGLLEASRANEWVVTEKLGGS------------TTSTPAAAT 453
TRIAE_CS42_7DS_TGACv PTAITVLYAVRNPSSIHFIPFWILFENVMSFHRTKATFIGLLELGSVNEWVVTEKLG-----------------SASNT 477
TRIAE_CS42_7AS_TGACv PTAITILYAVRNPSSIHFIPFWILFENVMSFHRTKATFIGLLELGSVNEWVVTEKLG-----------------SVSNT 483
TRIAE_CS42_7BS_TGACv PTAITILYAVRNPSSIHFIPFWILFENVMSFHRTKATFIGLLELGSVNEWVVTEKLG-----------------SVSNT 479
TRIAE_CS42_6DS_TGACv PTAITVMNAIRNPGSLHLMPFWILFENVMSMHRMRAALTGLLETAHVNDWVVTEKVG-----------------DLVKDD 461
TRIAE_CS42_6BS_TGACv PTAITIMNAIRNPGSLHLMPFWILFENVMSMHRMRAALTGLLETAHVNDWVVTEKVG-----------------DLVKDD 458
TRIAE_CS42_6AS_TGACv PTAITIMNAIRNPGSLHLMPFWILFENVMSMHRMRAALTGLLETAHVNDWVVTEKVG-----------------DVVKDD 458
TRIAE_CS42_2BS_TGACv PTVLLVVTAIRHPKNLHILPFWILFESVMTMHRMRAALSGLFELSEFNEWVVTKKTG-------------------NNFE 510
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TRIAE_CS42_2AS_TGACv PAVLLVVTAIRNPKNIHLLPFWILFESVMTIHRTRAALVGLFEFSEFNEWVVTKKTG-------------------NNFE 508
TRIAE_CS42_2DS_TGACv PTVLLVVTAIRNPKNIHLLPFWILFESVMTIHRTRAALVGLFELTEFDEWLVTKKTG-------------------NNFE 810

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TRIAE_CS42_3B_TGACv1 TPLVPKLEKR-------YNCSEIFVGTCIIICGCYDVLYA-NKGCYIYLFIQGVAFLVIGFEYIGTRPPGAE-------- 925
TRIAE_CS42_3B_TGACv1 TPLVPKLEKRRTRLWHKYNCSEIFVGTFIIICGCYDVLYA-KKGYYIYLFIQGLAFLVIGFEYIGTRPPSTE-------- 570
TRIAE_CS42_3DS_TGACv TPLVPKLEKRRTRLWDKYNCSEIFVGTCIIICGCYDVLYA-KKGYYIYLFIQGLAFLVIGFEYIGTRPPSIE-------- 568
TRIAE_CS42_3DS_TGACv TPLVPKLEKRRTRLWDKYNCSEIPVGTCIIICGCYDVLYA-KKGYYIYLFIQGLAFLVGFEYIGTRPPSAE-------- 570
TRIAE_CS42_3DL_TGACv TPLVPKLKKRRIRLWDKYNCSEIFVGTCIVICGFYDLFYA-NKGYYIYLFIQGLAFLVGFEYIGTRPPTPSA------- 572
TRIAE_CS42_3B_TGACv1 TPLVPKLKKRRIRLWDKYNCSEIFVGTCIIISGFYDLFYA-NKGYYIYLFIQGLAFLVVGFEYIGTRPPTPSAG------ 571
TRIAE_CS42_3AL_TGACv TPLVPKLKKRRIKLWDKYNCSEIFVGTSIIICGFYDLFYA-NKGYYIYLFIQGLAFLVVGFEYIGTRPPTPSAE------ 573
TRIAE_CS42_6BS_TGACv SANKASARKSFMRMWERLNVPELGVGAFLFSCGWYDVAFG-KDNFFIYLFFQSMAFFVVGVGYVGTIVPPS--------- 518
TRIAE_CS42_6AS_TGACv SANKASARKSFMRMWERLNVPELGVGAFLFSCGWYDVAFG-KDNFFIYLFFQSMAFFVVGVGYVGTIVPQS--------- 518
TRIAE_CS42_7AL_TGACv MPSKALK-KLRMRIGERLHLWELGVAAYLFLCGCYDISFG-NNRYFIFLFMQSIAFFIVGVGYVGTFVAQ---------- 518
TRIAE_CS42_U_TGACv1_ MPSKALR-KLRMRIGERLHLWELGVAAYLFLCGCYDISFG-NNRYFIFLFMQSIAFFIVGVGYVGTFVAQ---------- 512
TRIAE_CS42_U_TGACv1_ MPSKALK-KLRIGIGERLHLWELGVAAYLFICGCYSISFG-NNHYFIFLLMQSIAFFIVGVGYVGTFVTQ---------- 522
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TRIAE_CS42_7BL_TGACv VQSKVTK-KLRMRIRERLQLLELGVAAYIFFCGSYDLLFG-KRYYYIFLFMQSIAFFVVGVGFVGTLVPN---------- 515
TRIAE_CS42_7DL_TGACv VQSKVTK-KLRMRIRERYIIIIGLLMCISQLSYLNFNMES-GCSFWSLVLQPISSFVEVTTFCLAKDITISFSSCNPSLS 525
TRIAE_CS42_2AL_TGACv TMATNKGAMKKKKSQSSILAPEIVMGLCLLYCAVYDIFFG-HDHFYVYLLMQSAAAFVIGFGYVGSQ------------- 527
TRIAE_CS42_2DL_TGACv TMATNKGATKKKKSQSSILAPEIVMGLCLLYCAVYDIVFG-HDHFYVYLLMQSAAAFVIGFGYVGSQ------------- 548
TRIAE_CS42_2BL_TGACv TMAANKGAMKKKKSQSSILAPEIVMGLCLLYCAVYDIVFG-HDHFYVYLLMQSAAAFVIGFGYVGSQ------------- 528
TRIAE_CS42_1AS_TGACv TTMVAK----KKKSSSSFLAPEIVMGLFLLYCALYDIVFG-HDHFYVYLLMQSAAAFVIGFGYVGSQ------------- 515
TRIAE_CS42_7DS_TGACv KPVPQILERPRCRFWDRWTVSELLFAVFLFVCATYNLVYG-SDFYFIYIYLQAITFIIVGTGFCGTSNS----------- 545
TRIAE_CS42_7AS_TGACv KPVPQILERPRCRFWDRWTVSELLFAVFLFVCATYNLVYG-SDFYFIYIYLQAITFIIVGTGFCGTSNS----------- 551
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TRIAE_CS42_6DS_TGACv FDVPLLEPLKPTECVERIYIPELLLALYLLICASYDYVLG-SQTYFMYIYLQALAFIVLGFGFVGMKTPCS--------- 531
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TRIAE_CS42_6AS_TGACv FEVPLLEPLKPTECVERIYIPELLLALYLLICASYDYVLG-SQTYFMYIYLQALAFIVLGFGFVGMKTPCS--------- 528
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TRIAE_CS42_6AS_TGACv ------------------------------- 518
TRIAE_CS42_7AL_TGACv ------------------------------- 518
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TRIAE_CS42_U_TGACv1_ ------------------------------- 522
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TRIAE_CS42_2DL_TGACv ------------------------------- 548
TRIAE_CS42_2BL_TGACv ------------------------------- 528
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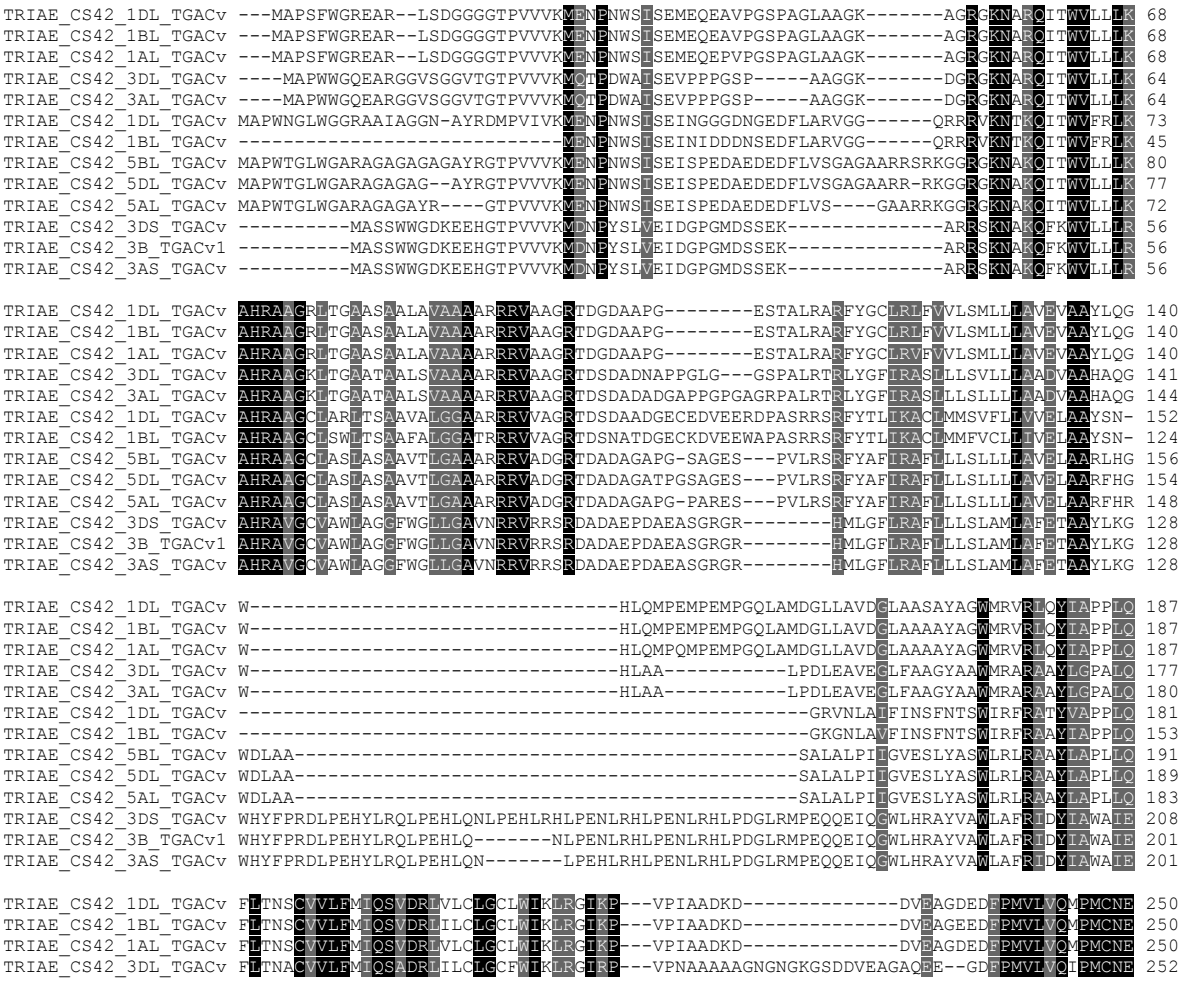
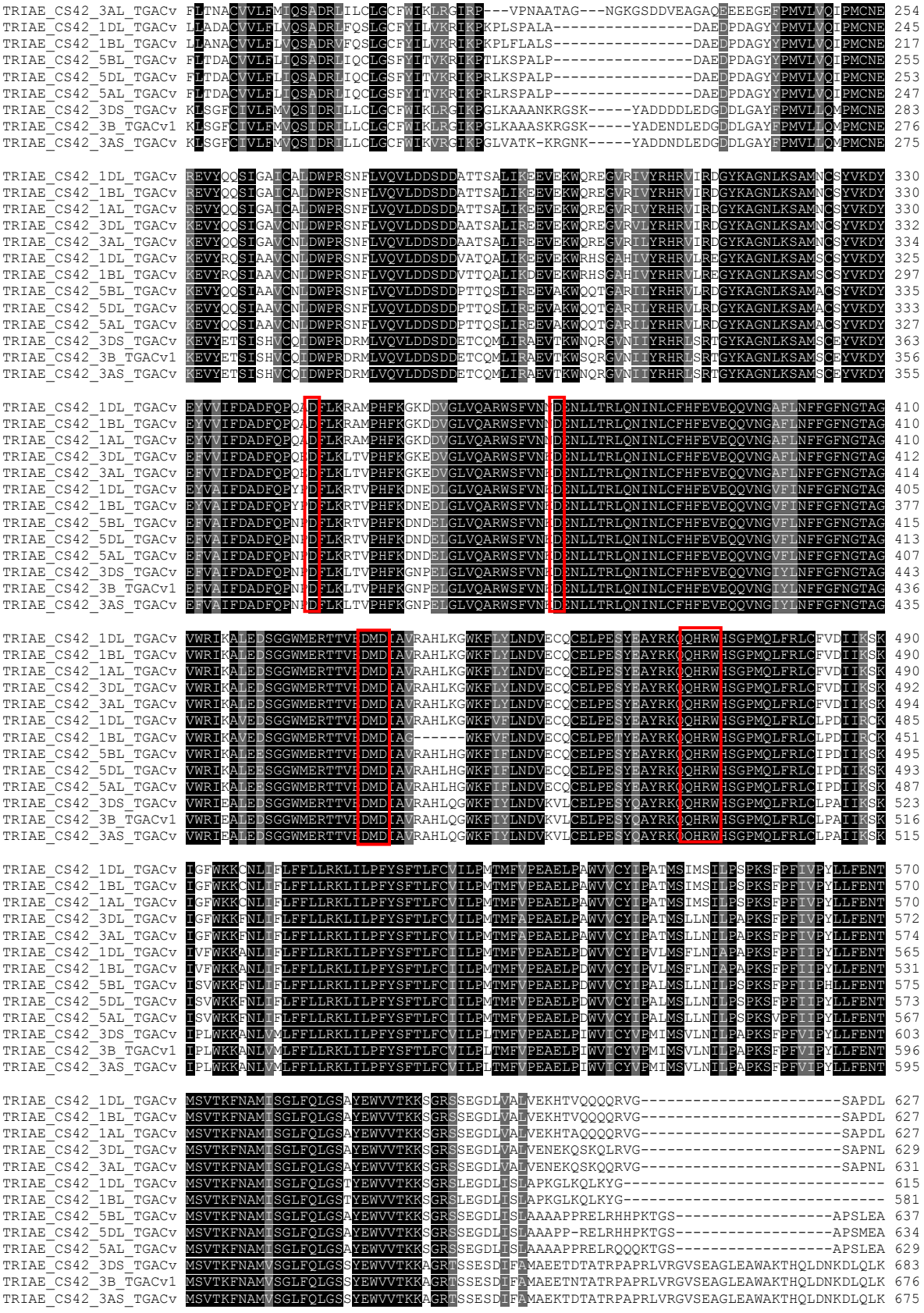
TRIAE_CS42_7DS_TGACv ------------------------------- 545
TRIAE_CS42_7AS_TGACv ------------------------------- 551
TRIAE_CS42_7BS_TGACv ------------------------------- 547
TRIAE_CS42_6DS_TGACv ------------------------------- 531
TRIAE_CS42_6BS_TGACv ------------------------------- 528
TRIAE_CS42_6AS_TGACv ------------------------------- 528
TRIAE_CS42_2BS_TGACv ------------------------------- 581
TRIAE_CS42_2DS_TGACv ------------------------------- 581
TRIAE_CS42_2AS_TGACv ------------------------------- 580
TRIAE_CS42_2AS_TGACv ------------------------------- 579
TRIAE_CS42_2DS_TGACv ------------------------------- 881
```

Appendix 6.2 List of *CslC* subfamily genes, their protein length (amino acids) and multiple sequence alignment of amino acids showing the conserved motifs (D, D, DXD, QXXRW) specific to *Cellulose synthase like* (*Csl*) family (highlighted with red boxes).

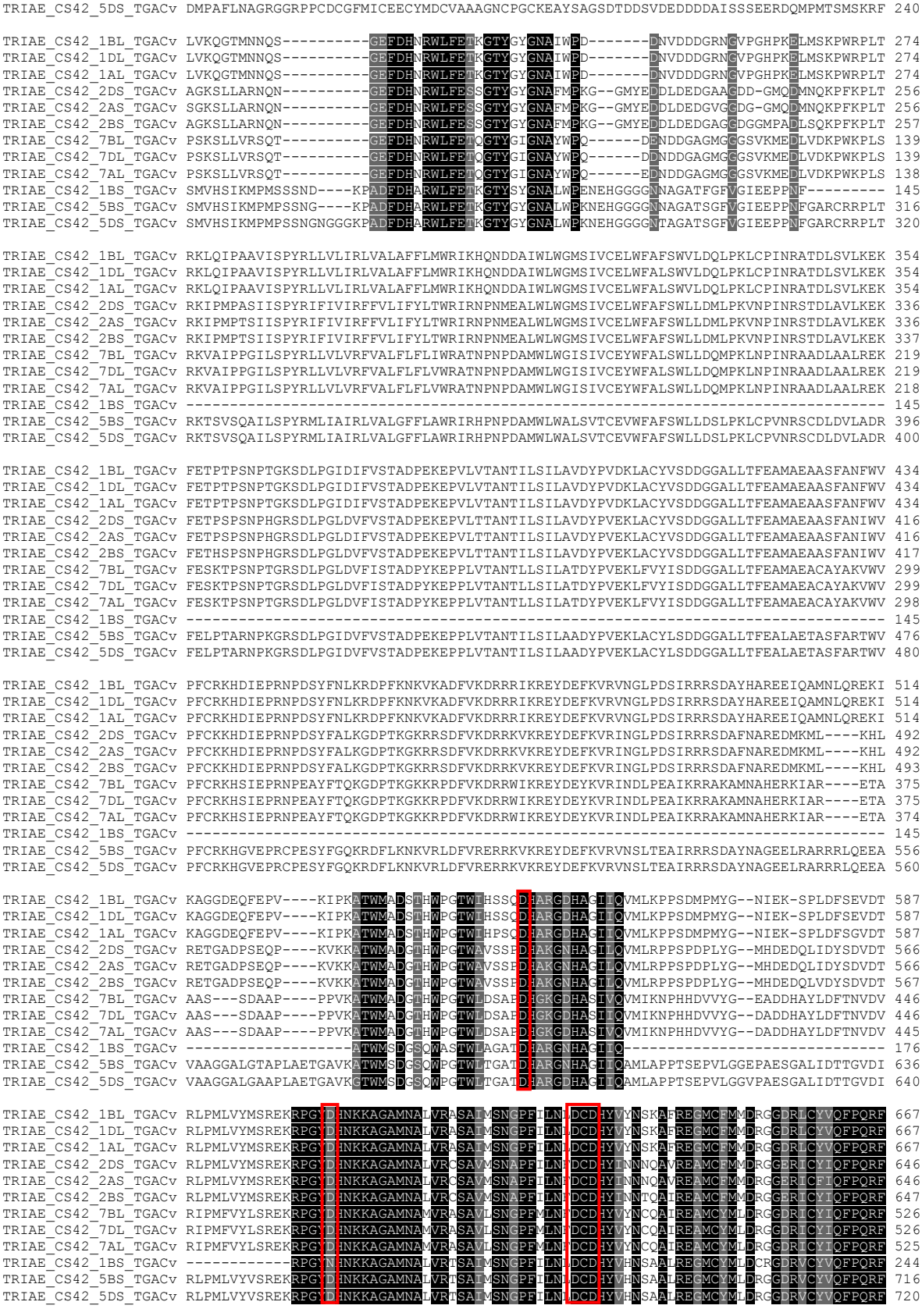
S.No	Gene name with number of splice variants (CslC)	No. of amino acids (aa)
1	TRIAE_CS42_1DL_TGACv1_062162_AA0209740.1	690 aa
2	TRIAE_CS42_1BL_TGACv1_030501_AA0092480.1	656 aa
3	TRIAE_CS42_5BL_TGACv1_404820_AA1311790.1	712 aa
4	TRIAE_CS42_5DL_TGACv1_435778_AA1454840.1	708 aa
5	TRIAE_CS42_5AL_TGACv1_374268_AA1195590.3	703 aa
6	TRIAE_CS42_1DL_TGACv1_061928_AA0205730.1	702 aa
7	TRIAE_CS42_1BL_TGACv1_030750_AA0099830.1	702 aa
8	TRIAE_CS42_1AL_TGACv1_001272_AA0028090.1	702 aa
9	TRIAE_CS42_3DL_TGACv1_251593_AA0882850.1	704 aa
10	TRIAE_CS42_3AL_TGACv1_197197_AA0665370.1	704 aa
11	TRIAE_CS42_3DS_TGACv1_271926_AA0910940.1	758 aa
12	TRIAE_CS42_3B_TGACv1_220758_AA0718310.2	751 aa
13	TRIAE_CS42_3AS_TGACv1_211225_AA0686890.2	750 aa

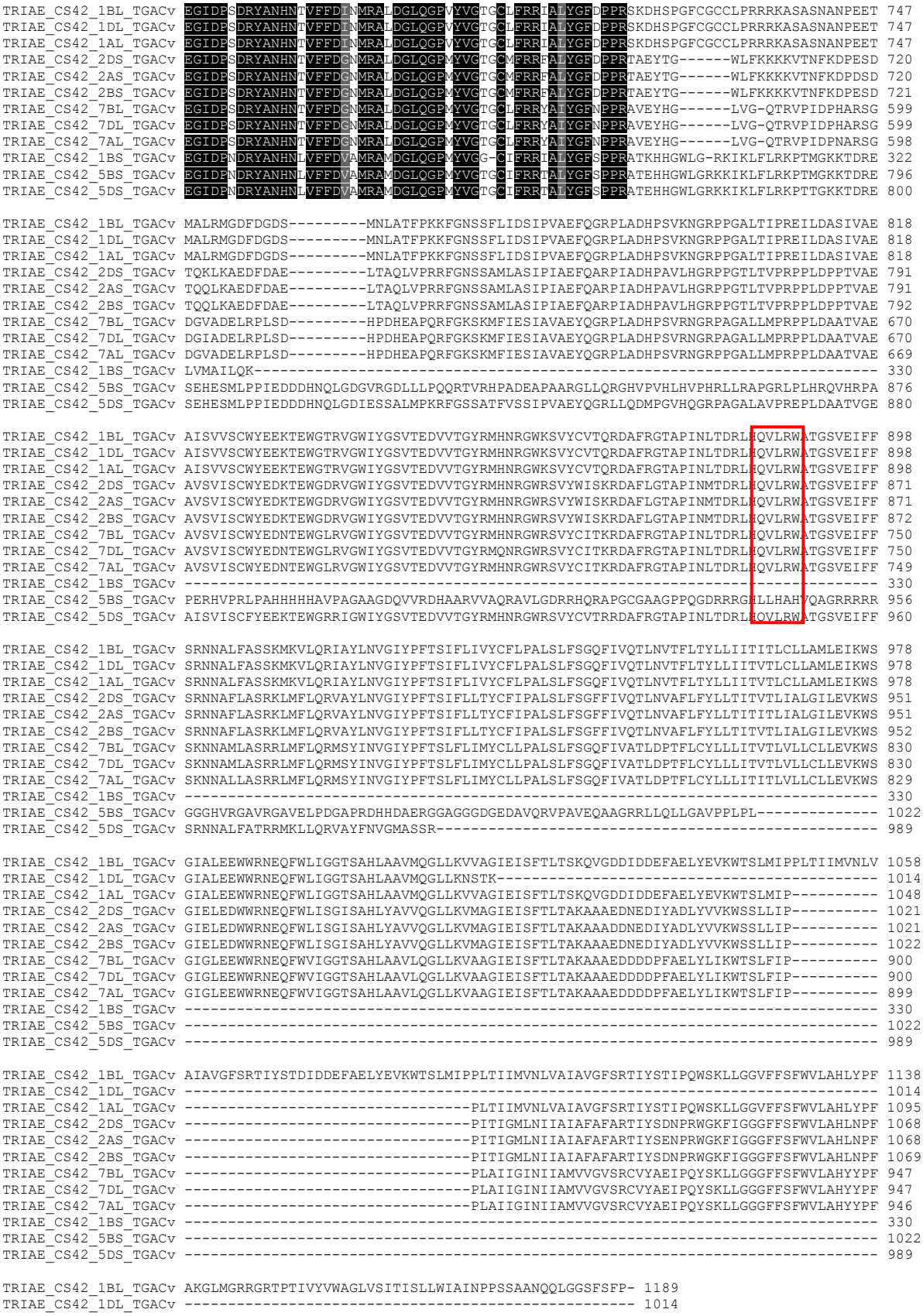



TRIAE_CS42_3AL_TGACv
TRIAE_CS42_1DL_TGACv
TRIAE_CS42_1BL_TGACv
TRIAE_CS42_5BL_TGACv
TRIAE_CS42_5DL_TGACv
TRIAE_CS42_5AL_TGACv
TRIAE_CS42_3DS_TGACv
TRIAE_CS42_3B_TGACv1
TRIAE_CS42_3AS_TGACv

Appendix 6.3 List of *CslD* subfamily genes, their protein length (amino acids) and multiple sequence alignment of amino acids showing the conserved motifs (D, D, DXD, QXXRW) specific to *Cellulose synthase like* (*Csl*) family (highlighted with red boxes).

S.No	Gene name with number of splice variants (CslD)	No. of amino acids (aa)
1	TRIAE_CS42_2BS_TGACv1_148683_AA0494520.1	1121 aa
2	TRIAE_CS42_2DS_TGACv1_177279_AA0572180.1	1120 aa
3	TRIAE_CS42_2AS_TGACv1_114244_AA0365360.1	1120 aa
4	TRIAE_CS42_1BL_TGACv1_030586_AA0094860.1	1189 aa
5	TRIAE_CS42_1AL_TGACv1_001700_AA0034150.2	1146 aa
6	TRIAE_CS42_1DL_TGACv1_063091_AA0223780.1	1014 aa
7	TRIAE_CS42_1BS_TGACv1_049706_AA0160220.1	330 aa
8	TRIAE_CS42_5BS_TGACv1_425241_AA1392650.1	1022 aa
9	TRIAE_CS42_5DS_TGACv1_457675_AA1488780.1	989 aa
10	TRIAE_CS42_7BL_TGACv1_577301_AA1871610.1	994 aa
11	TRIAE_CS42_7AL_TGACv1_559436_AA1799630.1	993 aa
12	TRIAE_CS42_7DL_TGACv1_603510_AA1985050.1	994 aa


TRIAE_CS42_5DS_TGACv DMPAFLNAGRGGRPPCDCGFMICEECYMDCVAAAGNCPGCKEAYSAGSDTDDSVDEDDDDAISSSEERDQMPMTSMSKRF 240
TRIAE_CS42_1BL_TGACv LVKQGTMNNQS----------GEFDHNRWLFETKGTYGYGNAIWPD-------DNVDDDGRNGVPGHPKELMSKPWRPLT 274
TRIAE_CS42_1DL_TGACv LVKQGTMNNQS----------GEFDHNRWLFETKGTYGYGNAIWPD-------DNVDDDGRNGVPGHPKELMSKPWRPLT 274
TRIAE_CS42_1AL_TGACv LVKQGTMNNQS----------GEFDHNRWLFETKGTYGYGNAIWPD-------DNVDDDGRNGVPGHPKELMSKPWRPLT 274
TRIAE_CS42_2DS_TGACv AGKSLLARNQN----------GEFDHNRWLFESSGTYGYGNAFMPKG--GMYEDDLDEDGAAGDD-GMQDMNQKPFKPLT 256
TRIAE_CS42_2AS_TGACv SGKSLLARNQN----------GEFDHNRWLFESSGTYGYGNAFMPKG--GMYEDDLDEDGVGGDG-GMQDMNQKPFKPLT 256
TRIAE_CS42_2BS_TGACv AGKSLLARNQN----------GEFDHNRWLFESSGTYGYGNAFMPKG--GMYEDDLDEDGAGGDGGMPADLSQKPFKPLT 257
TRIAE_CS42_7BL_TGACv PSKSLLVRSQT----------GEFDHNRWLFETQGTYGIGNAYWPQ------DENDDGAGMGGGSVKMEDLVDKPWKPLS 139
TRIAE_CS42_7DL_TGACv PSKSLLVRSQT----------GEFDHNRWLFETQGTYGIGNAYWPQ------DDNDDGAGMGGGSVKMEDLVDKPWKPLS 139
TRIAE_CS42_7AL_TGACv PSKSLLVRSQT----------GEFDHNRWLFETQGTYGIGNAYWPQ------EDNDDGAGMGGGSVKMEDLVDKPWKPLS 138
TRIAE_CS42_1BS_TGACv SMVHSIKMPMSSSND----KPADFDHARWLFETKGTYSYGNALWPENEHGGGGNNAGATFGFVGIEEPPNF--------- 145
TRIAE_CS42_5BS_TGACv SMVHSIKMPMPSSNG----KPADFDHARWLFETKGTYGYGNALWPKNEHGGGGNNAGATSGFVGIEEPPNFGARCRRPLT 316
TRIAE_CS42_5DS_TGACv SMVHSIKMPMPSSNGNGGGKPADFDHARWLFETKGTYGYGNALWPKNEHGGGGNTAGATSGFVGIEEPPNFGARCRRPLT 320
TRIAE_CS42_1BL_TGACv RKLQIPAAVISPYRLLVLIRLVALAFFLMWRIKHQNDDAIWLWGMSIVCELWFAFSWVLDQLPKLCPINRATDLSVLKEK 354
TRIAE_CS42_1DL_TGACv RKLQIPAAVISPYRLLVLIRLVALAFFLMWRIKHQNDDAIWLWGMSIVCELWFALSWVLDQLPKLCPINRATDLSVLKEK 354
TRIAE_CS42_1AL_TGACv RKLQIPAAVISPYRLLVLIRLVALAFFLMWRIKHQNDDAIWLWGMSIVCELWFALSWVLDQLPKLCPINRATDLSVLKEK 354
TRIAE_CS42_2DS_TGACv RKIPMPASIISPYRIFIVIRFFVLIFYLTWRIRNPNMEALWLWGMSIVCELWFAFSWLLDMLPKVNPINRSTDLAVLKEK 336
TRIAE_CS42_2AS_TGACv RKIPMPTSIISPYRIFIVIRFFVLIFYLTWRIRNPNMEALWLWGMSIVCELWFAFSWLLDMLPKVNPINRSTDLAVLKEK 336
TRIAE_CS42_2BS_TGACv RKIPMPTSIISPYRIFIVIRFFVLIFYLTWRIRNPNMEALWLWGMSIVCELWFAFSWLLDMLPKVNPINRSTDLAVLKEK 337
TRIAE_CS42_7BL_TGACv RKVAIPPGILSPYRLLVLVRFVALFLFLIWRATNPNPDAMWLWGISIVCEYWFALSWLLDQMPKLNPINRAADLAALREK 219
TRIAE_CS42_7DL_TGACv RKVAIPPGILSPYRLLVLVRFVALFLFLVWRATNPNPDAMWLWGISIVCEYWFALSWLLDQMPKLNPINRAADLAALREK 219
TRIAE_CS42_7AL_TGACv RKVAIPPGILSPYRLLVLVRFVALFLFLVWRATNPNPDAMWLWGISIVCEYWFALSWLLDQMPKLNPINRAADLAALREK 218
TRIAE_CS42_1BS_TGACv ---------- 145
TRIAE_CS42_5BS_TGACv RKTSVSQAILSPYRMLIAIRLVALGFFLAWRIRHPNPDAMWLWALSVTCEVWFAFSWLLDSLPKLCPVNRSCDLDVLADR 396
TRIAE_CS42_5DS_TGACv RKTSVSQAILSPYRMLIAIRLVALGFFLAWRIRHPNPDAMWLWALSVTCEVWFAFSWLLDSLPKLCPVNRSCDLDVLADR 400
TRIAE_CS42_1BL_TGACv FETPTPSNPTGKSDLPGIDIFVSTADPEKEPVLVTANTILSILAVDYPVDKLACYVSDDGGALLTFEAMAEAASFANFWV 434
TRIAE_CS42_1DL_TGACv FETPTPSNPTGKSDLPGIDIFVSTADPEKEPVLVTANTILSILAVDYPVDKLACYVSDDGGALLTFEAMAEAASFANFWV 434
TRIAE_CS42_1AL_TGACv FETPTPSNPTGKSDLPGIDIFVSTADPEKEPVLVTANTILSILAVDYPVDKLACYVSDDGGALLTFEAMAEAASFANFWV 434
TRIAE_CS42_2DS_TGACv FETPSPSNPHGRSDLPGLDVFVSTADPEKEPVLTTANTILSILAVDYPVEKLACYVSDDGGALLTFEAMAEAASFANIWV 416
TRIAE_CS42_2AS_TGACv FETPSPSNPHGRSDLPGLDIFVSTADPEKEPVLTTANTILSILAVDYPVEKLACYVSDDGGALLTFEAMAEAASFANIWV 416
TRIAE_CS42_2BS_TGACv FETHSPSNPHGRSDLPGLDVFVSTADPEKEPVLTTANTILSILAVDYPVEKLACYVSDDGGALLTFEAMAEAASFANIWV 417
TRIAE_CS42_7BL_TGACv FESKTPSNPTGRSDLPGLDVFISTADPYKEPPLVTANTLLSILATDYPVEKLFVYISDDGGALLTFEAMAEACAYAKVWV 299
TRIAE_CS42_7DL_TGACv FESKTPSNPTGRSDLPGLDVFISTADPYKEPPLVTANTLLSILATDYPVEKLFVYISDDGGALLTFEAMAEACAYAKVWV 299
TRIAE_CS42_7AL_TGACv FESKTPSNPTGRSDLPGLDVFISTADPYKEPPLVTANTLLSILATDYPVEKLFVYISDDGGALLTFEAMAEACAYAKVWV 298
TRIAE_CS42_1BS_TGACv ---------- 145
TRIAE_CS42_5BS_TGACv FELPTARNPKGRSDLPGIDVFVSTADPEKEPPLVTANTILSILAADYPVEKLACYLSDDGGALLTFEALAETASFARTWV 476
TRIAE_CS42_5DS_TGACv FELPTARNPKGRSDLPGIDVFVSTADPEKEPPLVTANTILSILAADYPVEKLACYLSDDGGALLTFEALAETASFARTWV 480
TRIAE_CS42_1BL_TGACv PFCRKHDIEPRNPDSYFNLKRDPFKNKVKADFVKDRRRIKREYDEFKVRVNGLPDSIRRRSDAYHAREEIQAMNLQREKI 514
TRIAE_CS42_1DL_TGACv PFCRKHDIEPRNPDSYFNLKRDPFKNKVKADFVKDRRRIKREYDEFKVRVNGLPDSIRRRSDAYHAREEIQAMNLQREKI 514
TRIAE_CS42_1AL_TGACv PFCRKHDIEPRNPDSYFNLKRDPFKNKVKADFVKDRRRIKREYDEFKVRVNGLPDSIRRRSDAYHAREEIQAMNLQREKI 514
TRIAE_CS42_2DS_TGACv PFCKKHDIEPRNPDSYFALKGDPTKGKRRSDFVKDRRKVKREYDEFKVRINGLPDSIRRRSDAFNAREDMKML----KHL 492
TRIAE_CS42_2AS_TGACv PFCKKHDIEPRNPDSYFALKGDPTKGKRRSDFVKDRRKVKREYDEFKVRINGLPDSIRRRSDAFNAREDMKML----KHL 492
TRIAE_CS42_2BS_TGACv PFCKKHDIEPRNPDSYFALKGDPTKGKRRSDFVKDRRKVKREYDEFKVRINGLPDSIRRRSDAFNAREDMKML----KHL 493
TRIAE_CS42_7BL_TGACv PFCRKHSIEPRNPEAYFTQKGDPTKGKKRPDFVKDRRWIKREYDEYKVRINDLPEAIKRRAKAMNAHERKIAR----ETA 375
TRIAE_CS42_7DL_TGACv PFCRKHSIEPRNPEAYFTQKGDPTKGKKRPDFVKDRRWIKREYDEYKVRINDLPEAIKRRAKAMNAHERKIAR----ETA 375
TRIAE_CS42_7AL_TGACv PFCRKHSIEPRNPEAYFTQKGDPTKGKKRPDFVKDRRWIKREYDEYKVRINDLPEAIKRRAKAMNAHERKIAR----ETA 374
TRIAE_CS42_1BS_TGACv ---------- 145
TRIAE_CS42_5BS_TGACv PFCRKHGVEPRCPESYFGQKRDFLKNKVRLDFVRERRKVKREYDEFKVRVNSLTEAIRRRSDAYNAGEELRARRRLQEEA 556
TRIAE_CS42_5DS_TGACv PFCRKHGVEPRCPESYFGQKRDFLKNKVRLDFVRERRKVKREYDEFKVRVNSLTEAIRRRSDAYNAGEELRARRRLQEEA 560
TRIAE_CS42_1BL_TGACv KAGGDEQFEPV----KIPKATWMADSTHWPGTWIHSSQDHARGDHAGIIQVMLKPPSDMPMYG--NIEK-SPLDFSEVDT 587
TRIAE_CS42_1DL_TGACv KAGGDEQFEPV----KIPKATWMADSTHWPGTWIHSSQDHARGDHAGIIQVMLKPPSDMPMYG--NIEK-SPLDFSEVDT 587
TRIAE_CS42_1AL_TGACv KAGGDEQFEPV----KIPKATWMADSTHWPGTWIHPSQDHARGDHAGIIQVMLKPPSDMPMYG--NIEK-SPLDFSGVDT 587
TRIAE_CS42_2DS_TGACv RETGADPSEQP----KVKKATWMADGTHWPGTWAVSSPDHAKGNHAGILQVMLRPPSPDPLYG--MHDEDQLIDYSDVDT 566
TRIAE_CS42_2AS_TGACv RETGADPSEQP----KVKKATWMADGTHWPGTWAVSSPDHAKGNHAGILQVMLRPPSPDPLYG--MHDEDQLIDYSDVDT 566
TRIAE_CS42_2BS_TGACv RETGADPSEQP----KVKKATWMADGTHWPGTWAVSSPDHAKGNHAGILQVMLRPPSPDPLYG--MHDEDQLVDYSDVDT 567
TRIAE_CS42_7BL_TGACv AAS---SDAAP----PPVKATWMADGTHWPGTWLDSAPDHGKGDHASIVQVMIKNPHHDVVYG--EADDHAYLDFTNVDV 446
TRIAE_CS42_7DL_TGACv AAS---SDAAP----PPVKATWMADGTHWPGTWLDSAPDHGKGDHASIIQVMIKNPHHDVVYG--DADDHAYLDFTNVDV 446
TRIAE_CS42_7AL_TGACv AAS---SDAAP----PPVKATWMADGTHWPGTWLDSAPDHGKGDHASIVQVMIKNPHHDVVYG--DADDHAYLDFTNVDV 445
TRIAE_CS42_1BS_TGACv ----------ATWMSDGSQWASTWLAGATDHARGNHAGIIQ---------- 176
TRIAE_CS42_5BS_TGACv VAAGGALGTAPLAETGAVKATWMSDGSQWPGTWLTGATDHARGNHAGIIQAMLAPPTSEPVLGGEPAESGALIDTTGVDI 636
TRIAE_CS42_5DS_TGACv VAAGGALGAAPLAETGAVKGTWMSDGSQWPGTWLTGATDHARGDHAGIIQAMLAPPTSEPVLGGVPAESGALIDTTGVDI 640
TRIAE_CS42_1BL_TGACv RLPMLVYMSREKRPGYDHNKKAGAMNALVRASAIMSNGPFILNLDCDHYVYNSKAFREGMCFMMDRGGDRLCYVQFPQRF 667
TRIAE_CS42_1DL_TGACv RLPMLVYMSREKRPGYDHNKKAGAMNALVRASAIMSNGPFILNLDCDHYVYNSKAFREGMCFMMDRGGDRLCYVQFPQRF 667
TRIAE_CS42_1AL_TGACv RLPMLVYMSREKRPGYDHNKKAGAMNALVRASAIMSNGPFILNLDCDHYVYNSKAFREGMCFMMDRGGDRLCYVQFPQRF 667
TRIAE_CS42_2DS_TGACv RLPMLVYMSREKRPGYDHNKKAGAMNALVRCSAVMSNAPFILNLDCDHYINNNQAVREAMCFMMDRGGERICYIQFPQRF 646
TRIAE_CS42_2AS_TGACv RLPMLVYMSREKRPGYDHNKKAGAMNALVRCSAVMSNAPFILNLDCDHYINNNQAVREAMCFMMDRGGERICFIQFPQRF 646
TRIAE_CS42_2BS_TGACv RLPMLVYMSREKRPGYDHNKKAGAMNALVRCSAVMSNAPFILNLDCDHYINNTQAIREAMCFMMDRGGERICYIQFPQRF 647
TRIAE_CS42_7BL_TGACv RIPMFVYLSREKRPGYDHNKKAGAMNAMVRASAVLSNGPFMLNLDCDHYVYNCQAIREAMCYMLDRGGDRICYIQFPQRF 526
TRIAE_CS42_7DL_TGACv RIPMFVYLSREKRPGYDHNKKAGAMNAMVRASAVLSNGPFMLNLDCDHYVYNCQAIREAMCYMLDRGGDRICYIQFPQRF 526
TRIAE_CS42_7AL_TGACv RIPMFVYLSREKRPGYDHNKKAGAMNAMVRASAVLSNGPFMLNLDCDHYVYNCQAIREAMCYMLDRGGDRICYIQFPQRF 525
TRIAE_CS42_1BS_TGACv ----------RPGYNHNKKAGAMNALVRTSAIMSNGPFILNLDCDHYVHNSAALREGMCYMLDCRGDRVCYVQFPQRF 244
TRIAE_CS42_5BS_TGACv RLPMLVYVSREKRPGYDHNKKAGAMNALVRTSAIMSNGPFILNLDCDHYVHNSAALREGMCYMLDRGGDRVCYVQFPQRF 716
TRIAE_CS42_5DS_TGACv RLPMLVYVSREKRPGYDHNKKAGAMNALVRTSAIMSNGPFILNLDCDHYVHNSAALREGMCYMLDRGGDRVCYVQFPQRF 720



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TRIAE_CS42_2AS_TGACv AKGLMGRRGKTPTIIFVWSGLISITISLLWVALSPPEANSTGGARSGGFQFP 1120
TRIAE_CS42_2BS_TGACv AKGLMGRRGKTPTIIFVWSGLISITISLLWVALSPPEANSTGGARGGGFQFP 1121
TRIAE_CS42_7BL_TGACv AKGLMGRRGRTPTIVYVWAGLISITVSLLWITISPPDDRVSQSGIEV----- 994
TRIAE_CS42_7DL_TGACv AKGLMGRRGRTPTIVYVWAGLISITVSLLWITISPPDDRVSQSGIEV----- 994
TRIAE_CS42_7AL_TGACv AKGLMGRRGRTPTIVYVWAGLISITVSLLWITISPPDDRVSQSGIEV----- 993
TRIAE_CS42_1BS_TGACv -------------------------------------------------- 330
TRIAE_CS42_5BS_TGACv -------------------------------------------------- 1022
TRIAE_CS42_5DS_TGACv -------------------------------------------------- 989
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Appendix 6.4 List of *CslE* subfamily genes, their protein length (amino acids) and multiple sequence alignment of amino acids showing the conserved motifs (D, D, DXD, QXXRW) specific to *Cellulose synthase like* (*Csl*) family (highlighted with red boxes).

S.No	Gene name with number of splice variants (CslE)	No. of amino acids (aa)
1	TRIAE_CS42_6DL_TGACv1_526558_AA1687090.1	738 aa
2	TRIAE_CS42_6AL_TGACv1_471004_AA1500600.1	737 aa
3	TRIAE_CS42_6BL_TGACv1_499967_AA1596110.2	736 aa
4	TRIAE_CS42_U_TGACv1_683314_AA2158770.1	446 aa
5	TRIAE_CS42_5DL_TGACv1_433536_AA1415840.1	756 aa
6	TRIAE_CS42_5BL_TGACv1_406235_AA1342610.1	728 aa
7	TRIAE_CS42_5AL_TGACv1_376126_AA1232370.2	728 aa
8	TRIAE_CS42_5DL_TGACv1_433536_AA1415830.1	728 aa
9	TRIAE_CS42_5BL_TGACv1_406235_AA1342600.1	734 aa
10	TRIAE_CS42_6DS_TGACv1_543277_AA1737920.1	725 aa

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Color Align Conservation results
TRIAE_CS42_5DL_TGACv MVAIGRRTGQQHGHWRLAAESPPYLGPRDGEEHEAVRDGDSRGPGGVQAPRRHGGRRILLLLYYRATRVPAAGEGRAAWL 80
TRIAE_CS42_5BL_TGACv ----------------------------MERSRRLFETETHGGRAAYRLHAVTVAAGILLVLYYRATHVPAAGEGRATWL 52
TRIAE_CS42_U_TGACv1_ ---------------------------------------------------------------------------- 0
TRIAE_CS42_5AL_TGACv ----------------------------MERTRLFETETHGGRAAYRLHAVTVAAGILLLLYYRATRVPAAGEGRAAWL 51
TRIAE_CS42_6DS_TGACv ----------------------------MERRLFETVRHGGRALYRLHAVTVAASTLLVLYYRATRVPGSGGRRAAWL 50
TRIAE_CS42_5DL_TGACv ----------------------------MERTRRLFETETHGGRAAYRLHAVTVAAGILLLLYYRATHVPAAGEGRAAWL 52
TRIAE_CS42_5BL_TGACv ----------------------------MERSRRLFETETHGGRAVYRLHAVTVAAGILLLLYYRATRVPAAGEGRAAWL 52
TRIAE_CS42_6AL_TGACv --------------------MAGSSVSGGGGRPPLFATEKPKRVLAYRVYAGTIFAGILLIWFYRATHIPARGSSSLGWR 60
TRIAE_CS42_6BL_TGACv ----------------------MAGSSVSGGGRPPLFATEKPKRVLAYRLYAGTIFAGILLIWFYRATHIPERGDSSLGWR 59
TRIAE_CS42_6DL_TGACv -------------------MAGSSVSGGGGGRPPLFATEKPKRVLAYRLYAGTIFAGILLIWFYRATHIPARGSSSLGWR 61

TRIAE_CS42_5DL_TGACv G---MLAAELWYAAYWVVTQSVRWSPVRRRPFIDRLAARHG-ETLPCVDIFVCTADPYSEPPSLVVSTILSLMAYNYPPE 156
TRIAE_CS42_5BL_TGACv G---MLAAELWYAAYWVVTQSVRWSPVRRRPFIDRLAARHG-ERLPCVDIFVCTADPYSEPPSLVVSTILSLMAYNYPPE 128
TRIAE_CS42_U_TGACv1_ ---------------------------------------------------------------------------- 0
TRIAE_CS42_5AL_TGACv G---MLAAELWYAAYWVVTQSVRWSPVRRRPFRDRLAARHG-ERLPSVDIFVCTADPYSEPPSLVVSTILSLMAYNYPPE 127
TRIAE_CS42_6DS_TGACv G---MLAAELWYAAYWVVTQSVRWSPVRRCTFRDRLTARYG-DRLPGVDIFVCTADPLSEPPSLVISTILSVMAYNYLAE 126
TRIAE_CS42_5DL_TGACv G---MLAAELWYAAYWAVTQSVRWSPVRRLPFIDRLAARYG-ERLPCVDIFVCTADPHSEPPSLVISTVLSLMAYNYPAE 128
TRIAE_CS42_5BL_TGACv G---MLAAELCYAAYWVVTQSVRWSPLHRRPCRDRLAARYG-ERLPCVDIFVCTADPHSEPPSLVISTVLSLMAYNYPAE 128
TRIAE_CS42_6AL_TGACv AGLGLLVAEILFGLYWVLTLSVRWNPVRRTTFKDRLSERYDDDQLPGVDIFVCTADPALEPPMLVISTVLSVMAYDYPPE 140
TRIAE_CS42_6BL_TGACv AGLGLLVAELLFGLYWVLTLSVRWNPVRRTTFKDRLSERYDDDQLPGVDIFVCTADPALEPPMLVISTVLSVMAYDYPPE 139
TRIAE_CS42_6DL_TGACv AGLGLLVAELWFGLYWVLTLSVRWNPVRRATFKDRLSERYDDDQLPGVDIFVCTADPALEPPMLVISTVLSVMAYDYPPE 141

TRIAE_CS42_5DL_TGACv KLSVYLSDDGGSILTFYGMWEASLFAKHWLPFCKRYNIEPRSPAAYFSESDGHQELCNPKEWSLIKDMFDKMTERIDTVV 236
TRIAE_CS42_5BL_TGACv KLSVYLSDDGGSILTLYGMWEASLFAKHWLPFCKRYNIEPRSPAAYFSESDGHQELCTPKEWSLIKDMFDKMTERIDTAV 208
TRIAE_CS42_U_TGACv1_ ---------------------------------------------------------------------------- 0
TRIAE_CS42_5AL_TGACv KLSVYLSDDGGSILTYYGMWEASLFAKHWLPFCKRYNIEPRSPAAYFSQSDGHQELCTPKEWSLIKDMFDEMTERIDTAV 207
TRIAE_CS42_6DS_TGACv KLSVYLSDDGGSVLTFYAMWEASLFAKHWLPFCKRYNIEPRSPAAYFSES--YQDLCTPKEWSFIKDMYDEMTERIDTAV 204
TRIAE_CS42_5DL_TGACv KISVYLSDDGGSVLTFYALWEASLFAKHWIPFCKRYNIEPRSPAAYFSESDGHQDLCSPKEWSLIREMYEDMTERIDTAV 208
TRIAE_CS42_5BL_TGACv KISVYLSDDGGSILTFYALWEASLFAKHWIPFCKRYNIEPRSPATYFSESDGHQDMCTPKEWSLIREMYEDMTERIDTAA 208
TRIAE_CS42_6AL_TGACv KLNIYLSDDAGSAVTFYALHEASEFAKHWIPFCKNYKVEPRSPAAYFAEGATPHDACSPQELLRMKELYKDLTDRVNSVV 220
TRIAE_CS42_6BL_TGACv KLNIYLSDDAGSAVTFYALHEASEFAKHWIPFCKNYKVEPMSPAAYFAEGATPHDACSPQELLRMKELYKDLTDRVNSVV 219
TRIAE_CS42_6DL_TGACv KLNIYLSDDAGSAVTFYALHEASEFAKHWIPFCKNYKVEPRSPAAYFAKGATPHDACSPQEFLRMKELYKDLTDRMNSVV 221

TRIAE_CS42_5DL_TGACv MSGKVPEEIKASHKGFYEWNQEITSKNHQPIVQILIDSKDQNAVDNEGKVLPTLVYMAREKRPQHHHNFKAGAMNALIRV 316
TRIAE_CS42_5BL_TGACv MSGKVPEEIKARHKGFYEWNQEISSKNHQPIVQILIDGKDQNAVDNEGKVLPTLVYMAREKRPQHHHNFKAGAMNALIRV 288
TRIAE_CS42_U_TGACv1_ ----------------------------------------------------------------------MQIRV 5
TRIAE_CS42_5AL_TGACv MSGKVPEEIKARQKGFHEWNQEITSKNHQPIVQILIDGKDQNAVDNEGNVLPTLVYMAREKRPQHHHNFKAGAMNALIRV 287
TRIAE_CS42_6DS_TGACv ISRKIPEEIRSNHKGFYEWNPEITSKNHQPIVQVLIDGKDQKGVDSEGNVLPTLVYMAREKRPQHHHNFKAGAMNALIRV 284
TRIAE_CS42_5DL_TGACv LSGKISEEVKANHKGFHEWDQENTSKNHQPIVQILIEGKDKNANDDEGNVLPTLVYMAREKRPQHHHNFKAGAMNALIRV 288
TRIAE_CS42_5BL_TGACv LSGKISEEVKENHKGFHEWDQENTSKNHQPIVQILIEGKDKNANDDEGNVLPTLVYMAREKRPQHHHNFKAGAMNALIRV 288
TRIAE_CS42_6AL_TGACv HSGKIPEVPECNHRGSSEWNEMITSGDHPSIVQILIDRNKRKAVDVDGNALPKLVYMSREKRPQEQHHFKAGSLNALIRV 300
```

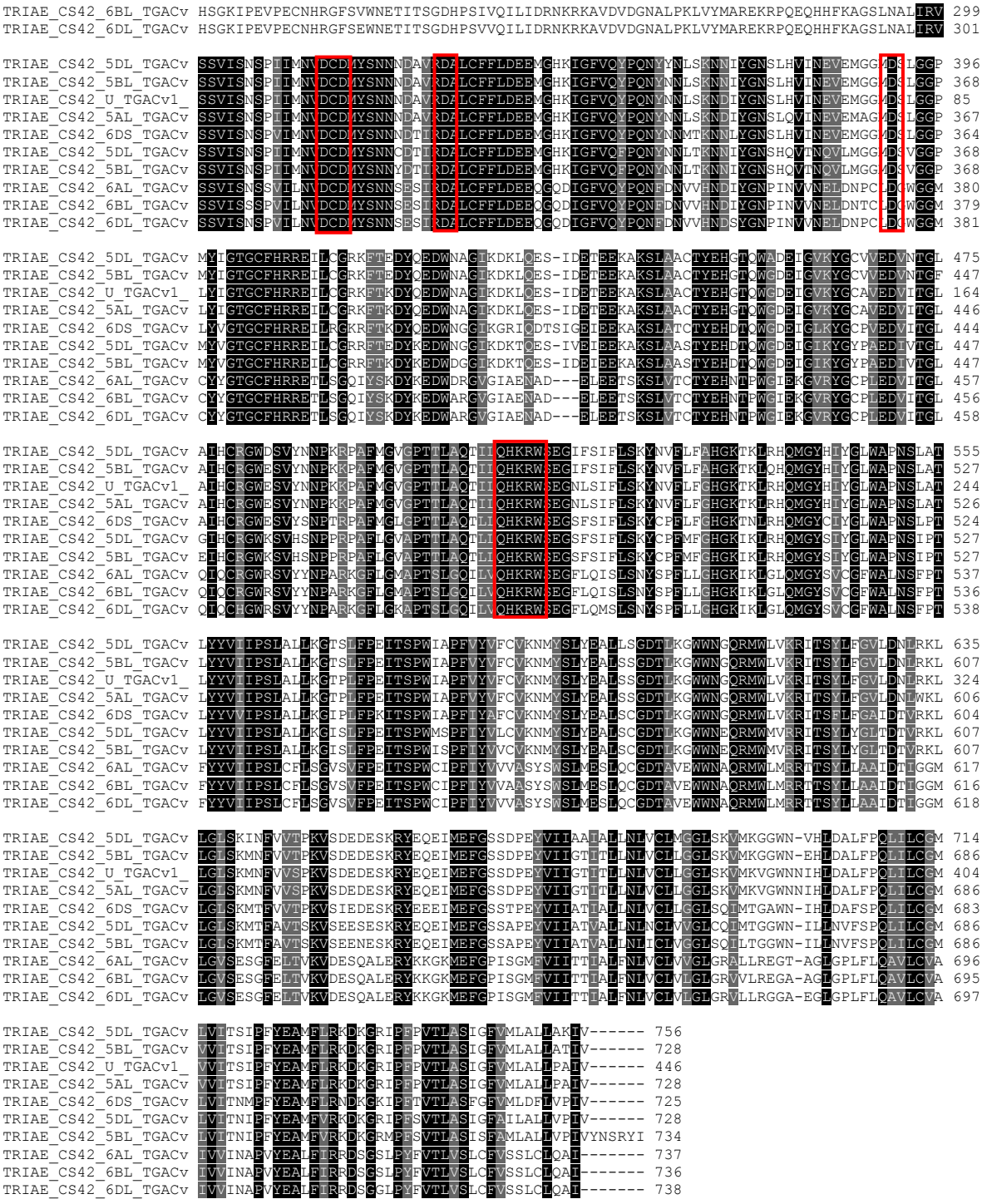
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TRIAE_CS42_6DL_TGACv HSGKIPEVPECNHRGFSEWNETITSGDHPSVVQILIDRNKRKAVDVDGNALPKLVYMAREKRPQEQHHFKAGSLNALIRV 301
TRIAE_CS42_5DL_TGACv SSVISNSPIIMNVDCDMYSNNNDAVRDALCFFLDEEMGHKIGFVQYPQNYNNLSKNNIYGNSLHVINEVEMGGMDSLGGP 396
TRIAE_CS42_5BL_TGACv SSVISNSPIIMNVDCDMYSNNNDAVRDALCFFLDEEMGHKIGFVQYPQNYNNLSKNNIYGNSLHVINEVEMGGMDSLGGP 368
TRIAE_CS42_U_TGACv1_ SSVISNSPIIMNVDCDMYSNNNDAVRDALCFFLDEEMGHKIGFVQYPQNYNNLSKNDIYGNSLHVINEVEMGGMDSLGGP 85
TRIAE_CS42_5AL_TGACv SSVISNSPIIMNVDCDMYSNNNDAVRDALCFFLDEEMGHKIGFVQYPQNYNNLSKNDIYGNSLQVINEVEMAGMDSLGGP 367
TRIAE_CS42_6DS_TGACv SSVISNSPVIMNVDCDMYSNNNDTIRDALCFFLDEEMGHKIGFVQYPQNYNNMTKNNLYGNSLHVINEVEMGGMDSLGGP 364
TRIAE_CS42_5DL_TGACv SSVISNSPIIMNVDCDMYSNNCDTIRDALCFFLDEEMGHKIGFVQFPQNYNNLTKNNIYGNSHQVINQVLMGGMDSVGGP 368
TRIAE_CS42_5BL_TGACv SSVISNSPIIMNVDCDMYSNNYDTIRDALCFFLDEEMGHKIGFVQFPQNYNNLTKNNIYGNSHQVINQVLMGGMDSVGGP 368
TRIAE_CS42_6AL_TGACv SSVISNSSVILNVDCDMYSNNSESIRDALCFFLDEEQGQDIGFVQYPQNFDNVVHNDIYGNPINVVNELDNPCLDQWGGM 380
TRIAE_CS42_6BL_TGACv SSVISSSPVILNVDCDMYSNNSESIRDALCFFLDEEQGQDIGFVQYPQNFDNVVHNDIYGNPINVVNELDNTCLDQWGGM 379
TRIAE_CS42_6DL_TGACv SSVISNSPVILNVDCDMYSNNSESIRDALCFFLDEEQGQDIGFVQYPQNFDNVVHNDSYGNPINVVNELDNPCLDQWGGM 381
TRIAE_CS42_5DL_TGACv MYIGTGCFHRREILCGRKFTEDYQEDWNAGIKDKLQESIDETEEKAKSLAACTYEHGTQWADEIGVKYGCVVEDVNTGL 475
TRIAE_CS42_5BL_TGACv MYIGTGCFHRREILCGRKFTEDYQEDWNAGIKDKLQESIDETEEKAKSLAACTYEHGTQWGDEIGVKYGCVVEDVNTGF 447
TRIAE_CS42_U_TGACv1_ LYIGTGCFHRREILCGRKFTKDYQEDWNAGIKDKLQESIDETEEKAKSLAACTYEHGTQWGDEIGVKYGCAVEDVITGL 164
TRIAE_CS42_5AL_TGACv LYIGTGCFHRREILCGRKFTKDYQEDWNAGIKDKLQESIDETEEKAKSLAACTYEHGTQWGDEIGVKYGCAVEDVITGL 446
TRIAE_CS42_6DS_TGACv LYVGTGCFHRREILRGKRFTKDYQEDWNGGIKGRIQDTSIGEIEEKAKSLATCTYEHDTQWGDEIGLKYGCPVEDVITGL 444
TRIAE_CS42_5DL_TGACv MYVGTGCFHRREILCGRRFTEDYKEDWNGGIKDKTQESIVEIEEKAKSLAASTYEHDTQWGDEIGIKYGYPAEDIVTGL 447
TRIAE_CS42_5BL_TGACv MYVGTGCFHRREILCGRRFTKDYKEDWDGGIKDKTQESIDEIEEKAKSLAASTYEHDTQWGDEIGIKYGYPAEDIVTGL 447
TRIAE_CS42_6AL_TGACv CYYGTGCFHRRETLSGQIYSKDYKEDWDRGVGIAENAD---ELEETSKSLVTCTYEHNTPWGIEKGVRYGCPLEDVITGL 457
TRIAE_CS42_6BL_TGACv CYYGTGCFHRRETLSGQIYSKDYKEDWARGVGIAENAD---ELEETSKSLVTCTYEHNTPWGIEKGVRYGCPLEDVITGL 456
TRIAE_CS42_6DL_TGACv CYYGTGCFHRRETLSGQIYSKDYKEDWARGVGIAENAD---ELEETSKSLVTCTYEHNTPWGIEKGVRYGCPLEDVITGL 458
TRIAE_CS42_5DL_TGACv AIHCRGWDSVYNNPKKPAFMGVGPTTLAQTIIQHKRWSEGIFSIFLSKYNVFLFAHGKTKLRHQMGYHIYGLWAPNSLAT 555
TRIAE_CS42_5BL_TGACv AIHCRGWESVYNNPKKPAFMGVGPTTLAQTIIQHKRWSEGIFSIFLSKYNVFLFAHGKTKLQHQMGYHIYGLWAPNSLAT 527
TRIAE_CS42_U_TGACv1_ AIHCRGWESVYNNPKKPAFMGVGPTTLAQTIIQHKRWSEGNLSIFLSKYNVFLFGHGKTKLRHQMGYHIYGLWAPNSLAT 244
TRIAE_CS42_5AL_TGACv AIHCRGWESVYNNPKKPAFMGVGPTTLAQTIIQHKRWSEGNLSIFLSKYNVFLFGHGKTKLRHQMGYHIYGLWAPNSLAT 526
TRIAE_CS42_6DS_TGACv AIHCRGWESVYSNPTTPAFMGLGPTTLAQTLIQHKRWSEGSFSIFLSKYCPFLFGHGKTNLRHQMGYCIYGLWAPNSLPT 524
TRIAE_CS42_5DL_TGACv GIHCRGWKSVHSNPPPPAFLGVAPTTLAQTLIQHKRWSEGSFSIFLSKYCPFMFGHGKIKLRHQMGYSIYGLWAPNSIPT 527
TRIAE_CS42_5BL_TGACv EIHCRGWKSVHSNPPPPAFLGVAPTTLAQTLIQHKRWSEGSFSIFLSKYCPFMFGHGKIKLRHQMGYSIYGLWAPNSIPT 527
TRIAE_CS42_6AL_TGACv QIQCRGWRSVYYNPAKKGFLGMAPTSLGQILVQHKRWSEGFLQISLSNYSPFLLGHGKIKLGLQMGYSVCGFWALNSFPT 537
TRIAE_CS42_6BL_TGACv QIQCRGWRSVYYNPAKKGFLGMAPTSLGQILVQHKRWSEGFLQISLSNYSPFLLGHGKIKLGLQMGYSVCGFWALNSFPT 536
TRIAE_CS42_6DL_TGACv QIQCHGWRSVYYNPAKKGFLGKAPTSLGQILVQHKRWSEGFLQMSLSNYSPFLLGHGKIKLGLQMGYSVCGFWALNSFPT 538
TRIAE_CS42_5DL_TGACv LYYVIIPSLALLKGTSLFPEITSPWIAPFVYVFCVKNMYSIYEALSGDTIKGWWNGQRMWLVKRITSYLFGVLDNLRKL 635
TRIAE_CS42_5BL_TGACv LYYVIIPSLALLKGTSLFPEITSPWIAPFVYVFCVKNMYSIYEALSSGDTIKGWWNGQRMWLVKRITSYLFGVLDNLRKL 607
TRIAE_CS42_U_TGACv1_ LYYVIIPSLALLKGTPLFPEITSPWIAPFVYVFCVKNMYSIYEALSSGDTIKGWWNGQRMWLVKRITSYLFGVLDNLRKL 324
TRIAE_CS42_5AL_TGACv LYYVIIPSLALLKGTPLFPEITSPWIAPFVYVFCVKNMYSIYEALSSGDTIKGWWNGQRMWLVKRITSYLFGVLDNLWKL 606
TRIAE_CS42_6DS_TGACv LYYVVIPSLALLKGIPLFPKITSPWIAPFIYAFCVKNMYSIYEALSCGDTIKGWWNGQRMWLVKRITSFLFGAIDTVRKL 604
TRIAE_CS42_5DL_TGACv LYYVIIPSLALLKGISLFPEITSPWMSPFIYVLCVKNMYSIYEALSCGDTIKGWWNEQRMWMVRRITSYLYGLTDTVRKL 607
TRIAE_CS42_5BL_TGACv LYYVIIPSLALLKGISLFPEITSPWISPFIYVVCVKNMYSIYEALSCGDTIKGWWNEQRMWMVRRITSYLYGLTDTVRKL 607
TRIAE_CS42_6AL_TGACv FYYVIIPSLCFLSGVSVFPEITSPWCIPFIYVVVASYSWSIMESLQCGDTAVEWWNAQRMWLMRRTTSYLLAAIDTIGGM 617
TRIAE_CS42_6BL_TGACv FYYVIIPSLCFLSGVSVFPEITSPWCIPFIYVVASYSWSIMESLQCGDTAVEWWNAQRMWLMRRTTSYLLAAIDTIGGM 616
TRIAE_CS42_6DL_TGACv FYYVIIPSLCFLSGVSVFPEITSPWCIPFIYVVVASYSWSIMESLQCGDTAVEWWNAQRMWLMRRTTSYLLAAIDTIGGM 618
TRIAE_CS42_5DL_TGACv LGLSKINFVVTPKVSDEDESKRYEQEIMEFGSSDPEYVIIAAIALLNLVCIMGGLSKVMKGGWN-VHLDALFPQLILCGM 714
TRIAE_CS42_5BL_TGACv LGLSKMNFVVTPKVSDEDESKRYEQEIMEFGSSDPEYVIIGTITLLNLVCILGGLSKVMKGGWN-EHLDALFPQLILCGM 686
TRIAE_CS42_U_TGACv1_ LGLSKMNFVVSPKVSDEDESKRYEQEIMEFGSSDPEYVIIGTITLLNLVCILGGLSKVMKVGWNNIHLDALFPQLILCGM 404
TRIAE_CS42_5AL_TGACv LGLSKMNFVVSPKVSDEDESKRYEQEIMEFGSSDPEYVIIGTITLLNLVCILGGLSKVMKVGWNNIHLDALFPQLILCGM 686
TRIAE_CS42_6DS_TGACv LGLSKMTFVVTPKVSIEDESKRYEEEIMEFGSSTPEYVIIATIALLNLVCILGGLSQIMTGAWN-IHLDAFSPQLILCGM 683
TRIAE_CS42_5DL_TGACv LGLSKMTFAVTSKVSEESESKRYEQEIMEFGSSAPEYVIIATVALLNLNCIVVGLCQIMTGGWN-ILLNVFSPQLILCGM 686
TRIAE_CS42_5BL_TGACv LGLSKMTFAVTSKVSEENESKRYEQEIMEFGSSAPEYVIIATVALLNLICIVGGLSQILTGGWN-ILLNVFSPQLILCGM 686
TRIAE_CS42_6AL_TGACv LGVSESGFELTVKVDESQALERYKKGKMEFGPISGMEVIITTIALPNLVCIVVGLGRALLREGT-AGLGPLFLQAVLCVA 696
TRIAE_CS42_6BL_TGACv LGVSESGFELTVKVDESQALERYKKGKMEFGPISGMEVIITTIALPNLVCIVLGLGRVVLREGA-AGLGPLFLQAVLCVA 695
TRIAE_CS42_6DL_TGACv LGVSESGFELTVKVDESQALERYKKGKMEFGPISGMEVIITTIALPNLVCIVLGLGRVLLRGGA-EGLGPLFLQAVLCVA 697
TRIAE_CS42_5DL_TGACv LVITSIPFYEAMFLRKDKGRIPFPVTLASIGFVMLALLAKHV------ 756
TRIAE_CS42_5BL_TGACv VVITSIPFYEAMFLRKDKGRIPFPVTLASIGFVMLALLATHV------ 728
TRIAE_CS42_U_TGACv1_ VVITSIPFYEAMFLRKDKGRIPFPVTLASIGFVMLALLPAHV------ 446
TRIAE_CS42_5AL_TGACv VVITSIPFYEAMFLRKDKGRIPFPVTLASIGFVMLALLPAHV------ 728
TRIAE_CS42_6DS_TGACv LVITNMPFYEAMFLRNDKGKIPFTVTLASFGFVMLDFLVPHV------ 725
TRIAE_CS42_5DL_TGACv LVITNIPFYEAMFVRKDKGRIPFSVTLASIGFAILALLVPHV------ 728
TRIAE_CS42_5BL_TGACv LVITNIPFYEAMFVRKDKGRMPFSVTLASISFAMLALLVPHVYYNSRYI 734
TRIAE_CS42_6AL_TGACv LVVINAPVYEALFIRRDSGSLPYFVTLVSLCFVSSLCLQAH------- 737
TRIAE_CS42_6BL_TGACv LVVINAPVYEALFIRRDSGSLPYFVTLVSLCFVSSLCLQAH------- 736
TRIAE_CS42_6DL_TGACv LVVINAPVYEALFIRRDSGGLPYFVTLVSLCFVSSLCLQAH------- 738

Appendix 6.5 List of *CslF* subfamily genes, their protein length (amino acids) and multiple sequence alignment of amino acids showing the conserved motifs (D, D, DXD, QXXRW) specific to *Cellulose synthase like* (*Csl*) family (highlighted with red boxes).

S.No	Gene name with number of splice variants (CslF)	No. of amino acids (aa)
1	TRIAE_CS42_2DL_TGACv1_159781_AA0542640.1	845 aa
2	TRIAE_CS42_7BL_TGACv1_580651_AA1914920.1	614 aa
3	TRIAE_CS42_7AL_TGACv1_557532_AA1782680.1	837 aa
4	TRIAE_CS42_7DL_TGACv1_602590_AA1961740.1	835 aa
5	TRIAE_CS42_2AL_TGACv1_094713_AA0301960.1	865 aa
6	TRIAE_CS42_2DL_TGACv1_160109_AA0546890.1	862 aa
7	TRIAE_CS42_2BL_TGACv1_130934_AA0420130.1	663 aa
8	TRIAE_CS42_2DS_TGACv1_178985_AA0603230.1	870 aa
9	TRIAE_CS42_2AS_TGACv1_112790_AA0345230.1	878 aa
10	TRIAE_CS42_2BS_TGACv1_148027_AA0489970.1	877 aa
11	TRIAE_CS42_2AS_TGACv1_113659_AA0359050.1	847 aa
12	TRIAE_CS42_2DS_TGACv1_177641_AA0581710.2	847 aa
13	TRIAE_CS42_2BS_TGACv1_148608_AA0494060.1	851 aa
14	TRIAE_CS42_U_TGACv1_641498_AA2096480.1	857 aa
15	TRIAE_CS42_2BS_TGACv1_146146_AA0456710.1	754 aa
16	TRIAE_CS42_2DS_TGACv1_179076_AA0604160.1	783 aa
17	TRIAE_CS42_2AS_TGACv1_112322_AA0335290.1	878 aa
18	TRIAE_CS42_2BS_TGACv1_147667_AA0486240.1	877 aa
19	TRIAE_CS42_2DS_TGACv1_177329_AA0573830.1	875 aa
20	TRIAE_CS42_2BS_TGACv1_148916_AA0495580.1	701 aa
21	TRIAE_CS42_2DS_TGACv1_178471_AA0596060.1	701 aa
22	TRIAE_CS42_2AS_TGACv1_112322_AA0335280.1	897 aa
23	TRIAE_CS42_5BL_TGACv1_409916_AA1366600.2	815 aa
24	TRIAE_CS42_5DL_TGACv1_433902_AA1424880.1	808 aa
25	TRIAE_CS42_5AL_TGACv1_374191_AA1193100.1	807 aa
26	TRIAE_CS42_7BL_TGACv1_577473_AA1876170.1	941 aa
27	TRIAE_CS42_7AL_TGACv1_555973_AA1751470.1	899 aa
28	TRIAE_CS42_7DL_TGACv1_607937_AA2011180.1	498 aa
29	TRIAE_CS42_1BS_TGACv1_049866_AA0163180.1	856 aa

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Color Align Conservation results
TRIAE_CS42_5DL_TGACv ------------------------------------------------------------MSMTYITKKHDYAATLDEKEP 21
TRIAE_CS42_5AL_TGACv ------------------------------------------------------------MSMTYITKKHDYVASLDGKES 21
TRIAE_CS42_5BL_TGACv ------------------------------------------------------------MSMTYISKKHDYAATLDEKEQ 21
TRIAE_CS42_2AS_TGACv -----------MTTSPATHDGAATGLSEPLLPNRNGVHAGALVVTPVVANGHGGGDKLKGDLKAKDKYWKDVDQPDDVAA 69
TRIAE_CS42_2BS_TGACv -----------MTTSPATAAGAATGLSEPLLSNGNGVHAGALVVTPVVANGHGG-DKLKGDLKAKDKYWKDVDQPDDVAA 68
TRIAE_CS42_2DS_TGACv -----------MTTSPATDAGAATGLSEPLLSNRNGVHAGALVVTPVAANGHGG--------KAKDKYWKDVDQPGDMAV 61
TRIAE_CS42_2AS_TGACv ------------MASAAGAGGANAGLADPLLAS------------------------AKKPVGAKGKHWVAADK-DQRRA 43
TRIAE_CS42_2DS_TGACv ------------MASAAGAGGANAGLADPLLAS------------------------AKKPVGAKGKHWVAADK-DQRRA 43
TRIAE_CS42_2BS_TGACv ------------MASAVGAGGANAGLADPLLASRD--------------------GGAKKPVGAKGKHWVAADK-DQRRA 47
TRIAE_CS42_2DL_TGACv --------------------------------MAAAVTRRSNALRVDVPGGEAVAVSVAADSPVAKRGLGAKDDVWVAADE 49
TRIAE_CS42_2BL_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_2AL_TGACv ------------------------------MMAAAVTRRSNALRVDVPDGDAVAVSVVADSPVAKRGLGAKEDVWVAVDE 50
TRIAE_CS42_2DL_TGACv ------------------------------MAAAVTRRVNALRVEVPDG-----NADTANAPAAKRILDAKDDVWVSAD 44
TRIAE_CS42_2BS_TGACv --------------------------MAAAVTRRANALRVEAPDGNTESGRASLAADSPVAKRAVDAKDDVWVAADEGEA 54
TRIAE_CS42_2DS_TGACv --------------------------MAAAVTRRANALRAEAPDGNAESGRASLAADSPAAKRAVDAKDDVWVAADEGDT 54
TRIAE_CS42_7AL_TGACv ----------------------------------------------MPLRVEALVATDTASAAAAEGRRAKDDVWVAEEGDM 36
TRIAE_CS42_7DL_TGACv ---------------------------------------------MALRVEALVATDTAAAEGR--RAKDDVWVAAEEGDM 34
TRIAE_CS42_7BL_TGACv ------------------------------------------------------MATDTVADAAEGRRARDDVWVAAEEGDM 28
TRIAE_CS42_U_TGACv1_ ------------------------------MPSPAAVGGGRLADPLLAAD-------VVVGAKDKYWVPADEREILASQK 43
TRIAE_CS42_1BS_TGACv ------------------------------MASPAAGGGGRLADPLLATD-------VVVGPKDKYWVPADEREILASHR 43
TRIAE_CS42_2AS_TGACv --------------------------MVSPATGGGRGGNAGLAEPLLATNDDSDGAKHVFGAKAKHWVPADEKEMAASRE 54
TRIAE_CS42_2BS_TGACv --------------------------MVSPATSGGRGGNAGLADPLLATNDDSDGARHVFGAKAKYWAPADEKEMTASRE 54
TRIAE_CS42_2DS_TGACv ---------------------------MVSPAASGGGNAGLADPLLATNDNSEGARHVFGAKAKYWVPADEKEIAASRE 52
TRIAE_CS42_2BS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_2DS_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_2AS_TGACv ----MGSLAAANGAGHASNGAGVADQALALENGTGNGHKAGVANRATPPLQANGGSKVAKKISPKDKYWVAADEGEMAAA 76
TRIAE_CS42_7AL_TGACv MAPAVAGGGRVRSNEPAAAAAPAAASGKPCVCGFQVCACTGSAAVASAASSLDMDIVAMGQIGAVNDESWVGVELGEDGE 80
TRIAE_CS42_7DL_TGACv ------------------------------------------------------------------------------- 0
TRIAE_CS42_7BL_TGACv MAPAVAGGGRVRSNEP----AAAAASDKPCVCGFQVCACTGSAAVASAASSLDMDIVAMGQIGAVNDESWVGVELGEDGE 76

TRIAE_CS42_5DL_TGACv SEDQKSASVKNLLVRTTKLTTVTIKLYRLMVFVRLTIFVLFFKWRVSTALTVISDGTTTARAMWTMSIAGELWFALMWVL 101
TRIAE_CS42_5AL_TGACv PEHEKSASVERLLVRTTKLTTVTIKLYRLVVFVRMIIFVLFFKWRSSTALAMISDGTTTVRAMWTMSIAGELWFALMWVL 101
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TRIAE_CS42_2AS_TGACv APDLENGGGRPLLFSNRRVKNIILYPYRVLILIRVIAVILFVGWRIK-------HNNSDVMWFWVMSVVADVWFSLSWLS 142
TRIAE_CS42_2BS_TGACv APDLENGGGRPLLFSNRRVKNIILYPYRVLILIRVIAVILFVGWRIK-------HNNSDVMWFWVMSVVADVWFSLSWLS 141
TRIAE_CS42_2DS_TGACv APDLENGGGRPLLFSNRRVKNIILYPYRVLILIRVIAVILFVGWRIK-------NNNSDVMWFWVISVVADVWFSLSWLS 134
TRIAE_CS42_2AS_TGACv AKESGGEDGRPLLFRTYKVKGTLLHPYRALIFIRLIAVLLFFVWRIK-------HNKSDVMWFWTMSVVGDVWFGFSWLL 116
TRIAE_CS42_2DS_TGACv AKESGGEDGRPLLFRTYKVKGTLLHPYRALIFIRLIAVLLFFVWRIK-------HNKSDIMWFWTLSVVGDVWFGFSWLL 116
TRIAE_CS42_2BS_TGACv AKESGGEEGRPLLFRTYKVKGTLLHPYRALIFIRLIAVLLFFVWRIK-------HNKSDIMWFWTMSVVGDVWFGFSWLL 120
TRIAE_CS42_2DL_TGACv GGIMSGDGNRPLLFRTMKVKGSILHPYRFLMLVRLVAVVAFFKWHVE-------HKNQDSVWLWTASMTADPWFGFSWLL 122
TRIAE_CS42_2BL_TGACv ---------------------------------------------------------------------------- 0
TRIAE_CS42_2AL_TGACv GG-MSGDGNRPLLFRTMKVKGSILHPYRFLMLMRLVAVVIFFKWRME-------HKNHDGVWLWTVSMTADVWFGFSWLL 122
TRIAE_CS42_2DL_TGACv DGTSAGNGNQPLLFRTMKVKGSILHPYRFLILVRLVAVAAFFAWRLE-------HRNHDGTWLWATSMVADAWFGFSWLL 117
TRIAE_CS42_2BS_TGACv SGSIAGDGNRTPLFRTFKVKGSILHPYRFMILVRLVAIVAFFAWRVK-------HKNHDGVWLWATSMVADVWFGFSWLL 127
TRIAE_CS42_2DS_TGACv SGAIAGDGNRPPLFRTFKVKGSILHPYRFMILVRLVAIVAFFAWRVK-------HKNHDGVWLWATSMVADVWFGFSWLL 127
TRIAE_CS42_7AL_TGACv SGASAG---RPLLFRTMKVKGSILHPYRFLILVRLVAIVAFFAWRVE-------HRNHDGTWLWATSMVADAWFGFSWLL 106
TRIAE_CS42_7DL_TGACv SGASAG---RPLLFRTMKVKGSILHPYRFLILVRLVAIIAFFAWRVE-------HRNHDGMWLWATSMVADAWFGFSWLL 104
TRIAE_CS42_7BL_TGACv PEASAG---RPLLFRTMKVKGSILHPYRFLILVRLVAIVAFFAWRVE-------HRNHDGVWLWATSMVADAWFGFSWLL 98
TRIAE_CS42_U_TGACv1_ SGAG-EDGRAPLLYRTFRVKGPLINLYRLLTLVRVIVVILFFTWRMR-------HRDSDAMWLWWISVVGDLWFGVTWLL 115
TRIAE_CS42_1BS_TGACv SGAGGDDGRAPLLYRTFRVKGPLINLYRLLTLVRVIVVILFFTWRMR-------HRDSDAMWLWWISVVGDLWFGVTWLL 116
TRIAE_CS42_2AS_TGACv CGGE---DGRPLLYRTFKVRGFLVNTYRFLNLARLTAVIVFFAWRVQ-------HPDSDAMWLWWISVVGDFWFGLSWWL 124
TRIAE_CS42_2BS_TGACv CSGE---DGRPLLYRTFKVKGFLVNTYRFLNLARLTAVIVFFAWRVQ-------HPDSDAMWLWWISVVGDFWFGLSWWL 124
TRIAE_CS42_2DS_TGACv CGGE---DGRPLLYRTFKVKGMLVNTYRFLNLARLTAVIVFFAWRVQ-------HPDSDAMWLWWISVVGDFWFGLSWWL 122
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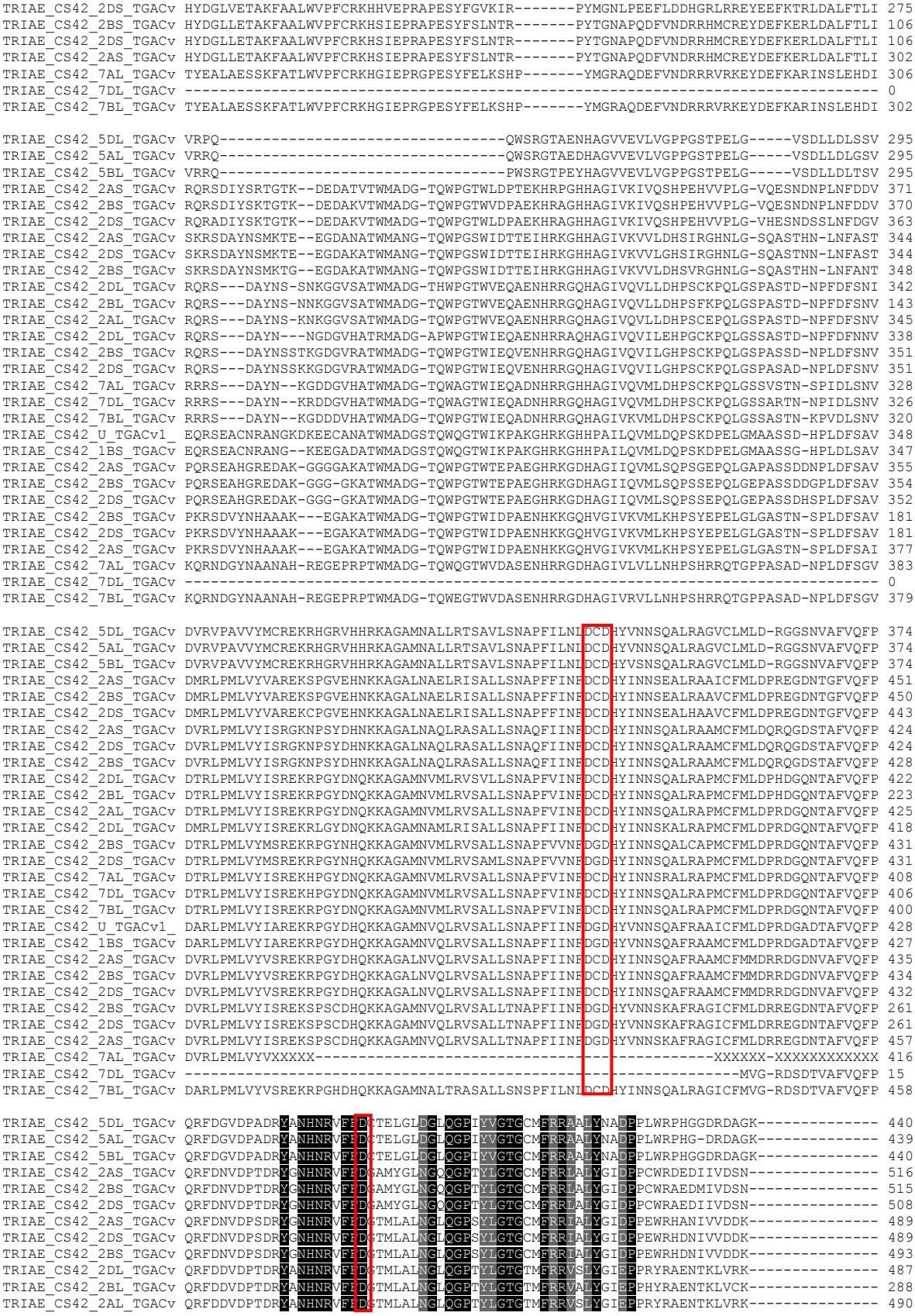
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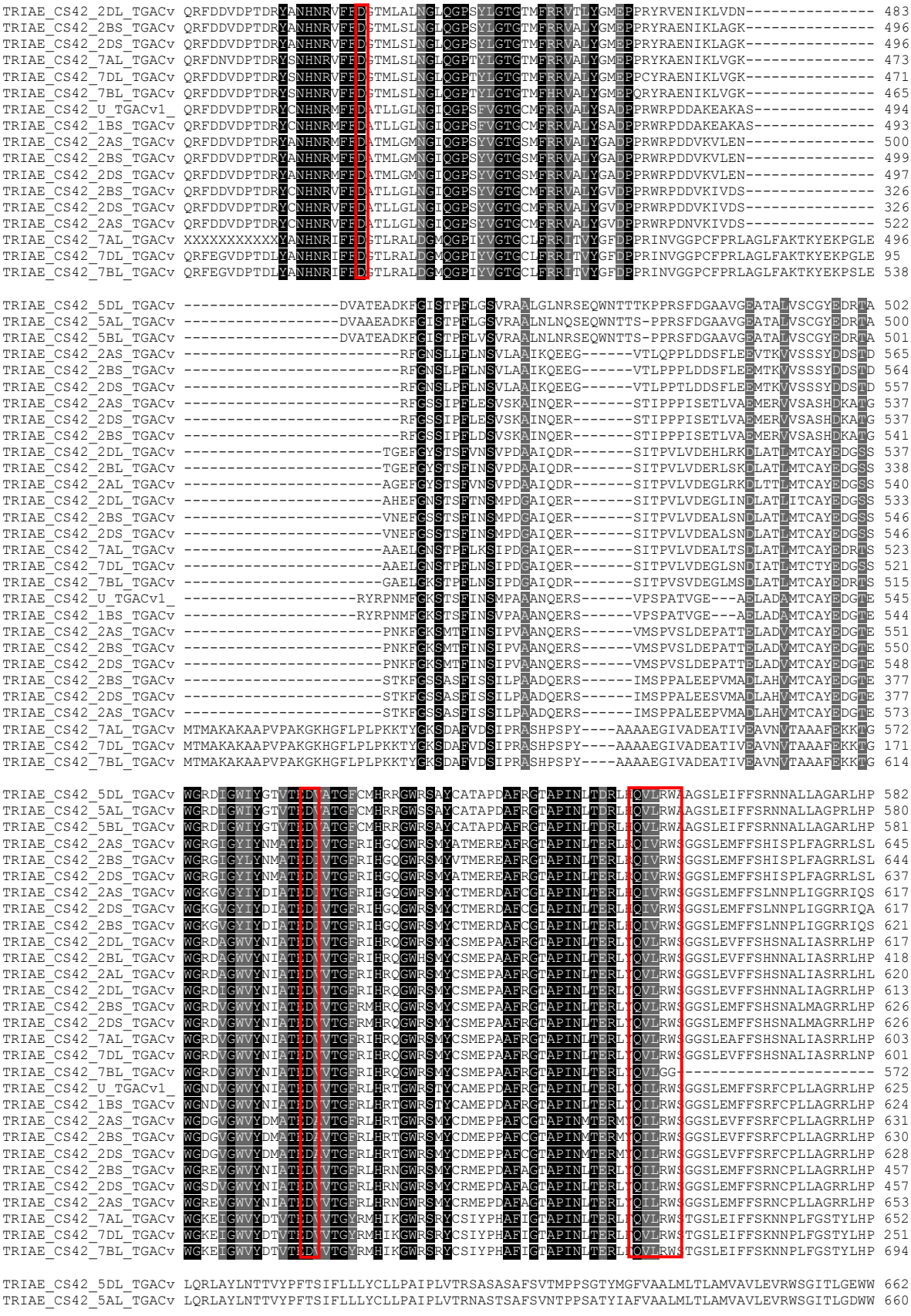

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TRIAE_CS42_2DS_TGACv VQRLSYINFTIYPLTSLFILMYAFCPVMWLLP------TEILVQRPYTRYIVYLIIVIVMIHVIGMFEIMWAGITWLDWW 711
TRIAE_CS42_2AS_TGACv LQRVSYLNMTVYPVTSLFILLYALSPVMWLIP------DEVYIQRPFTKYVVFLLVIILMIHVIGWLEIKWAGVTWLDYW 691
TRIAE_CS42_2DS_TGACv LQRVSYLNMTVYPVTSLFILLYALSPVMWLIP------DEVYIQRPFTKYVVFLLVIILMIHVIGWLEIKWAGVTWLDYW 691
TRIAE_CS42_2BS_TGACv LQRVSYLNMTVYPVTSLFILLYALSPVMWLIP------DEVYIQRPFTKYVVFLLVIILMIHVIGWLEIKWAGVTWLDYW 695
TRIAE_CS42_2DL_TGACv LQRIAYLNMSTHPIVTVFILSYNFFPVMWLFS------EQLYIQRPFGMYMGYLVAIIAMVHLIGMFEVRWSGITLLDWF 691
TRIAE_CS42_2BL_TGACv LQRITYLNMSTYPIVTVFILSYNFFPVMWLFS------EQLYIQRPFGTYMAYLVGIIAMVHLIGMFEVRWSGITLLDWF 492
TRIAE_CS42_2AL_TGACv LQRIAYLNMSTYPIVTVFILSYNFFPVMWLFS------EQLYIQRPFGTYMAYLVAIIAMVHLIGMFEVRWSGITLLDWF 694
TRIAE_CS42_2DL_TGACv LQRIAYFNMSTYPIVTVFILAYNFFPVMWLFS------EQLYIQRPFGTYIAYLVAVIAMMHVIGMFEVKWAGITLLDWC 687
TRIAE_CS42_2BS_TGACv LQRIAYLNMSTYPIVTVFILAYNLFPVLWLFS------EQFYIQRPFAWGFFTDQARHVLLGML-------FNVWILVLL 693
TRIAE_CS42_2DS_TGACv LQRVAYLNMSTYPIVTVFILAYNLFPVLWLFS------EQFYIQRPFGTYIMYLVAVIAMIHVIGMFEVKWAGITLLDWC 700
TRIAE_CS42_7AL_TGACv LQRIAYLNMSIYPIATMFILAYSFFPVMWLFSE-----ESYYIQRPFGTFIMYLVAVIAMMHVIGMFEVKWAGITLQDWW 678
TRIAE_CS42_7DL_TGACv LQRIAYLNMSIYPIATMFILAYSFFPVMWLFSE-----QSYYIQRPFGTFIMYLVVVIAMMHVIGMFEVKWAGITLQDWW 676
TRIAE_CS42_7BL_TGACv ---------------------------------------------------------------------------- 572
TRIAE_CS42_U_TGACv1_ MQRVAYINMTTYPVSTFFICMYYLYPVMWLFQ------GEFYIQRPFQTFALFVVVIIATVELIGMVEIRWAGLTLLDWV 699
TRIAE_CS42_1BS_TGACv MQRIAYINMTTYPVSTFFICMYYFYPVMWLFQ------GEFYIQRPFQTFALFVVIVIATVELIGMVEIRWAGLTPLDWF 698
TRIAE_CS42_2AS_TGACv MQRVAYTNMTFYPLSALFVVCYHLLPLMWVFN------GRFYIQKPYPTYVMYVLVIIVSNEVIGMVEIVWAGLTLLDWF 705
TRIAE_CS42_2BS_TGACv MQRVAYTNMTFYPLSALFVVCYHLLPLMWVFN------GRFYIQKPYPTYVMYVLIIIISNEVIGMVEIVWAGLTLLDWF 704
TRIAE_CS42_2DS_TGACv MQRVAYTNMTFYPLSALFVVCYHLLPLMWVFN------GQFYIQKPYPTYVMYVLIIIVSNEVIGMVEIVWAGLTLLDWF 702
TRIAE_CS42_2BS_TGACv MQRIAYANMTAYPVSSVFLVFYLLFPVIWIFR------GQFYIQKPFPTYVLYLVIVIALTELIGMVEIKWAGLTLLDWI 531
TRIAE_CS42_2DS_TGACv MQRIAYANMTAYPVSSVFLVFYLLFPVIWIFR------GQFYIQKPFPTYVLYLVIVIALTELIGMVEIKWAGLTLLDWI 531
TRIAE_CS42_2AS_TGACv MQRIAYANMTAYPVSSVFLVFYLLFPVIWIFR------GQFYIQKPFPTYVLYLVIVIALTELIGMVEIKWAGLTLLDWI 727
TRIAE_CS42_7AL_TGACv LQRVAYINITTYPFTAIFLIFYTTVPALSFVTG------HFIVQRPTTMFYVYLGIVLSTLLVIAVLEVKWAGVTVFEWF 726
TRIAE_CS42_7DL_TGACv LQRVAYINITTYPFTAIFLIFYTTVPALSFVTG------HFIVQRPTTMFYVYLGIVLSTLLVIAVLEVKWAGVTVFEWF 325
TRIAE_CS42_7BL_TGACv LQRVAYINITTYPFTAIFLIFYTTVPALSFVTG------HFIVQRPTTMFYVYLGIVLSTLLVIAVLEVKWAGVTVFEWF 768

TRIAE_CS42_5DL_TGACv RNEQFWMVSATSAYAAAVVQVALKVSAGKEIAFKLTSKQRAS-SPGGGVKERFAELYAVRWTVLMVPTAVVLAVNVMSMA 741
TRIAE_CS42_5AL_TGACv RNEQFWMVSATSAYAAAVVQVALKVAAGKEIAFKLTSKHRASNSGGGVVKDRFAELYAVRWTVLMVPTAVVLAVNVTSMA 740
TRIAE_CS42_5BL_TGACv RNEQFWMVSATSAYAAAVVQVALKVAAGKEIAFKLTSKQRAPSAGGGVVKRQVRGAVRREMDGADGSDGGGADGERGVHG 741
TRIAE_CS42_2AS_TGACv RNEQFFMIGSVTAYPTAVLHMVVNLLTKKGIHFRVTTKQPVADTDDK-----YAEMYEVHWVPMMVPAVVILFSNILAIG 794
TRIAE_CS42_2BS_TGACv RNEQFFMIGSVTAYPTAVLHMVVNLLTKKGIHFRVTTKQPVADTDDK-----YAEMYEVHWVPMMVPAVVVLFSNILAIG 793
TRIAE_CS42_2DS_TGACv RNEQFFMIGSVTAYPTAVLHMVVNILTKKGIHFRVTTKQPVADTDDK-----YAEMYEVHWVPMMIPAVVVLFSNILAIG 786
TRIAE_CS42_2AS_TGACv RNEQFFMIGSTSAYPAAVLHMVVNLLTKKGIHFRVTSKQTAADTNDK-----FADLYDMRWVPMLIPTTVVLIANVGAIG 766
TRIAE_CS42_2DS_TGACv RNEQFFMIGSTSAYPAAVLHMVVNLLTKKGIHFRVTSKQTAADTNDK-----FADLYDMRWVPMLIPTTVVLIANVGAIG 766
TRIAE_CS42_2BS_TGACv RNEQFFMIGSTSAYPAAVLHMVVNLLTKKGIHFRVTSKQTAADTNDK-----FADLYDMRWVPMLIPTTVVLIANVGAIG 770
TRIAE_CS42_2DL_TGACv RNEQFYMIGATGVYPTAVLYMLLKLATGKGIYFRLTSKQTEACSNDK-----FADLYTVRWVPLLIPTTAVIIVNVAAVG 766
TRIAE_CS42_2BL_TGACv RNEQFYMIGATGVYPTAVLYMLLKLVTGKGIYFRLTSKQTEGCSNDK-----FADLYTVRWVPLLIPTAAVIIVNVAAIG 567
TRIAE_CS42_2AL_TGACv RNEQFYMIGATGVYPTAVLYMLLKLVTGKGIYFRLTSKQTEACSNDK-----FADLYTVRWVPLLIPTTAVIIVNVAAVG 769
TRIAE_CS42_2DL_TGACv RNEQFYLIAATGVYPTAVLYMALKLVTGKGMHFRLTSKQTEACSRDK-----FANLYTVRWVPLLIPTTAVLVVNVAAVG 762
TRIAE_CS42_2BS_TGACv YPFALGIMGKWGKRPVILFVMLVMAVGAVGLLYVAFHAPYPADFSEV-----AASLGEASLTGPSG-------------- 754
TRIAE_CS42_2DS_TGACv RNEQFYMIGATGVYPTAVLYMALKLVTGKGIYFRLTSKQTDACSNDK-----XXXXXXXXXXXXXXXXXXXXXXGCRCC 775
TRIAE_CS42_7AL_TGACv RNEQFYMITATGVYPTAVLYMALKLIRGKGIYFRLTSKQTEACSGEK-----FADLYTVRWVPLLIPTVAVLVVNIAAIG 753
TRIAE_CS42_7DL_TGACv RNEQFYMIAATGVYPTAVLYMALKLIRGKGIYFRLTSKQTEACSDEK-----FADLYTVRWVPLLIPTVAVLIVNVTAVG 751
TRIAE_CS42_7BL_TGACv ----------------------------QAAPSRCSSPTATLSSPAV-----GSTLYSASLTSTCRSTRSPRCSS----- 614
TRIAE_CS42_U_TGACv1_ RNEQFYIIGTTGVYPMAMLHILLRSLGIKGVSFKLTAKKLTGGARER-----LAELYDVQWVPLLVPTVVVMAVNVAAIG 774
TRIAE_CS42_1BS_TGACv RNEQFYIIGTTGVYPMAMLHIILRSLGIKGVSFKLTAKKLTSGTRER-----LAELYDVQWVPLLVPTVVVMAVNVAAIG 773
TRIAE_CS42_2AS_TGACv RNEQFYMICATGVYPTAVLHVVLRSLGLKGMSFKMTAKQLATGARER-----FAELYNVQWAPLLIPTLVVIAVNVVAIG 780
TRIAE_CS42_2BS_TGACv RNEQFYMICATGVYPTAVLHVVLRSLGLKGISFKMTAKQLATGARER-----FAELYDVQWAPLLIPTLVVIAVNVVAIG 779
TRIAE_CS42_2DS_TGACv RNEQFYMICATGVYPTAVLHVVLRSLGLKGMSFKMTAKQLATGARER-----FAELYDVQWAPLLIPTLVVIAVNVVAIG 777
TRIAE_CS42_2BS_TGACv RNEQFYIIGATAVYPTAVFHIVLKLFGLKGVSFKLTAKQVASSTSDK-----FAELYAVQWAPMLIPTMVVIAVNVCAIG 606
TRIAE_CS42_2DS_TGACv RNEQFYIIGATAVYPTAVFHIVLKLFGLKGVSFKLTAKQVASSTSDK-----FAELYAVQWAPMLIPTMVVIAVNVCAIG 606
TRIAE_CS42_2AS_TGACv RNEQFYIIGATAVYPTAVFHIVLKLFGLKGVSFKLTAKQVASSTSDK-----FAELYAVQWAPMLIPTMVVIAVNVCAIG 802
TRIAE_CS42_7AL_TGACv RNGQFWMTASCSAYLAAVCQVLTKVIFRRDISFKLTSKLPSGDEKKD----PYADLYVVRWTPLMITPIIIIFVNIIGSA 802
TRIAE_CS42_7DL_TGACv RNGQFWMTASCSAYLAAVCQVLTKVIFRRDISFKLTSKLPSGDEKKD----PYADLYVVRWTPLMITPIIIIFVNIIGSA 401
TRIAE_CS42_7BL_TGACv RNGQFWMTASCSAYLAAVCQVLTKVIFRRDISFKLTSKLPSGDEKKD----PYADLYVVRWTPLMITPIIIIFVNIIGSA 844

TRIAE_CS42_5DL_TGACv AAVQEGRWRK-------GPAAVLAMAFNAWVVVHLHPFALGLMGRWSKTLSPLLLLVVGFTVLSLCFVLHLHML------ 808
TRIAE_CS42_5AL_TGACv AAVQEGRWRK-------GPAAVLAMAFNAWVVVHLYPFALGLMGRWSKTLSPLLLLVVVFTVLSLCFVLHLHML------ 807
TRIAE_CS42_5BL_TGACv SSGTRGTVEE-------RPRGGARDGVQRVGGGASPPVRPWSHGPLEQDVEPPALARRSVHSSITMFCPPFAYALIWLLF 814
TRIAE_CS42_2AS_TGACv VAIGKSVLYMGTWSAAQRRHGALGLLFNLWIMVLLYPFALAIIGRWAKRTGILFILLPIAFLSTALMYIGIHTFLLHFFP 874
TRIAE_CS42_2BS_TGACv VAIGKSVLYMGTWSAAQKRHGALGLLFNMWIMVLLYPFALAIIGRWAKRTGILFILLPIAFLSTALMYIGIHTFLLHFFP 873
TRIAE_CS42_2DS_TGACv VAIGKSILYMGTWSAAQKRHGALGLLFNLWIMVLLYPFALAIIGRWAKRTGILFILLPIAFLSTSLMYIGVHTFLLHFFP 866
TRIAE_CS42_2AS_TGACv VAMGKTIVYMGAWTIAQKTHAALGLLFNVWIMVLLYPFALAIMGRWAKRPVILVVLLPVAFTIVCLVYVAVHILLLSYLT 846
TRIAE_CS42_2DS_TGACv VAMGKTIVYMGAWTIAQKTHAALGLLFNVWIMVLLYPFALAIMGRWAKRPVILLVLLPVAFTIVCLVYVAVHILLLSYLT 846
TRIAE_CS42_2BS_TGACv VAMGKTIVYMGAWTIAQKTHAALGLLFNVWIMVLLYPFALAIMGRWAKRPVILLVLLPVAFTIVCLVYVAVHILLLSYLT 850
TRIAE_CS42_2DL_TGACv AAIGKAATWG--FFTDEARHALLGMVFNMGILVLLYPFALGIMGKWAKRPIILFIVLVMAISVVGLLYVSLHAPYTGEWS 844
TRIAE_CS42_2BL_TGACv AAIGKAATWG--FFTDEARHALLGMVFNMGILVLLYPFALGIMGKWGKRPIILFIVLVMAISVVGLLYVTLHAPYTGEWS 645
TRIAE_CS42_2AL_TGACv AAIGKAATWG--FFTDEARHALLGMVFNMGILVLLYPFALGIMGKWGKRPIILFIVLVMAISAVGLLYVMLHAPYTGEWS 847
TRIAE_CS42_2DL_TGACv AAIGKAAAWG--FSTDQARHVLLGMVFNVGTLMLLYPFALGIMGKRGKTPVILFVLLLMAIAAVGLLYVTLYAPYPQESL 840
TRIAE_CS42_2BS_TGACv ---------------------------------------------------------------------------- 754
TRIAE_CS42_2DS_TGACv SRPSWCSS---------------------------------------------------------------------- 783
TRIAE_CS42_7AL_TGACv AAIGKAATWG--FFTDQAWHAVLGMVFNVGTLVLLYPFALGIMGQWGKRPGILLVMLVMAIGTVGLLYVTLQQDGHRMSF 831
TRIAE_CS42_7DL_TGACv AAIGKAATWG--FFTDQAWHAVLGMVFNVGTLVLLYPFALGIMGQWGKRPGILLVMLVMAIGTVGLLYVTLQQDGHRMSF 829
TRIAE_CS42_7BL_TGACv ---------------------------------------------------------------------------- 614
TRIAE_CS42_U_TGACv1_ AAAGKAIVGR--WSAAQVAGAASGLVFNVWMLLLLYPFALGIMGRWSKRPYILFIVLVTAVAATASMYVALAGSLPYLHS 852
TRIAE_CS42_1BS_TGACv AAAGKAIAGR--WSAAQVAGAASGLVFNVWMLLLLYPFALGIMGRWSKRPYILFIVLVTAVAATASVYVALAGSLPYLHS 851
TRIAE_CS42_2AS_TGACv AAVGKAITWG--WSAGQVVEAASGLMFNVWILLMFYPFALGVIGRWGKRPYVLFAMFVAAFAAIAAVYVAVQAALAGNLL 858
TRIAE_CS42_2BS_TGACv AAVGKAITWG--WSAGQVVEAASGLMFNVWILLMFYPFALGVIGRWGKKPYVLFAMFVAAFAAIAAVYVAVQAALAGNLP 857
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TRIAE_CS42_2DS_TGACv VAVGKAITWG--WSAGQVVEAASGLMFNVWILLMFYPFALGVIGRWGKRPFVLFAMFVAAFAAIAAVYVAVQAALAGNLP 855
TRIAE_CS42_2BS_TGACv ASIGKAIVGG--WSLMQMADAGLGLVFNAWILVLIYPFALGMIGRWSKRPYILFILFVIAFILIALVDIAIQAMRSGFVR 684
TRIAE_CS42_2DS_TGACv ASIGKAIVGG--WSLMQMADAGLGLVFNAWILVLIYPFALGMIGRWSKRPYILFILFVIAFILIALVDIAIQAMRSGFVR 684
TRIAE_CS42_2AS_TGACv ASIGKAIVGG--WSLMQMADAGLGLVFNAWILVLIYPFALGMIGRWSKRPYILFILFVIAFILIALVDIAIQAMRSGFVR 880
TRIAE_CS42_7AL_TGACv VAFAKVLDGEW----THWLKVAGGVFFNFWVLFHLYPFAKGILGKHGKTPVVVLVWWAFTFVITAVLYINIPHMHSSGGK 878
TRIAE_CS42_7DL_TGACv VAFAKVLDGEW----THWLKVAGGVFFNFWVLFHLYPFAKGILGKHGKTPVVVLVWWAFTFVITAVLYINIPHMHSSGGK 477
TRIAE_CS42_7BL_TGACv VAFAKVLDGEW----THWLKVAGGVFFNFWVLFHLYPFAKGILGKHGKTPVVVLVWWAFTFVITAVLYINIPHMHSSGGK 920

TRIAE_CS42_5DL_TGACv ---------------------- 808
TRIAE_CS42_5AL_TGACv ---------------------- 807
TRIAE_CS42_5BL_TGACv G--------------------- 815
TRIAE_CS42_2AS_TGACv SMLI----------------- 878
TRIAE_CS42_2BS_TGACv SMLI----------------- 877
TRIAE_CS42_2DS_TGACv SMLI----------------- 870
TRIAE_CS42_2AS_TGACv F--------------------- 847
TRIAE_CS42_2DS_TGACv F--------------------- 847
TRIAE_CS42_2BS_TGACv F--------------------- 851
TRIAE_CS42_2DL_TGACv QVAVSLGKASLTGPSGSG--- 862
TRIAE_CS42_2BL_TGACv QVAVSLGKASLTGPSGSG--- 663
TRIAE_CS42_2AL_TGACv QVAVSLGKASLTGPSGSG--- 865
TRIAE_CS42_2DL_TGACv TFLSW---------------- 845
TRIAE_CS42_2BS_TGACv ---------------------- 754
TRIAE_CS42_2DS_TGACv ---------------------- 783
TRIAE_CS42_7AL_TGACv LTRPSG--------------- 837
TRIAE_CS42_7DL_TGACv LTRPSG--------------- 835
TRIAE_CS42_7BL_TGACv ---------------------- 614
TRIAE_CS42_U_TGACv1_ GIKLV---------------- 857
TRIAE_CS42_1BS_TGACv GIKLV---------------- 856
TRIAE_CS42_2AS_TGACv YFQLGHWSIGGAVSLPSRRV- 878
TRIAE_CS42_2BS_TGACv YFQLGHWSIGGAVSLPSRRV- 877
TRIAE_CS42_2DS_TGACv YFQLGHRSIGGAVSLASRRV- 875
TRIAE_CS42_2BS_TGACv FHFKSSGGATFPTSWGL---- 701
TRIAE_CS42_2DS_TGACv FHFKSSGGATFPTSWGL---- 701
TRIAE_CS42_2AS_TGACv FHFKSSGGATFPTSWGL---- 897
TRIAE_CS42_7AL_TGACv HTTVHGHHGKKFVDAGYYNWP 899
TRIAE_CS42_7DL_TGACv HTTVHGHHGKKFVDAGYYNWP 498
TRIAE_CS42_7BL_TGACv HTTVHGHHGKKFVDAGYYNWP 941
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Appendix 6.6 List of *CslH* subfamily genes, their protein length (amino acids) and multiple sequence alignment of amino acids showing the conserved motifs (D, D, DXD, QXXRW) specific to *Cellulose synthase like* (*Csl*) family (highlighted with red boxes).

S.No	Gene name with number of splice variants (CslH)	No. of amino acids (aa)
1	TRIAE_CS42_3DS_TGACv1_271739_AA0907200.1	714 aa
2	TRIAE_CS42_3AS_TGACv1_212952_AA0704280.1	331 aa
3	TRIAE_CS42_3B_TGACv1_222234_AA0760340.1	751 aa
4	TRIAE_CS42_3B_TGACv1_221049_AA0728260.1	458 aa
5	TRIAE_CS42_3DS_TGACv1_273502_AA0931770.1	579 aa
6	TRIAE_CS42_2AL_TGACv1_094351_AA0296300.3	752 aa
7	TRIAE_CS42_2DL_TGACv1_158387_AA0517170.1	752 aa
8	TRIAE_CS42_2BL_TGACv1_129372_AA0380770.1	799 aa

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TRIAE_CS42_2AL_TGACv ---------------------------------------------MAGGKKLHERVALGRTAWMLADFVILLLLLALV 33
TRIAE_CS42_2BL_TGACv MHRGEDSLSGLYKCTLAFVACGCGWSCGVVLLASLLLLVASYLSATAMAGGKKLQERVALGRSAWMLADFVILFLVLALV 80
TRIAE_CS42_2DL_TGACv ---------------------------------------------MAGGKKLQERVALGRTAWMLADFVILLLLLALV 33
TRIAE_CS42_3AS_TGACv ------------------------------------------------------------------------------ 0
TRIAE_CS42_3B_TGACv1 -----------------------------------------------MSSAMKLQERVSVPRTAWKLADIFILCLLF 30
TRIAE_CS42_3DS_TGACv -----------------------------------------------MSSAMKLQERVIVPRTAWKLADIFILCLLFALL 33
TRIAE_CS42_3B_TGACv1 -----------------------------------------------MSSAMKLQERVTVPRTAWKLADIFILCLLLVLL 33
TRIAE_CS42_3DS_TGACv -----------------------------------------------MGSAMKLQERVILPRTAWKLADIFILCLLFALL 33

TRIAE_CS42_2AL_TGACv ARRAASLGE--RGGTWLAALVCEAWFAFVWILNMNGKWSPVRFDTYPENLSHRLEELPAVDMFVTTADPALEPPLITVNT 111
TRIAE_CS42_2BL_TGACv ARRAASLGE--RGGTWLAALVCEAWFAFVWILNMNGKWSPVRFDTYPENLSHRMEELPAVDMFVTTADPALEPPLITVNT 158
TRIAE_CS42_2DL_TGACv ARRAASLGE--RGGTWLAALVCEAWFAFVWILNMNGKWSPVRFDTYPDNLSHRMEELPAVDMFVTTADPALEPPLITVNT 111
TRIAE_CS42_3AS_TGACv ------------------------------------------------------------------------------ 0
TRIAE_CS42_3B_TGACv1 ALLSCRVASLREGGASVAALVCEAWFTFVWIINMNIKWNPVRFNTYPENLSQRTDELPAVDMLVTTADPELEPPLMTVNT 110
TRIAE_CS42_3DS_TGACv SCRVLSLGEGGAGAASVAALVCEAWFTFVWILNMNIRWNPVRFHTYPENLSQRMDGLPAVDMLVTTADPELEPPLMTVNT 113
TRIAE_CS42_3B_TGACv1 SCRVASLGEGGAG---AAALVCEAWFTFVWILNMNIKWNPVRFHTYPENLSQRMDELPAVDMLVTTADPELEPPLMTVNT 110
TRIAE_CS42_3DS_TGACv SCRVASLGDGGAGAASVAALVCEAWFTFVWILNMNIKWNPVRFHTYPENLSQRMDELPAVDMLVTTADPELEPPLMTVNT 113

TRIAE_CS42_2AL_TGACv VLSLLALDYPPDVGKLACYVSDDGCSPVTCYALREAAKFASLWIPFCKRYDVGVRAPFMYFSSAPEVGTGTADHEFLESWA 191
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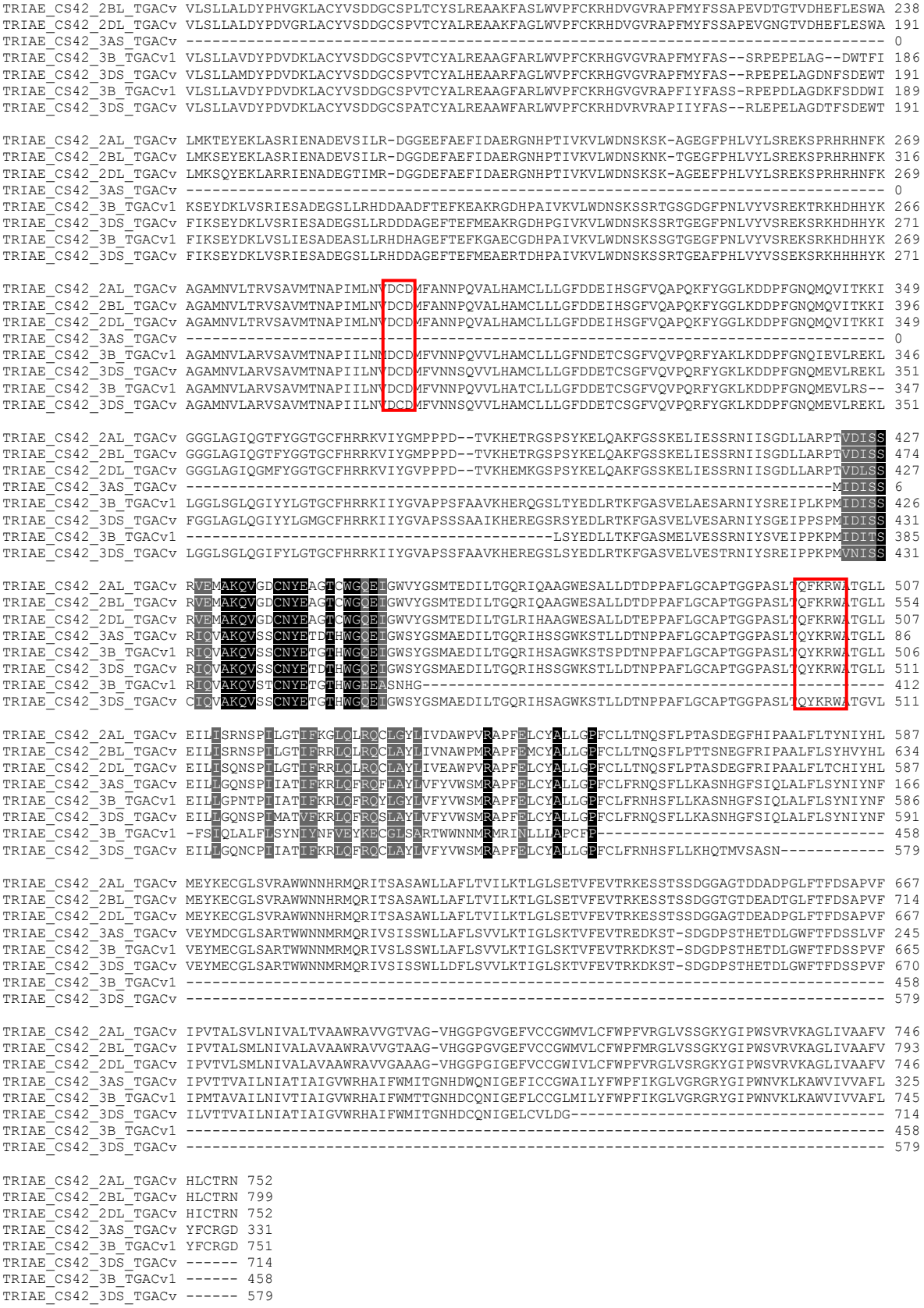
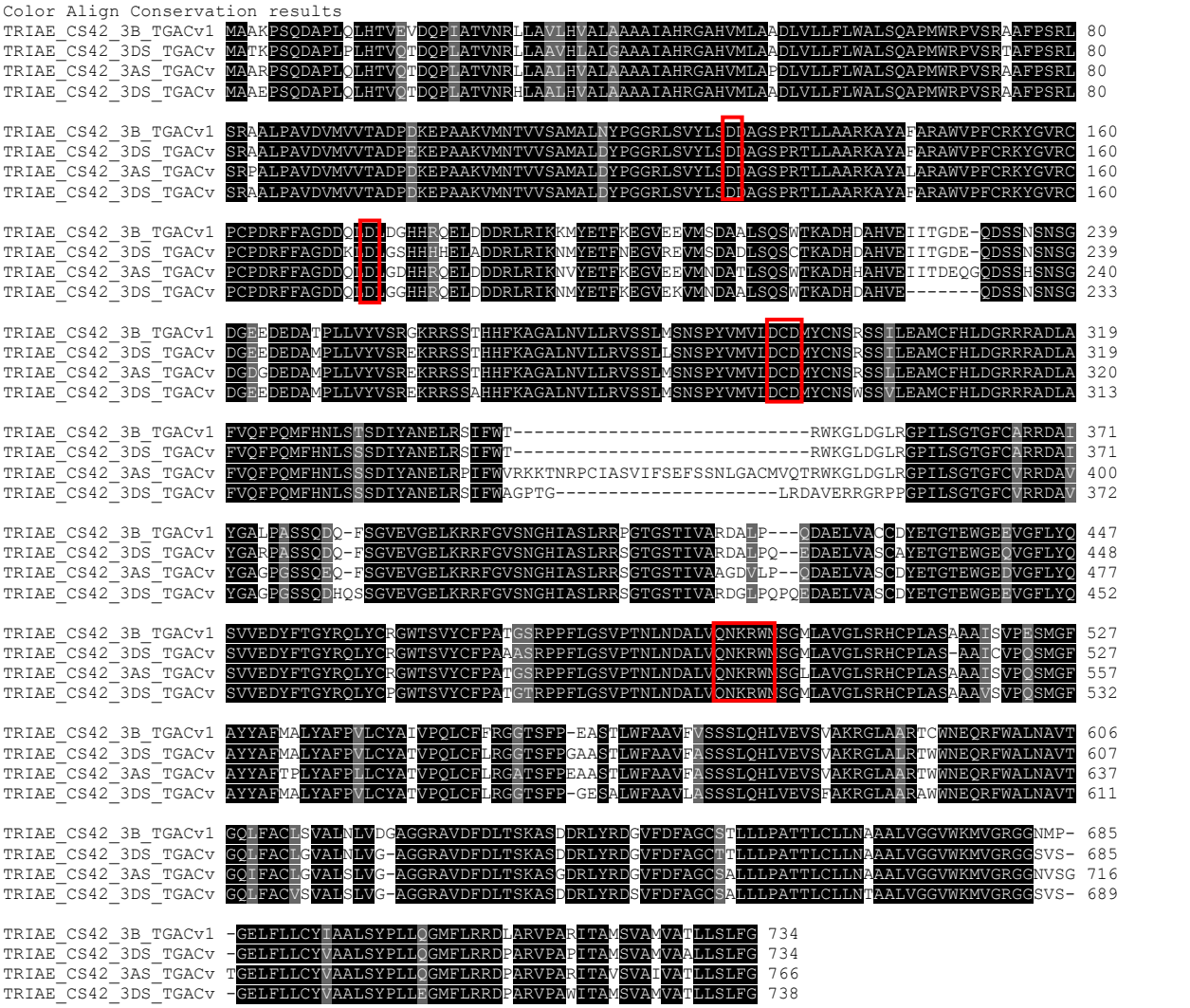


Appendix 6.7 List of *CslJ* subfamily genes, their protein length (amino acids) and multiple sequence alignment of amino acids showing the conserved motifs (D, D, DXD, QXXRW) specific to *Cellulose synthase like* (*Csl*) family (highlighted with red boxes).

S.No	Gene name with number of splice variants (CslJ)	No. of amino acids (aa)
1	TRIAE_CS42_3DS_TGACv1_272297_AA0918580.1	738 aa
2	TRIAE_CS42_3AS_TGACv1_210908_AA0681280.2	766 aa
3	TRIAE_CS42_3B_TGACv1_221705_AA0747940.1	734 aa
4	TRIAE_CS42_3DS_TGACv1_272756_AA0924850.1	734 aa



IX. REFERENCES

Achyuthan KE, Achyuthan AM, Adams PD, Dirk SM, Harper JC, Simmons BA, Singh AK. 2010. Supramolecular self-assembled chaos: polyphenolic lignin's barrier to cost-effective lignocellulosic biofuels. *Molecules* **15**: 8641-8688.

Ahmad S, Kaur S, Lamb-Palmer ND, Lefsrud M, Singh J. 2015. Genetic diversity and population structure of Pisum sativum accessions for marker-trait association of lipid content. *The Crop Journal* **3**: 238-245.

Andersen JR, Lubberstedt T. 2003. Functional markers in plants. *Trends Plant Sci* **8**: 554-560.

Appenzeller L, Doblin M, Barreiro R, Wang H, Niu X, Kollipara K, Carrigan L, Tomes D, Chapman M, Dhugga KS. 2004. Cellulose synthesis in maize: isolation and expression analysis of the cellulose synthase (CesA) gene family. *Cellulose* **11**: 287-299.

Aramrak A, Kidwell KK, Steber CM, Burke IC. 2015. Molecular and phylogenetic characterization of the homoeologous EPSP Synthase genes of allohexaploid wheat, Triticum aestivum (L.). *BMC genomics* **16**: 1.

Arioli T, Peng L, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Höfte H, Plazinski J, Birch R. 1998. Molecular analysis of cellulose biosynthesis in Arabidopsis. *Science* **279**: 717-720.

Baenziger PS, Bakhsh A, Lorenz A, Walia H. 2014. Bridging Conventional Breeding and Genomics for A More Sustainable Wheat Production. doi:10.1007/978-94-007-7575-6_7: 185-209.

Baldwin L, Głazowska S, Mravec J, Fangel J, Zhang H, Felby C, Willats WG, Schjoerring JK. 2017. External nitrogen input affects pre-and post-harvest cell wall composition but not the enzymatic saccharification of wheat straw. *Biomass and Bioenergy* **98**: 70-79.

Bassi FM, Bentley AR, Charmet G, Ortiz R, Crossa J. 2016. Breeding schemes for the implementation of genomic selection in wheat (Triticum spp.). *Plant Science* **242**: 23-36.

Battenfield SD, Guzmán C, Gaynor RC, Singh RP, Peña RJ, Dreisigacker S, Fritz AK, Poland JA. 2016. Genomic selection for processing and end-use quality traits in the CIMMYT spring bread wheat breeding program. *The Plant Genome* **9**.

Bennypaul HS, Mutti JS, Rustgi S, Kumar N, Okubara PA, Gill KS. 2012a. Virus-induced gene silencing (VIGS) of genes expressed in root, leaf, and meiotic tissues of wheat. *Functional & integrative genomics* **12**: 143-156.

Bennypaul HS, Mutti JS, Rustgi S, Kumar N, Okubara PA, Gill KS. 2012b. Virus-induced gene silencing (VIGS) of genes expressed in root, leaf, and meiotic tissues of wheat. *Funct Integr Genomics* **12**: 143-156.

Bentsen NS, Felby C, Thorsen BJ. 2014. Agricultural residue production and potentials for energy and materials services. *Progress in energy and combustion science* **40**: 59-73.

Bernal AJ, Jensen JK, Harholt J, Sørensen S, Moller I, Blaukopf C, Johansen B, De Lotto R, Pauly M, Scheller HV. 2007. Disruption of ATCSLD5 results in reduced growth, reduced xylan and homogalacturonan synthase activity and altered xylan occurrence in Arabidopsis. *The Plant Journal* **52**: 791-802.

Bernardo R, Yu J. 2007. Prospects for genomewide selection for quantitative traits in maize. *Crop Science* **47**: 1082-1090.

Bhullar R, Nagarajan R, Bennypaul H, Sidhu GK, Sidhu G, Rustgi S, von Wettstein D, Gill KS. 2014. Silencing of a metaphase I-specific gene results in a phenotype similar to that of the Pairing homeologous 1 (Ph1) gene mutations. *Proceedings of the National Academy of Sciences* **111**: 14187-14192.

Boerjan W, Ralph J, Baucher M. 2003. Lignin biosynthesis. *Annual review of plant biology* **54**: 519-546.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*: btu170.

Bolot S, Abrouk M, Masood-Quraishi U, Stein N, Messing J, Feuillet C, Salse J. 2009. The 'inner circle' of the cereal genomes. *Curr Opin Plant Biol* **12**: 119-125.

Bolser DM, Kerhornou A, Walts B, Kersey P. 2015. Triticeae resources in ensembl plants. *Plant and Cell Physiology* **56**: e3-e3.

Bottley A, Xia G, Koebner R. 2006. Homoeologous gene silencing in hexaploid wheat. *The Plant Journal* **47**: 897-906.

Brachi B, Morris GP, Borevitz JO. 2011. Genome-wide association studies in plants: the missing heritability is in the field. *Genome Biol* **12**: 232.

Bradbury LM, Niehaus TD, Hanson AD. 2013. Comparative genomics approaches to understanding and manipulating plant metabolism. *Curr Opin Biotechnol* **24**: 278-284.

Bray N, Pimentel H, Melsted P, Pachter L. 2015. Near-optimal RNA-Seq quantification. *arXiv preprint arXiv:150502710*.

Bregitzer P, Cooper LD, Hayes PM, Lemaux PG, Singh J, Sturbaum AK. 2007. Viability and bar expression are negatively correlated in Oregon Wolfe Barley Dominant hybrids. *Plant biotechnology journal* **5**: 381-388.

Breton C, Šnajdrová L, Jeanneau C, Koča J, Imberty A. 2006. Structures and mechanisms of glycosyltransferases. *Glycobiology* **16**: 29R-37R.

Broom D, Galindo F, Murgueitio E. 2013. Sustainable, efficient livestock production with high biodiversity and good welfare for animals. *Proceedings of the Royal Society of London B: Biological Sciences* **280**: 20132025.

Buhrow LM, Clark SM, Loewen MC. 2016. Identification of an attenuated barley stripe mosaic virus for the virus-induced gene silencing of pathogenesis-related wheat genes. *Plant methods* **12**: 12.

Burton RA, Collins HM, Kibble NA, Smith JA, Shirley NJ, Jobling SA, Henderson M, Singh RR, Pettolino F, Wilson SM. 2011a. Over-expression of specific HVCSLF cellulose synthase-like genes in transgenic barley increases the levels of cell wall (1, 3; 1, 4)-β-D-glucans and alters their fine structure. *Plant Biotechnology Journal* **9**: 117-135.

Burton RA, Collins HM, Kibble NA, Smith JA, Shirley NJ, Jobling SA, Henderson M, Singh RR, Pettolino F, Wilson SM et al. 2011b. Over-expression of specific HvCslF cellulose synthase-like genes in transgenic barley increases the levels of cell wall (1,3;1,4)-beta-d-glucans and alters their fine structure. *Plant Biotechnol J* **9**: 117-135.

Burton RA, Fincher GB. 2014a. Evolution and development of cell walls in cereal grains.

Burton RA, Fincher GB. 2014b. Plant cell wall engineering: applications in biofuel production and improved human health. *Current Opinion in Biotechnology* **26**: 79-84.

Burton RA, Gibeaut DM, Bacic A, Findlay K, Roberts K, Hamilton A, Baulcombe DC, Fincher GB. 2000. Virus-induced silencing of a plant cellulose synthase gene. *The Plant Cell* **12**: 691-705.

Burton RA, Jobling SA, Harvey AJ, Shirley NJ, Mather DE, Bacic A, Fincher GB. 2008. The genetics and transcriptional profiles of the cellulose synthase-like HvCslF gene family in barley. *Plant Physiol* **146**: 1821-1833.

Burton RA, Shirley NJ, King BJ, Harvey AJ, Fincher GB. 2004. The CesA gene family of barley. Quantitative analysis of transcripts reveals two groups of co-expressed genes. *Plant Physiol* **134**: 224-236.

Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Medhurst A, Stone BA, Newbigin EJ, Bacic A, Fincher GB. 2006a. Cellulose synthase-like CslF genes mediate the synthesis of cell wall (1,3;1,4)-beta-D-glucans. *Science* **311**: 1940-1942.

Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Medhurst A, Stone BA, Newbigin EJ, Bacic A, Fincher GB. 2006b. Cellulose synthase-like CslF genes mediate the synthesis of cell wall (1, 3; 1, 4)-ß-D-glucans. *Science* **311**: 1940-1942.

Cakir C, Tör M. 2010. Factors influencing Barley Stripe Mosaic Virus-mediated gene silencing in wheat. *Physiological and Molecular Plant Pathology* **74**: 246-253.

Carpita NC. 1996. Structure and biogenesis of the cell walls of grasses. *Annual review of plant biology* **47**: 445-476.

Carroll A, Mansoori N, Li S, Lei L, Vernhettes S, Visser RG, Somerville C, Gu Y, Trindade LM. 2012. Complexes with mixed primary and secondary cellulose synthases are functional in Arabidopsis plants. *Plant Physiol* **160**: 726-737.

Chan J, Calder G, Fox S, Lloyd C. 2007. Cortical microtubule arrays undergo rotary movements in Arabidopsis hypocotyl epidermal cells. *Nature Cell Biology* **9**: 171-175.

Chan J, Crowell E, Eder M, Calder G, Bunnewell S, Findlay K, Vernhettes S, Höfte H, Lloyd C. 2010. The rotation of cellulose synthase trajectories is microtubule dependent and influences the texture of epidermal cell walls in Arabidopsis hypocotyls. *Journal of cell science* **123**: 3490-3495.

Chandra R, Takeuchi H, Hasegawa T. 2012. Methane production from lignocellulosic agricultural crop wastes: A review in context to second generation of biofuel production. *Renewable and Sustainable Energy Reviews* **16**: 1462-1476.

Chantreau M, Chabbert B, Billiard S, Hawkins S, Neutelings G. 2015. Functional analyses of cellulose synthase genes in flax (Linum usitatissimum) by virus-induced gene silencing. *Plant biotechnology journal*.

Chen L, Hao L, Parry MA, Phillips AL, Hu YG. 2014. Progress in TILLING as a Tool for Functional Genomics and Improvement of Crops. *J Integr Plant Biol* doi:10.1111/jipb.12192.

Chen Y, Cao J. 2014. Comparative genomic analysis of the Sm gene family in rice and maize. *Gene* **539**: 238-249.

Ching A, Dhugga KS, Appenzeller L, Meeley R, Bourett TM, Howard RJ, Rafalski A. 2006. Brittle stalk 2 encodes a putative glycosylphosphatidylinositol-anchored protein that affects mechanical strength of maize tissues by altering the composition and structure of secondary cell walls. *Planta* **224**: 1174-1184.

Cho S, Garvin DF, Muehlbauer GJ. 2006. Transcriptome analysis and physical mapping of barley genes in wheat-barley chromosome addition lines. *Genetics* **172**: 1277-1285.

Choulet F, Alberti A, Theil S, Glover N, Barbe V, Daron J, Pingault L, Sourdille P, Couloux A, Paux E. 2014a. Structural and functional partitioning of bread wheat chromosome 3B. *Science* **345**: 1249721.

Choulet F, Caccamo M, Wright J, Alaux M, Šimková H, Šafář J, Leroy P, Doležel J, Rogers J, Eversole K et al. 2014b. The Wheat Black Jack: Advances Towards Sequencing the 21 Chromosomes of Bread Wheat. doi:10.1007/978-94-007-7572-5_17: 405-438.

Ciesielski PN, Resch MG, Hewetson B, Killgore JP, Curtin A, Anderson N, Chiaramonti AN, Hurley DC, Sanders A, Himmel ME et al. 2014. Engineering plant cell walls: tuning lignin monomer composition for deconstructable biofuel feedstocks or resilient biomaterials. *Green Chemistry* **16**: 2627.

Cockram J, White J, Zuluaga DL, Smith D, Comadran J, Macaulay M, Luo Z, Kearsey MJ, Werner P, Harrap D et al. 2010. Genome-wide association mapping to candidate polymorphism resolution in the unsequenced barley genome. *Proc Natl Acad Sci U S A* **107**: 21611-21616.

Cocuron JC, Lerouxel O, Drakakaki G, Alonso AP, Liepman AH, Keegstra K, Raikhel N, Wilkerson CG. 2007. A gene from the cellulose synthase-like C family encodes a beta-1,4 glucan synthase. *Proc Natl Acad Sci U S A* **104**: 8550-8555.

Cohen J. 1992. Statistical power analysis. *Current directions in psychological science* **1**: 98-101.

Consortium IWGS. 2014. A chromosome-based draft sequence of the hexaploid bread wheat (Triticum aestivum) genome. *Science* **345**: 1251788.

Coomey JH, Hazen SP. 2015. Brachypodium distachyon as a Model Species to Understand Grass Cell Walls. In *Genetics and Genomics of Brachypodium*, pp. 197-217. Springer.

Cosgrove DJ. 2000. Expansive growth of plant cell walls. *Plant Physiology and Biochemistry* **38**: 109-124.

Cosgrove DJ. 2005. Growth of the plant cell wall. *Nature reviews molecular cell biology* **6**: 850-861.

Crossa J, Pérez P, de los Campos G, Mahuku G, Dreisigacker S, Magorokosho C. 2011. Genomic selection and prediction in plant breeding. *Journal of Crop Improvement* **25**: 239-261.

Crowell EF, Bischoff V, Desprez T, Rolland A, Stierhof Y-D, Schumacher K, Gonneau M, Höfte H, Vernhettes S. 2009. Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in Arabidopsis. *The Plant Cell* **21**: 1141-1154.

Csuros M, Rogozin IB, Koonin EV. 2011. A detailed history of intron-rich eukaryotic ancestors inferred from a global survey of 100 complete genomes. *PLoS Comput Biol* **7**: e1002150.

Desprez T, Juraniec M, Crowell EF, Jouy H, Pochylova Z, Parcy F, Hofte H, Gonneau M, Vernhettes S. 2007a. Organization of cellulose synthase complexes involved in primary cell wall synthesis in Arabidopsis thaliana. *Proc Natl Acad Sci U S A* **104**: 15572-15577.

Desprez T, Juraniec M, Crowell EF, Jouy H, Pochylova Z, Parcy F, Höfte H, Gonneau M, Vernhettes S. 2007b. Organization of cellulose synthase complexes involved in primary cell wall synthesis in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences* **104**: 15572-15577.

Devos KM, Wu X, Qi P. 2017. Genome Structure and Comparative Genomics. In *Genetics and Genomics of Setaria*, pp. 135-147. Springer.

Dhaliwal AK, Mohan A, Gill KS. 2014. Comparative analysis of ABCB1 reveals novel structural and functional conservation between monocots and dicots. *Frontiers in plant science* **5**: 657.

Dhugga KS. 2001. Building the wall: genes and enzyme complexes for polysaccharide synthases. *Current opinion in plant biology* **4**: 488-493.

Dhugga KS. 2007. Maize biomass yield and composition for biofuels. *Crop Science* **47**: 2211-2227.

Dhugga KS. 2012. Biosynthesis of non-cellulosic polysaccharides of plant cell walls. *Phytochemistry* **74**: 8-19.

Dhugga KS, Barreiro R, Whitten B, Stecca K, Hazebroek J, Randhawa GS, Dolan M, Kinney AJ, Tomes D, Nichols S. 2004a. Guar seed ß-mannan synthase is a member of the cellulose synthase super gene family. *Science* **303**: 363-366.

Dhugga KS, Barreiro R, Whitten B, Stecca K, Hazebroek J, Randhawa GS, Dolan M, Kinney AJ, Tomes D, Nichols S et al. 2004b. Guar seed beta-mannan synthase is a member of the cellulose synthase super gene family. *Science* **303**: 363-366.

Ding S-Y, Zhao S, Zeng Y. 2013. Size, shape, and arrangement of native cellulose fibrils in maize cell walls. *Cellulose* **21**: 863-871.

Doblin MS, De Melis L, Newbigin E, Bacic A, Read SM. 2001. Pollen tubes of Nicotiana alata express two genes from different β-glucan synthase families. *Plant Physiology* **125**: 2040-2052.

Doblin MS, Pettolino F, Bacic A. 2010. Plant cell walls: the skeleton of the plant world. *Functional Plant Biology* **37**: 357-381.

Doblin MS, Pettolino FA, Wilson SM, Campbell R, Burton RA, Fincher GB, Newbigin E, Bacic A. 2009. A barley cellulose synthase-like CSLH gene mediates (1,3;1,4)-beta-D-glucan synthesis in transgenic Arabidopsis. *Proc Natl Acad Sci U S A* **106**: 5996-6001.

Douche T, San Clemente H, Burlat V, Roujol D, Valot B, Zivy M, Pont-Lezica R, Jamet E. 2013. Brachypodium distachyon as a model plant toward improved biofuel crops: Search for secreted proteins involved in biogenesis and disassembly of cell wall polymers. *Proteomics* **13**: 2438-2454.

Douchkov D, Lueck S, Hensel G, Kumlehn J, Rajaraman J, Johrde A, Doblin MS, Beahan CT, Kopischke M, Fuchs R. 2016. The barley (Hordeum vulgare) cellulose synthase-like D2

gene (HvCslD2) mediates penetration resistance to host-adapted and nonhost isolates of the powdery mildew fungus. *New Phytologist*.

Ebringerová A, Hromádková Z, Heinze T. 2005. Hemicellulose. **186**: 1-67.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research* **32**: 1792-1797.

Endler A, Kesten C, Schneider R, Zhang Y, Ivakov A, Froehlich A, Funke N, Persson S. 2015. A mechanism for sustained cellulose synthesis during salt stress. *Cell* **162**: 1353-1364.

Endler A, Persson S. 2011. Cellulose synthases and synthesis in Arabidopsis. *Mol Plant* **4**: 199-211.

Endo T, Fedorov A, de Souza SJ, Gilbert W. 2002. Do introns favor or avoid regions of amino acid conservation? *Molecular biology and evolution* **19**: 521-252.

Eudes A, Liang Y, Mitra P, Loque D. 2014. Lignin bioengineering. *Curr Opin Biotechnol* **26**: 189-198.

Famoso AN, Zhao K, Clark RT, Tung CW, Wright MH, Bustamante C, Kochian LV, McCouch SR. 2011. Genetic architecture of aluminum tolerance in rice (Oryza sativa) determined through genome-wide association analysis and QTL mapping. *PLoS Genet* **7**: e1002221.

FAN W-x, HOU Y-x, FENG S-w, ZHU F-k, RU Z-g. 2012. Study on Cellulose and Lodging Resistance of Wheat Straw. *Journal of Henan Agricultural Sciences* **9**: 010.

Faris JD. 2014. Wheat domestication: Key to agricultural revolutions past and future. *Genomics of Plant Genetic Resources*: 439.

Farrokhi N, Burton RA, Brownfield L, Hrmova M, Wilson SM, Bacic A, Fincher GB. 2006. Plant cell wall biosynthesis: genetic, biochemical and functional genomics approaches to the identification of key genes. *Plant Biotechnol J* **4**: 145-167.

Fincher GB. 2009. Revolutionary times in our understanding of cell wall biosynthesis and remodeling in the grasses. *Plant physiology* **149**: 27-37.

Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, Potter SC, Punta M, Qureshi M, Sangrador-Vegas A. 2016. The Pfam protein families database: towards a more sustainable future. *Nucleic acids research* **44**: D279-D285.

Fry SC. 2001. Plant cell walls. *eLS*.

Fry SC. 2004. Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. *New phytologist* **161**: 641-675.

Fujii S, Hayashi T, Mizuno K. 2010. Sucrose synthase is an integral component of the cellulose synthesis machinery. *Plant and cell physiology* **51**: 294-301.

Gabhane J, William SP, Gadhe A, Rath R, Vaidya AN, Wate S. 2014. Pretreatment of banana agricultural waste for bio-ethanol production: individual and interactive effects of acid and alkali pretreatments with autoclaving, microwave heating and ultrasonication. *Waste Manag* **34**: 498-503.

Gil-Humanes J, Pistón F, Barro F, Rosell CM. 2014. The Shutdown of Celiac Disease-Related Gliadin Epitopes in Bread Wheat by RNAi Provides Flours with Increased Stability and Better Tolerance to Over-Mixing. *PloS one* **9**: e91931.

Girio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Lukasik R. 2010. Hemicelluloses for fuel ethanol: A review. *Bioresour Technol* **101**: 4775-4800.

Goodstein DM, Batra S, Carlson J, Hayes R, Phillips J, Shu S, Schmutz J, Rokhsar D. 2014. Phytozome Comparative Plant Genomics Portal. Ernest Orlando Lawrence Berkeley National Laboratory, Berkeley, CA (US).

Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Mitros T, Dirks W, Hellsten U, Putnam N. 2012. Phytozome: a comparative platform for green plant genomics. *Nucleic acids research* **40**: D1178-D1186.

Goubet F, Barton CJ, Mortimer JC, Yu X, Zhang Z, Miles GP, Richens J, Liepman AH, Seffen K, Dupree P. 2009. Cell wall glucomannan in Arabidopsis is synthesised by CSLA glycosyltransferases, and influences the progression of embryogenesis. *Plant J* **60**: 527-538.

Guerriero G, Hausman JF, Strauss J, Ertan H, Siddiqui KS. 2016. Lignocellulosic biomass: Biosynthesis, degradation, and industrial utilization. *Engineering in Life Sciences* **16**: 1-16.

Gupta P, Rustgi S. 2004. Molecular markers from the transcribed/expressed region of the genome in higher plants. *Functional & integrative genomics* **4**: 139-162.

Gupta PK, Mir RR, Mohan A, Kumar J. 2008. Wheat genomics: present status and future prospects. *Int J Plant Genomics* **2008**: 896451.

Gurung S, Mamidi S, Bonman JM, Xiong M, Brown-Guedira G, Adhikari TB. 2014. Genome-wide association study reveals novel quantitative trait Loci associated with resistance to multiple leaf spot diseases of spring wheat. *PLoS One* **9**: e108179.

Gutierrez R, Lindeboom JJ, Paredez AR, Emons AMC, Ehrhardt DW. 2009. Arabidopsis cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. *Nature Cell Biology* **11**: 797-806.

Haigler CH, Davis JK, Slabaugh E, Kubicki JD. 2016. Biosynthesis and Assembly of Cellulose. *Molecular Cell Biology of the Growth and Differentiation of Plant Cells*: 120.

Hamann T, Osborne E, Youngs HL, Misson J, Nussaume L, Somerville C. 2004. Global expression analysis of CESA and CSL genes in Arabidopsis. *Cellulose* **11**: 279-286.

Hatfield RD, Marita JM, Frost K, Grabber J, Ralph J, Lu F, Kim H. 2009. Grass lignin acylation: p-coumaroyl transferase activity and cell wall characteristics of C3 and C4 grasses. *Planta* **229**: 1253-1267.

Hazen SP, Scott-Craig JS, Walton JD. 2002. Cellulose synthase-like genes of rice. *Plant Physiol* **128**: 336-340.

He XY, Zhang YL, He ZH, Wu YP, Xiao YG, Ma CX, Xia XC. 2008. Characterization of phytoene synthase 1 gene (Psy1) located on common wheat chromosome 7A and development of a functional marker. *Theor Appl Genet* **116**: 213-221.

Held MA, Penning B, Brandt AS, Kessans SA, Yong W, Scofield SR, Carpita NC. 2008. Small-interfering RNAs from natural antisense transcripts derived from a cellulose synthase gene modulate cell wall biosynthesis in barley. *Proceedings of the National Academy of Sciences* **105**: 20534-20539.

Hernández-Blanco C, Feng DX, Hu J, Sánchez-Vallet A, Deslandes L, Llorente F, Berrocal-Lobo M, Keller H, Barlet X, Sánchez-Rodríguez C. 2007. Impairment of cellulose synthases required for Arabidopsis secondary cell wall formation enhances disease resistance. *The Plant Cell* **19**: 890-903.

Hill JL, Hammudi MB, Tien M. 2014. The Arabidopsis Cellulose Synthase Complex: A Proposed Hexamer of CESA Trimers in an Equimolar Stoichiometry. *The Plant Cell Online*: tpc. 114.131193.

Holland N, Holland D, Helentjaris T, Dhugga KS, Xoconostle-Cazares B, Delmer DP. 2000. A comparative analysis of the plant cellulose synthase (CesA) gene family. *Plant Physiology* **123**: 1313-1323.

Holzberg S, Brosio P, Gross C, Pogue GP. 2002. Barley stripe mosaic virus-induced gene silencing in a monocot plant. *The Plant Journal* **30**: 315-327.

Hong Y, Chen L, Du LP, Su Z, Wang J, Ye X, Qi L, Zhang Z. 2014. Transcript suppression of TaGW2 increased grain width and weight in bread wheat. *Funct Integr Genomics* **14**: 341-349.

Hood EE. 2016. Plant-based biofuels. *F1000Research* **5**.

Houston K, Burton RA, Sznajder B, Rafalski AJ, Dhugga KS, Mather DE, Taylor J, Steffenson BJ, Waugh R, Fincher GB. 2015. A Genome-Wide Association Study for Culm Cellulose Content in Barley Reveals Candidate Genes Co-Expressed with Members of the CELLULOSE SYNTHASE A Gene Family. *PloS one* **10**.

Hu Z, Song N, Xing J, Chen Y, Han Z, Yao Y, Peng H, Ni Z, Sun Q. 2013. Overexpression of three TaEXPA1 homoeologous genes with distinct expression divergence in hexaploid wheat exhibit functional retention in Arabidopsis. *PloS one* **8**: e63667.

Huang X, Wei X, Sang T, Zhao Q, Feng Q, Zhao Y, Li C, Zhu C, Lu T, Zhang Z. 2010. Genome-wide association studies of 14 agronomic traits in rice landraces. *Nature genetics* **42**: 961-967.

Hunter CT, Kirienko DH, Sylvester AW, Peter GF, McCarty DR, Koch KE. 2012. Cellulose Synthase-Like D1 is integral to normal cell division, expansion, and leaf development in maize. *Plant physiology* **158**: 708-724.

Iehisa JC, Okada M, Sato K, Takumi S. 2017. Detection of splicing variants in the leaf and spike transcripts of wild diploid wheat Aegilops tauschii and transmission of the splicing patterns to synthetic hexaploid wheat. *Plant Gene* **9**: 6-12.

Jaiswal V, Gahlaut V, Meher PK, Mir RR, Jaiswal JP, Rao AR, Balyan HS, Gupta PK. 2016. Genome Wide Single Locus Single Trait, Multi-Locus and Multi-Trait Association Mapping for Some Important Agronomic Traits in Common Wheat (T. aestivum L.). *PloS one* **11**: e0159343.

Jonas E, de Koning DJ. 2013. Does genomic selection have a future in plant breeding? *Trends Biotechnol* **31**: 497-504.

Jordan KW, Wang S, Lun Y, Gardiner L-J, MacLachlan R, Hucl P, Wiebe K, Wong D, Forrest KL, Sharpe AG. 2015. A haplotype map of allohexaploid wheat reveals distinct patterns of selection on homoeologous genomes. *Genome biology* **16**: 1.

Joshi CP, Mansfield SD. 2007. The cellulose paradox—simple molecule, complex biosynthesis. *Current opinion in plant biology* **10**: 220-226.

Kaur R, Singh K, Singh J. 2013. A root-specific wall-associated kinase gene, HvWAK1, regulates root growth and is highly divergent in barley and other cereals. *Functional & integrative genomics* **13**: 167-177.

Kaur S, Dhugga KS, Gill K, Singh J. 2016. Novel Structural and Functional Motifs in cellulose synthase (CesA) Genes of Bread Wheat (Triticum aestivum, L.). *PLoS One* **11**.

Keegstra K. 2010. Plant cell walls. *Plant physiology* **154**: 483-486.

Kim CM, Park SH, Je BI, Park SH, Park SJ, Piao HL, Eun MY, Dolan L, Han C-d. 2007a. OsCSLD1, a cellulose synthase-like D1 gene, is required for root hair morphogenesis in rice. *Plant Physiology* **143**: 1220-1230.

Kim E, Magen A, Ast G. 2007b. Different levels of alternative splicing among eukaryotes. *Nucleic acids research* **35**: 125-131.

Kollers S, Rodemann B, Ling J, Korzun V, Ebmeyer E, Argillier O, Hinze M, Plieske J, Kulosa D, Ganal MW. 2013. Genetic architecture of resistance to Septoria tritici blotch (Mycosphaerella graminicola) in European winter wheat. *Molecular breeding* **32**: 411-423.

Kotake T, Aohara T, Hirano K, Sato A, Kaneko Y, Tsumuraya Y, Takatsuji H, Kawasaki S. 2011. Rice Brittle culm 6 encodes a dominant-negative form of CesA protein that perturbs cellulose synthesis in secondary cell walls. *Journal of Experimental Botany* **62**: 2053-2062.

Krasileva KV, Vasquez-Gross HA, Howell T, Bailey P, Paraiso F, Clissold L, Simmonds J, Ramirez-Gonzalez RH, Wang X, Borrill P. 2017. Uncovering hidden variation in polyploid wheat. *Proceedings of the National Academy of Sciences*: 201619268.

Kumar M, Wightman R, Atanassov I, Gupta A, Hurst CH, Hemsley PA, Turner S. 2016. S-Acylation of the cellulose synthase complex is essential for its plasma membrane localization. *Science* **353**: 166-169.

Kurek I, Kawagoe Y, Jacob-Wilk D, Doblin M, Delmer D. 2002. Dimerization of cotton fiber cellulose synthase catalytic subunits occurs via oxidation of the zinc-binding domains. *Proceedings of the National Academy of Sciences* **99**: 11109-11114.

Laird NM, Lange C. 2011. *The fundamentals of modern statistical genetics*. Springer.

Lairson L, Henrissat B, Davies G, Withers S. 2008. Glycosyltransferases: structures, functions, and mechanisms. *Biochemistry* **77**: 521.

Lee WS, Hammond-Kosack KE, Kanyuka K. 2012. Barley stripe mosaic virus-mediated tools for investigating gene function in cereal plants and their pathogens: virus-induced gene

silencing, host-mediated gene silencing, and virus-mediated overexpression of heterologous protein. *Plant Physiol* **160**: 582-590.

Lei L, Zhang T, Strasser R, Lee CM, Gonneau M, Mach L, Vernhettes S, Kim SH, Cosgrove DJ, Li S. 2014. The jiaoyao1 mutant is an allele of korrigan1 that abolishes endoglucanase activity and affects the organization of both cellulose microfibrils and microtubules in Arabidopsis. *The Plant Cell* **26**: 2601-2616.

Li K, Wang H, Hu X, Liu Z, Wu Y, Huang C. 2016. Genome-wide association study reveals the genetic basis of stalk cell wall components in maize. *PloS one* **11**: e0158906.

Li M, Xiong G, Li R, Cui J, Tang D, Zhang B, Pauly M, Cheng Z, Zhou Y. 2009. Rice cellulose synthase-like D4 is essential for normal cell-wall biosynthesis and plant growth. *The Plant Journal* **60**: 1055-1069.

Li S, Bashline L, Lei L, Gu Y. 2014a. Cellulose synthesis and its regulation. *Arabidopsis Book* **12**: e0169.

Li S, Logan Bashline LL, Gu Y. 2014b. Cellulose Synthesis and Its Regulation. *The Arabidopsis book/American Society of Plant Biologists* **12**.

Li W, Godzik A. 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* **22**: 1658-1659.

Liepman AH, Cavalier DM. 2012. The CELLULOSE SYNTHASE-LIKE A and CELLULOSE SYNTHASE-LIKE C families: recent advances and future perspectives. *Frontiers in plant science* **3**: 109.

Liepman AH, Wilkerson CG, Keegstra K. 2005. Expression of cellulose synthase-like (Csl) genes in insect cells reveals that CslA family members encode mannan synthases. *Proc Natl Acad Sci U S A* **102**: 2221-2226.

Lin F, Manisseri C, Fagerström A, Peck ML, Vega-Sánchez ME, Williams B, Chiniquy DM, Saha P, Pattathil S, Conlin B. 2016. Cell Wall Composition and Candidate Biosynthesis Gene Expression Across Rice Development. *Plant and Cell Physiology*: pcw125.

Lin Y, Kao Y-Y, Chen Z-Z, Chu F-H, Chung J-D. 2014. cDNA cloning and molecular characterization of five cellulose synthase A genes from Eucalyptus camaldulensis. *Journal of plant biochemistry and biotechnology* **23**: 199-210.

Lindedam J, Andersen SB, DeMartini J, Bruun S, Jørgensen H, Felby C, Magid J, Yang B, Wyman C. 2012. Cultivar variation and selection potential relevant to the production of cellulosic ethanol from wheat straw. *biomass and bioenergy* **37**: 221-228.

Lipka AE, Tian F, Wang Q, Peiffer J, Li M, Bradbury PJ, Gore MA, Buckler ES, Zhang Z. 2012. GAPIT: genome association and prediction integrated tool. *Bioinformatics* **28**: 2397-2399.

Liu X, Huang M, Fan B, Buckler ES, Zhang Z. 2016. Iterative usage of fixed and random effect models for powerful and efficient genome-wide association studies. *PLoS Genet* **12**: e1005767.

Liu X, Wang Q, Chen P, Song F, Guan M, Jin L, Wang Y, Yang C. 2012. Four Novel Cellulose Synthase (CESA) Genes from Birch (Betula platyphylla Suk.) Involved in Primary and Secondary Cell Wall Biosynthesis. *Int J Mol Sci* **13**: 12195-12212.

Lopes M, Dreisigacker S, Peña R, Sukumaran S, Reynolds M. 2015. Genetic characterization of the wheat association mapping initiative (WAMI) panel for dissection of complex traits in spring wheat. *Theoretical and Applied Genetics* **128**: 453-464.

Ma J, Stiller J, Berkman PJ, Wei Y, Rogers J, Feuillet C, Dolezel J, Mayer KF, Eversole K, Zheng Y-L. 2013. Sequence-based analysis of translocations and inversions in bread wheat (Triticum aestivum L.). *PloS one* **8**: e79329.

Madramootoo C. 2015. *Emerging Technologies for Promoting Food Security: Overcoming the World Food Crisis*. Woodhead Publishing.

Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, Geer RC, He J, Gwadz M, Hurwitz DI. 2014. CDD: NCBI's conserved domain database. *Nucleic acids research*: gku1221.

Marcussen T, Sandve SR, Heier L, Spannagl M, Pfeifer M, Jakobsen KS, Wulff BB, Steuernagel B, Mayer KF, Olsen O-A. 2014. Ancient hybridizations among the ancestral genomes of bread wheat. *Science* **345**: 1250092.

Mayer KF, Rogers J, Doležel J, Pozniak C, Eversole K, Feuillet C, Gill B, Friebe B, Lukaszewski AJ, Sourdille P. 2014. A chromosome-based draft sequence of the hexaploid bread wheat (Triticum aestivum) genome. *Science* **345**: 1251788.

McFarlane HE, Doring A, Persson S. 2014. The cell biology of cellulose synthesis. *Annu Rev Plant Biol* **65**: 69-94.

McGrann GR, Smith PH, Burt C, Mateos GR, Chama TN, MacCormack R, Wessels E, Agenbag G, Boyd LA. 2014. Genomic and genetic analysis of the wheat race-specific yellow rust resistance gene Yr5. *Journal of Plant Science and Molecular Breeding* **3**.

McMullen MD, Kresovich S, Villeda HS, Bradbury P, Li H, Sun Q, Flint-Garcia S, Thornsberry J, Acharya C, Bottoms C et al. 2009. Genetic properties of the maize nested association mapping population. *Science* **325**: 737-740.

Meuwissen THE, Hayes BJ, Goddard ME. 2001. Prediction of total genetic value using genome-wide dense marker maps. *Genetics* **157**: 1819-1829.

Michel S, Ametz C, Gungor H, Epure D, Grausgruber H, Löschenberger F, Buerstmayr H. 2016. Genomic selection across multiple breeding cycles in applied bread wheat breeding. *Theoretical and Applied Genetics* **129**: 1179-1189.

Mittler R, Shulaev V. 2013. Functional genomics, challenges and perspectives for the future. *Physiologia Plantarum* **148**: 317-321.

Mochida K, Shinozaki K. 2010. Genomics and bioinformatics resources for crop improvement. *Plant Cell Physiol* **51**: 497-523.

Mochida K, Shinozaki K. 2013. Unlocking Triticeae genomics to sustainably feed the future. *Plant Cell Physiol* **54**: 1931-1950.

Mochida K, Yamazaki Y, Ogihara Y. 2004. Discrimination of homoeologous gene expression in hexaploid wheat by SNP analysis of contigs grouped from a large number of expressed sequence tags. *Molecular Genetics and Genomics* **270**: 371-377.

Mohan A, Schillinger WF, Gill KS. 2013. Wheat seedling emergence from deep planting depths and its relationship with coleoptile length. *PloS one* **8**: e73314.

Molnár I, Vrána J, Burešová V, Cápal P, Farkas A, Darkó É, Cseh A, Kubaláková M, Molnár-Láng M, Doležel J. 2016. Dissecting the U, M, S and C genomes of wild relatives of bread wheat (Aegilops spp.) into chromosomes and exploring their synteny with wheat. *The Plant Journal* **88**: 452-467.

Monaco MK, Stein J, Naithani S, Wei S, Dharmawardhana P, Kumari S, Amarasinghe V, Youens-Clark K, Thomason J, Preece J et al. 2014. Gramene 2013: comparative plant genomics resources. *Nucleic Acids Research* **42**: D1193-D1199.

Morgan JL, Strumillo J, Zimmer J. 2013. Crystallographic snapshot of cellulose synthesis and membrane translocation. *Nature* **493**: 181-186.

Moriarty P, Honnery D. 2016. Can renewable energy power the future? *Energy Policy* **93**: 3-7.

Motte JC, Escudié R, Beaufils N, Steyer JP, Bernet N, Delgenès JP, Dumas C. 2014. Morphological structures of wheat straw strongly impacts its anaerobic digestion. *Industrial Crops and Products* **52**: 695-701.

Muthamilarasan M, Theriappan P, Prasad M. 2013. Recent advances in crop genomics for ensuring food security. *Curr Sci* **105**: 155-158.

Nemeth C, Freeman J, Jones HD, Sparks C, Pellny TK, Wilkinson MD, Dunwell J, Andersson AAM, Aman P, Guillon F et al. 2010. Down-Regulation of the CSLF6 Gene Results in Decreased (1,3;1,4)-beta-D-Glucan in Endosperm of Wheat. *Plant Physiology* **152**: 1209-1218.

Neumann K, Kobiljski B, Denčić S, Varshney RK, Börner A. 2010. Genome-wide association mapping: a case study in bread wheat (Triticum aestivum L.). *Molecular Breeding* **27**: 37-58.

O'brien T, Feder N, McCully ME. 1964. Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma* **59**: 368-373.

Ong RG, Chundawat SPS, Hodge DB, Keskar S, Dale BE. 2014. Linking Plant Biology and Pretreatment: Understanding the Structure and Organization of the Plant Cell Wall and Interactions with Cellulosic Biofuel Production. doi:10.1007/978-1-4614-9329-7_14: 231-253.

Paredez AR, Somerville CR, Ehrhardt DW. 2006. Visualization of cellulose synthase demonstrates functional association with microtubules. *Science* **312**: 1491-1495.

Pauly M, Gille S, Liu L, Mansoori N, de Souza A, Schultink A, Xiong G. 2013. Hemicellulose biosynthesis. *Planta* **238**: 627-642.

Pauly M, Keegstra K. 2008. Cell-wall carbohydrates and their modification as a resource for biofuels. *The Plant Journal* **54**: 559-568.

Penning BW, Sykes RW, Babcock NC, Dugard CK, Klimek JF, Gamblin D, Davis M, Filley TR, Mosier NS, Weil CF et al. 2014. Validation of PyMBMS as a High-throughput Screen for Lignin Abundance in Lignocellulosic Biomass of Grasses. *BioEnergy Research* doi:10.1007/s12155-014-9410-3.

Perera FP. 2016. Multiple threats to child health from fossil fuel combustion: Impacts of air pollution and climate change. *Environ Health Perspect*.

Persson S, Paredez A, Carroll A, Palsdottir H, Doblin M, Poindexter P, Khitrov N, Auer M, Somerville CR. 2007. Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in Arabidopsis. *Proc Natl Acad Sci U S A* **104**: 15566-15571.

Petty I, Hunter B, Wei N, Jackson A. 1989. Infectious barley stripe mosaic virus RNA transcribed in vitro from full-length genomic cDNA clones. *Virology* **171**: 342-349.

Pingault L, Choulet F, Alberti A, Glover N, Wincker P, Feuillet C, Paux E. 2015. Deep transcriptome sequencing provides new insights into the structural and functional organization of the wheat genome. *Genome biology* **16**: 29.

Poczai P, Varga I, Laos M, Cseh A, Bell N, Valkonen JP, Hyvönen J. 2013. Advances in plant gene-targeted and functional markers: a review. *Plant methods* **9**: 6.

Porth I, Klapšte J, Skyba O, Hannemann J, McKown AD, Guy RD, DiFazio SP, Muchero W, Ranjan P, Tuskan GA. 2013. Genome-wide association mapping for wood characteristics in Populus identifies an array of candidate single nucleotide polymorphisms. *New Phytologist* **200**: 710-726.

Poursarebani N, Ariyadasa R, Zhou R, Schulte D, Steuernagel B, Martis MM, Graner A, Schweizer P, Scholz U, Mayer K et al. 2013. Conserved synteny-based anchoring of the barley genome physical map. *Funct Integr Genomics* **13**: 339-350.

Qiu Z, Wan L, Chen T, Wan Y, He X, Lu S, Wang Y, Lin J. 2013. The regulation of cambial activity in Chinese fir (Cunninghamia lanceolata) involves extensive transcriptome remodeling. *New Phytologist* **199**: 708-719.

Qureshi N, Saha BC, Cotta MA, Singh V. 2013. An economic evaluation of biological conversion of wheat straw to butanol: A biofuel. *Energy Conversion and Management* **65**: 456-462.

Ramegowda V, Mysore KS, Senthil-Kumar M. 2014. Virus-induced gene silencing is a versatile tool for unraveling the functional relevance of multiple abiotic-stress-responsive genes in crop plants. *Frontiers in plant science* **5**.

Ranik M, Myburg AA. 2006. Six new cellulose synthase genes from Eucalyptus are associated with primary and secondary cell wall biosynthesis. *Tree physiology* **26**: 545-556.

Ranocha P, Denancé N, Vanholme R, Freydier A, Martinez Y, Hoffmann L, Köhler L, Pouzet C, Renou JP, Sundberg B. 2010. Walls are thin 1 (WAT1), an Arabidopsis homolog of Medicago truncatula NODULIN21, is a tonoplast-localized protein required for secondary wall formation in fibers. *The Plant Journal* **63**: 469-483.

Rao G, Zeng Y, He C, Zhang J. 2016. Characterization and putative post-translational regulation of α-and β-tubulin gene families in Salix arbutifolia. *Scientific reports* **6**.

Rayon C, Olek AT, Carpita NC. 2014. Towards Redesigning Cellulose Biosynthesis for Improved Bioenergy Feedstocks. In *Plants and BioEnergy*, pp. 183-193. Springer.

Rejab NA, Nakano Y, Yoneda A, Ohtani M, Demura T. 2015. Possible contribution of TED6 and TED7, secondary cell wall-related membrane proteins, to evolution of tracheary element in angiosperm lineage. *Plant Biotechnology* **32**: 343-347.

Richmond T. 2000. Higher plant cellulose synthases. *Genome biology* **1**: REVIEWS3001-REVIEWS3001.

Richmond TA, Somerville CR. 2000. The cellulose synthase superfamily. *Plant physiology* **124**: 495-498.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139-140.

Ruiz HA, Cerqueira MA, Silva HD, Rodriguez-Jasso RM, Vicente AA, Teixeira JA. 2013. Biorefinery valorization of autohydrolysis wheat straw hemicellulose to be applied in a polymer-blend film. *Carbohydr Polym* **92**: 2154-2162.

Ruiz MT, Voinnet O, Baulcombe DC. 1998. Initiation and maintenance of virus-induced gene silencing. *The Plant Cell* **10**: 937-946.

Saini JK, Saini R, Tewari L. 2015. Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. *3 Biotech* **5**: 337-353.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution* **4**: 406-425.

Salgotra RK, Gupta BB, Stewart CN, Jr. 2014. From genomics to functional markers in the era of next-generation sequencing. *Biotechnol Lett* **36**: 417-426.

Sánchez-Rodríguez C, Bauer S, Hématy K, Saxe F, Ibáñez AB, Vodermaier V, Konlechner C, Sampathkumar A, Rüggeberg M, Aichinger E. 2012. Chitinase-like1/pom-pom1 and its

homolog CTL2 are glucan-interacting proteins important for cellulose biosynthesis in Arabidopsis. *The Plant Cell* **24**: 589-607.

Sarkar P, Bosneaga E, Auer M. 2009. Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *Journal of Experimental Botany* **60**: 3615-3635.

Saxena IM, Brown Jr RM, Fevre M, Geremia RA, Henrissat B. 1995. Multidomain architecture of beta-glycosyl transferases: implications for mechanism of action. *Journal of Bacteriology* **177**: 1419.

Saxena IM, Brown R. 1995. Identification of a second cellulose synthase gene (acsAII) in Acetobacter xylinum. *Journal of bacteriology* **177**: 5276-5283.

Saxena IM, Brown RM. 2005. Cellulose biosynthesis: current views and evolving concepts. *Annals of botany* **96**: 9-21.

Scholey D, Burton E, Williams P. 2016. The bio refinery; producing feed and fuel from grain. *Food Chemistry* **197**: 937-942.

Schreiber M, Wright F, MacKenzie K, Hedley PE, Schwerdt JG, Little A, Burton RA, Fincher GB, Marshall D, Waugh R. 2014a. The barley genome sequence assembly reveals three additional members of the CslF (1, 3; 1, 4)-β-glucan synthase gene family. *PloS one* **9**: e90888.

Schreiber M, Wright F, MacKenzie K, Hedley PE, Schwerdt JG, Little A, Burton RA, Fincher GB, Marshall D, Waugh R et al. 2014b. The barley genome sequence assembly reveals three additional members of the CslF (1,3;1,4)-beta-glucan synthase gene family. *PLoS One* **9**: e90888.

Schwerdt JG, MacKenzie K, Wright F, Oehme D, Wagner JM, Harvey AJ, Shirley NJ, Burton RA, Schreiber M, Halpin C. 2015. Evolutionary dynamics of the Cellulose synthase gene superfamily in grasses. *Plant physiology* **168**: 968-983.

Scofield SR, Huang L, Brandt AS, Gill BS. 2005. Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway. *Plant Physiology* **138**: 2165-2173.

Senthil-Kumar M, Mysore KS. 2011. New dimensions for VIGS in plant functional genomics. *Trends Plant Sci* **16**: 656-665.

Sethaphong L, Haigler CH, Kubicki JD, Zimmer J, Bonetta D, DeBolt S, Yingling YG. 2013. Tertiary model of a plant cellulose synthase. *Proceedings of the National Academy of Sciences* **110**: 7512-7517.

Shaveta, Bansal N, Singh P. 2014. F−/Cl− mediated microwave assisted breakdown of cellulose to glucose. *Tetrahedron Letters* **55**: 2467-2470.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology* **7**: 539.

Simeao Resende RM, Casler MD, Vilela de Resende MD. 2014. Genomic Selection in Forage Breeding: Accuracy and Methods. *Crop Science* **54**: 143-156.

Singh J, Zhang S, Chen C, Cooper L, Bregitzer P, Sturbaum A, Hayes PM, Lemaux PG. 2006. High-frequency Ds remobilization over multiple generations in barley facilitates gene tagging in large genome cereals. *Plant molecular biology* **62**: 937-950.

Singh M, Singh S, Randhawa H, Singh J. 2013. Polymorphic homoeolog of key gene of RdDM pathway, ARGONAUTE4_9 class is associated with pre-harvest sprouting in wheat (Triticum aestivum L.). *PLoS One* **8**: e77009.

Singh S, Tan HQ, Singh J. 2012. Mutagenesis of barley malting quality QTLs with Ds transposons. *Functional & Integrative Genomics* **12**: 131-141.

Singhania RR, Saini JK, Saini R, Adsul M, Mathur A, Gupta R, Tuli DK. 2014. Bioethanol production from wheat straw via enzymatic route employing Penicillium janthinellum cellulases. *Bioresource technology* **169**: 490-495.

Slabaugh E, Davis JK, Haigler CH, Yingling YG, Zimmer J. 2014. Cellulose synthases: new insights from crystallography and modeling. *Trends in Plant Science* **19**: 99-106.

Slavov GT, Nipper R, Robson P, Farrar K, Allison GG, Bosch M, Clifton-Brown JC, Donnison IS, Jensen E. 2014. Genome-wide association studies and prediction of 17 traits related to phenology, biomass and cell wall composition in the energy grass Miscanthus sinensis. *New Phytologist* **201**: 1227-1239.

Sokhansanj S, Mani S, Stumborg M, Samson R, Fenton J. 2006. Production and distribution of cereal straw on the Canadian Prairies. *Canadian Biosystems Engineering* **48**: 3.

Sorek N, Yeats TH, Szemenyei H, Youngs H, Somerville CR. 2014. The Implications of Lignocellulosic Biomass Chemical Composition for the Production of Advanced Biofuels. *BioScience* **64**: 192-201.

Stothard P. 2000. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques* **28**: 1102, 1104-1102, 1104.

Stratmann JW, Hind SR. 2011. Gene silencing goes viral and uncovers the private life of plants. *Entomologia Experimentalis Et Applicata* **140**: 91-102.

Strezov V, Evans TJ. 2014. *Biomass Processing Technologies*. CRC Press.

Su Z, Hao C, Wang L, Dong Y, Zhang X. 2011. Identification and development of a functional marker of TaGW2 associated with grain weight in bread wheat (Triticum aestivum L.). *Theor Appl Genet* **122**: 211-223.

Sukumaran S, Dreisigacker S, Lopes M, Chavez P, Reynolds MP. 2015. Genome-wide association study for grain yield and related traits in an elite spring wheat population grown in temperate irrigated environments. *Theoretical and Applied Genetics* **128**: 353-363.

Suzuki S, Li L, Sun Y-H, Chiang VL. 2006. The cellulose synthase gene superfamily and biochemical functions of xylem-specific cellulose synthase-like genes in Populus trichocarpa. *Plant physiology* **142**: 1233-1245.

Szyjanowicz PM, McKinnon I, Taylor NG, Gardiner J, Jarvis MC, Turner SR. 2004. The irregular xylem 2 mutant is an allele of korrigan that affects the secondary cell wall of Arabidopsis thaliana. *The Plant Journal* **37**: 730-740.

Tai Y, Bragg J, Edwards MC. 2005. Virus vector for gene silencing in wheat. *Biotechniques* **39**: 310.

Takata N, Taniguchi T. 2015. Expression divergence of cellulose synthase (CesA) genes after a recent whole genome duplication event in Populus. *Planta* **241**: 29-42.

Taketa S, Yuo T, Tonooka T, Tsumuraya Y, Inagaki Y, Haruyama N, Larroque O, Jobling SA. 2012. Functional characterization of barley betaglucanless mutants demonstrates a unique role for CslF6 in (1,3;1,4)-beta-D-glucan biosynthesis. *J Exp Bot* **63**: 381-392.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution* **30**: 2725-2729.

Tanaka K, Murata K, Yamazaki M, Onosato K, Miyao A, Hirochika H. 2003. Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthesis in the secondary wall. *Plant Physiology* **133**: 73-83.

Tanaka M, Tanaka H, Shitsukawa N, Kitagawa S, Takumi S, Murai K. 2015. Homoeologous copy-specific expression patterns of MADS-box genes for floral formation in allopolyploid wheat. *Genes & genetic systems*.

Tang Y, Liu X, Wang J, Li M, Wang Q, Tian F, Su Z, Pan Y, Liu D, Lipka AE. 2016. GAPIT Version 2: an enhanced integrated tool for genomic association and prediction. *The Plant Genome* **9**.

Taylor-Teeples M, Lin L, de Lucas M, Turco G, Toal T, Gaudinier A, Young N, Trabucco G, Veling M, Lamothe R. 2015. An Arabidopsis gene regulatory network for secondary cell wall synthesis. *Nature* **517**: 571-575.

Taylor NG, Howells RM, Huttly AK, Vickers K, Turner SR. 2003. Interactions among three distinct CesA proteins essential for cellulose synthesis. *Proc Natl Acad Sci U S A* **100**: 1450-1455.

Taylor NG, Scheible W-R, Cutler S, Somerville CR, Turner SR. 1999. The irregular xylem3 locus of Arabidopsis encodes a cellulose synthase required for secondary cell wall synthesis. *The plant cell* **11**: 769-779.

Team RC. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2013.

Thomas LH, Forsyth VT, Šturcová A, Kennedy CJ, May RP, Altaner CM, Apperley DC, Wess TJ, Jarvis MC. 2013. Structure of cellulose microfibrils in primary cell walls from collenchyma. *Plant physiology* **161**: 465-476.

Tian F, Bradbury PJ, Brown PJ, Hung H, Sun Q, Flint-Garcia S, Rocheford TR, McMullen MD, Holland JB, Buckler ES. 2011. Genome-wide association study of leaf architecture in the maize nested association mapping population. *Nat Genet* **43**: 159-162.

Tian J, Chang M, Du Q, Xu B, Zhang D. 2014. Single-nucleotide polymorphisms in PtoCesA7 and their association with growth and wood properties in Populus tomentosa. *Molecular Genetics and Genomics* **289**: 439-455.

Trapp SC, Croteau RB. 2001. Genomic organization of plant terpene synthases and molecular evolutionary implications. *Genetics* **158**: 811-832.

Tumuluru JS, Tabil LG, Song Y, Iroba KL, Meda V. 2014. Grinding energy and physical properties of chopped and hammer-milled barley, wheat, oat, and canola straws. *Biomass and Bioenergy* **60**: 58-67.

Turner SR, Somerville CR. 1997. Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. *The plant cell* **9**: 689-701.

Vain T, Crowell EF, Timpano H, Biot E, Desprez T, Mansoori N, Trindade LM, Pagant S, Robert S, Höfte H. 2014. The cellulase KORRIGAN is part of the cellulose synthase complex. *Plant physiology* **165**: 1521-1532.

Varshney RK, Mahendar T, Aggarwal RK, Börner A. 2007. Genic molecular markers in plants: development and applications. In *Genomics-assisted crop improvement*, pp. 13-29. Springer.

Verhertbruggen Y, Yin L, Oikawa A, Scheller HV. 2011. Mannan synthase activity in the CSLD family. *Plant signaling & behavior* **6**: 1620.

Vogel J. 2008. Unique aspects of the grass cell wall. *Current opinion in plant biology* **11**: 301-307.

Wang D, Yuan S, Yin L, Zhao J, Guo B, Lan J, Li X. 2012a. A missense mutation in the transmembrane domain of CESA9 affects cell wall biosynthesis and plant growth in rice. *Plant Science* **196**: 117-124.

Wang H, Smith KP, Combs E, Blake T, Horsley RD, Muehlbauer GJ. 2012b. Effect of population size and unbalanced data sets on QTL detection using genome-wide association mapping in barley breeding germplasm. *Theoretical and Applied Genetics* **124**: 111-124.

Wang L, Guo K, Li Y, Tu Y, Hu H, Wang B, Cui X, Peng L. 2010a. Expression profiling and integrative analysis of the CESA/CSL superfamily in rice. *BMC plant biology* **10**: 282.

Wang L, Guo K, Li Y, Tu Y, Hu H, Wang B, Cui X, Peng L. 2010b. Expression profiling and integrative analysis of the CESA/CSL superfamily in rice. *Bmc Plant Biology* **10**.

Wang W, Wang L, Chen C, Xiong G, Tan X-Y, Yang K-Z, Wang Z-C, Zhou Y, Ye D, Chen L-Q. 2011. Arabidopsis CSLD1 and CSLD4 are required for cellulose deposition and normal growth of pollen tubes. *Journal of experimental botany*: err221.

Wang Y, You FM, Lazo GR, Luo M-C, Thilmony R, Gordon S, Kianian SF, Gu YQ. 2013. PIECE: a database for plant gene structure comparison and evolution. *Nucleic acids research* **41**: D1159-D1166.

*Waste*neys GO. 2004. Progress in understanding the role of microtubules in plant cells. *Current opinion in plant biology* **7**: 651-660.

Wightman R, Turner SR. 2008. The roles of the cytoskeleton during cellulose deposition at the secondary cell wall. *The Plant Journal* **54**: 794-805.

Wong C, Bernardo R. 2008. Genomewide selection in oil palm: increasing selection gain per unit time and cost with small populations. *Theoretical and Applied Genetics* **116**: 815-824.

Xu Y, Xie C, Wan J, He Z, Prasanna BM. 2013. Marker-Assisted Selection in Cereals: Platforms, Strategies and Examples. doi:10.1007/978-94-007-6401-9_14: 375-411.

Yin L, Verhertbruggen Y, Oikawa A, Manisseri C, Knierim B, Prak L, Jensen JK, Knox JP, Auer M, Willats WG. 2011. The cooperative activities of CSLD2, CSLD3, and CSLD5 are required for normal Arabidopsis development. *Molecular plant* **4**: 1024-1037.

Yin Y, Huang J, Xu Y. 2009. The cellulose synthase superfamily in fully sequenced plants and algae. *BMC Plant Biol* **9**: 99.

Yin Y, Johns MA, Cao H, Rupani M. 2014. A survey of plant and algal genomes and transcriptomes reveals new insights into the evolution and function of the cellulose synthase superfamily. *BMC genomics* **15**: 1.

Yong W, Link B, O'Malley R, Tewari J, Hunter CT, Lu C-A, Li X, Bleecker AB, Koch KE, McCann MC. 2005. Genomics of plant cell wall biogenesis. *Planta* **221**: 747-751.

Yoshikawa T, Eiguchi M, Hibara K-I, Ito J-I, Nagato Y. 2013. Rice SLENDER LEAF 1 gene encodes cellulose synthase-like D4 and is specifically expressed in M-phase cells to regulate cell proliferation. *Journal of experimental botany* **64**: 2049-2061.

Yuo T, Shiotani K, Shitsukawa N, Miyao A, Hirochika H, Ichii M, Taketa S. 2011. Root hairless 2 (rth2) mutant represents a loss-of-function allele of the cellulose synthase-like gene OsCSLD1 in rice (Oryza sativa L.). *Breeding science* **61**: 225-233.

Zhang B, Deng L, Qian Q, Xiong G, Zeng D, Li R, Guo L, Li J, Zhou Y. 2009. A missense mutation in the transmembrane domain of CESA4 affects protein abundance in the plasma membrane and results in abnormal cell wall biosynthesis in rice. *Plant Molecular Biology* **71**: 509-524.

Zhang H, Fangel JU, Willats WGT, Selig MJ, Lindedam J, Jørgensen H, Felby C. 2014a. Assessment of leaf/stem ratio in wheat straw feedstock and impact on enzymatic conversion. *GCB Bioenergy* **6**: 90-96.

Zhang N, Huo W, Zhang L, Chen F, Cui D. 2016. Identification of Winter-Responsive Proteins in Bread Wheat Using Proteomics Analysis and Virus-Induced Gene Silencing (VIGS). *Molecular & Cellular Proteomics* **15**: 2954-2969.

Zhang Q, Cheetamun R, Dhugga KS, Rafalski JA, Tingey SV, Shirley NJ, Taylor J, Hayes K, Beatty M, Bacic A. 2014b. Spatial gradients in cell wall composition and transcriptional profiles along elongating maize internodes. *BMC plant biology* **14**: 27.

Zhang Y, Ghaly A, Li B. 2012. PHYSICAL PROPERTIES OF RICE RESIDUES AS AFFECTED BY VARIETY AND CLIMATIC AND CULTIVATION ONDITIONS IN THREE CONTINENTS. *American Journal of Applied Sciences* **9**: 1757.

Zhong R, Ye Z-H. 2014. Secondary cell walls: biosynthesis, patterned deposition and transcriptional regulation. *Plant and Cell Physiology*: pcu140.

Zhou Y, Zhou C, Ye L, Dong J, Xu H, Cai L, Zhang L, Wei L. 2003. Database and analyses of known alternatively spliced genes in plants. *Genomics* **82**: 584-595.

Zuckerkandl E, Pauling L. 1965. Evolutionary divergence and convergence in proteins. *Evolving genes and proteins* **97**: 97-166.