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Unravelling the Layers of Cell Wall Synthesis and Function in Rice

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

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**Abstract**

The plant cell wall is of critical importance to plant growth and survival, functioning in maintaining structural integrity, supporting cell expansion, and acting as the first line of defense in response to biotic and abiotic stresses. The major components of the cell wall are cellulose, hemicelluloses, lignin, and pectin. Recent focus on the transcriptional machinery regulating cell wall biosynthesis in plants has revealed many key transcription factors responsible for orchestrating cell wall deposition. However, many of these TFs act redundantly and work coherently with a suite of TFs to activate the cell wall biosynthetic machinery. Heterologous expression of TFs is an ideal way to characterize the conserved roles of a TF in a pathway.

Here, we investigate the process of cell wall formation in rice by overexpression of the heterologous TF *AtSHN2* from Arabidopsis, to unravel the process of cell wall biosynthesis in rice. Using Tandem Affinity Purification (TAP) enabled Chromatin Immunoprecipitation studies coupled with genome wide sequencing analysis, cis-regulatory elements bound by *AtSHN2* were identified. It was identified that *AtSHN2* can bind to GCC box elements present in the promoter sequence of the downstream MYB TFs and [GA]CAACA[TG][AT] element specific of AP2 TFs. Furthermore, transcriptomic profiling of *AtSHN2*-TAP rice transgenic lines was performed to identify direct and indirect global targets of *AtSHN2*. In addition, this dissertation also characterizes the role of *OsSHN2*, the ortholog of *AtSHN2* from rice in cell wall biosynthesis in rice using transcriptomic studies. Taken together, this dissertation seeks to unravel the mechanism of cell wall formation in rice using global genomic changes associated with *AtSHN2* overexpression in rice plants.

**Acknowledgments**

Coming into this program, I practically had zero knowledge about plant physiology, the complexity and the intricacies of this sessile organism that feeds us, lets us breathe and calms us down. As I began my journey, I tried learning the system and I am still in awe of the complexity and the dexterity the plant kingdom has developed in response to whatever has been thrown to them by the harsh environment around.

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injected in me a new motivation to learn about plant sciences. You have been more of a mentor, a guide and helped me in making decisions when I was at cross roads in my life. Long discussions with Dr. Pinto in her seminar class made me a better speaker, opened my horizons by learning and talking about everything under the sun. Dr. Korth, you have always been supportive and kind through all these years and your lab members have been very accommodating with every request I have made. Thank you for that!

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Kunj. Kamal, my kid brother, thank you for keeping my secrets and listening to my crappy electronic music and just being you! I am proud of the man you are becoming, and I wish you all the success and luck in this world.

## Dedication

This dissertation is dedicated to my mom and dad - Mrs. Sangita Mihani and Mr. Radheshyam Mihani for their love, faith, belief, and continuous support in all my endeavors throughout my life.

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## CHAPTER 1: Introduction

One of the key features that distinguish plant cells from animal cells is the presence of a strong rigid structure surrounding the cell, known as the cell wall. The cell wall is an important structure of the plant cell, the basic building block of plants, that is regulated by the cell type and developmental pattern it is part of. It forms the first line of defense against challenging pathogens, providing protection against invading foreign organisms and maintains the turgor pressure of the plant, thus abetting the plant to withstand mechanical forces.

An abundance of information is available on the different models of plant cell walls in a variety of species. Broadly, the cell wall has been classified into two types—the primary cell wall and the secondary cell wall. Typically, the primary cell wall is made of cellulose, hemicellulose, and pectin molecules. They differ mainly in the composition, biogenesis and amounts of these moieties. The primary cell wall is divided into two types; Type I (Carpita, et al., 1993) cell wall present in dicotyledonous plants and non-commelinid monocots, and Type II cell wall found in commelinid monocots. The Type I primary cell wall comprises of cellulose and hemicelluloses (mainly xyloglucans) in equal amounts, and is bound by a network of pectic molecules predominantly homogalacturonic acid, rhamnogalacturonan I (RGI) and rhamnogalactouronan II (RGII). The Type II (Carpita, 1996) cell walls present in monocots differ from the type I cell wall in the amount of xyloglucans. Instead of higher amounts of xyloglucans, these contain glucuronoarabinoxylans and β1,3: β1,4 mixed glucans. The other moiety predominant in the type II primary cell wall is the hydroxycinnamate compound, ferulic acid. The primary cell wall of plants has a major role in maintaining the structural integrity of the cell by contributing to the strengthening of the cell wall. This is done by crosslinking of homogalacturonans through calcium ions and dimerization of RGII by borate ions. These molecules also serve an important role in

cellular adhesion and act as a defense barrier for many pathogens via signal transduction. Many oligosaccharide molecules have also been implicated as signal transducers for plant defense mechanisms. Oligogalacturonides trigger the activity of reactive oxygen species and phytoalexins in response to pathogens, making the cell wall an indispensable structure for the plant to maintain proper growth, development, and immune response.

The other type of cell wall, known as the secondary cell wall, is deposited after cell maturation is complete, making it predominant in sclerenchymatous cells. The secondary cell wall differs from the primary cell wall by the presence of lignin, which accounts for approximately 30% non-fossil organic carbon in the atmosphere. The incorporation of lignin in the secondary cell wall in plants has been associated with the transition from an aquatic to terrestrial habitat. The emergence of lignin can be traced back to early pteridophytes, but its presence was predominant in gymnosperms exhibiting tall and woody phenotype. The presence of lignin in gymnosperms made it easier for these plants to sustain advanced and complex water transport. In addition, lignin being a recalcitrant molecule contributes to the tensile strength of the plant cell wall and provides protection against pathogens secreting cell wall degrading enzymes. Lignin, a complex phenylpropanoid polymer is made of three monolignols namely p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Many researchers have extensively studied the biosynthesis of lignin. In short, monolignols are produced by using the phenylpropanoid pathway which is common to many compounds such as flavonoids, acetosyringone etc. Hydroxycinnamoyl CoA esters are sequentially reduced by cinnamyl CoA reductase and cinnamyl alcohol dehydrogenase into the three monolignols. The monolignols are then transported to the cell wall followed by polymerization by enzymes such as laccases and peroxidases.

Cell wall synthesis is a complex process orchestrated by an interplay of numerous enzymes, transcription factors and other proteins, which are involved in the initiation, assembly, and disassembly of different components giving rise to varied cell walls depending on the cell type and development phases. Many studies have focused on the transcriptional machinery required for cell wall biosynthesis in the model plant Arabidopsis (Hall, et al., 2013; Nakano, et al., 2015). Numerous TFs have been implicated in the process of cell wall biosynthesis. Secondary cell wall NACs have been shown to be the primary initiators of the cell wall biosynthesis signals triggering a cascade of a suite of TFs, mainly belonging to the MYB family (Zhong, et al., 2008). It has been speculated that a similar transcriptional control mechanism operates in monocots although a comprehensive review of the recent studies carried out in rice has not been reviewed. In this chapter, we have addressed the recent advancements in the field of cell wall biosynthesis in rice. As most of studies pertaining to cell wall biosynthesis have been performed in Arabidopsis, we first review the mechanism of cell wall formation in Arabidopsis followed by comparative studies in rice. This review will provide the background towards an understanding of the complex process of cell wall biosynthesis and the recent advancements in the field.

**Section 1.1: Biosynthesis of cell wall components**

As outlined above, a variety of enzymes catalyze the formation of key components of the cell wall. The major enzymes responsible for cell wall polysaccharide biosynthesis are glycan synthases and the glycosyltransferases (GTs). These enzymes catalyze the addition of successive monomers by forming bonds to the growing polymers. The enzymes are classified as processive and non-processive enzymes based on their mechanism of monomer addition on the growing polymer and branching pattern (Carpita, 1993). For example, in case of cellulose formation, cellulose synthases repeatedly add a glucose residue to the non-reducing end of the growing

cellulose polymer through β-linkages forming a (1, 4)-β-glucosidic bonds. Thus, cellulose synthases belong to the processive group of enzymes. On the other hand, non-cellulosic polysaccharides such as hemicellulose and pectins are first synthesized by the processive glucan synthases and further addition of single sugar residues to branches occurs by various non-processive glycosyl transferases. While cellulose biosynthesis occurs at the plasma membrane (Delmer, 1999), hemicelluloses and pectins are generally synthesized in the endoplasmic reticulum or Golgi apparatus, transported into the cell surface via secretory vesicles and consequently assimilated into the cell wall (Carpita, et al., 1993).

**Cellulose**

Cellulose, the most abundant organic polymer on Earth, and the main load bearing molecule of the plant cell wall is a paracrystalline structure made up of β-(1→4)-D-glucan chains. These chains are arranged into a twisted, ribbon like cable structure known as microfibrils. The physico-chemical properties of the cell wall are determined by the orientation, angle, length, and crystallinity of these microfibrils. According to the model proposed by Somerville (2006), one microfibril is made up of 36 glucan chains, each chain constituting from 8000 (primary cell wall) to approximately 15000 (secondary cell wall) glucose subunits.

Freeze fractionation of cells between the plasma membrane and cell wall led to the discovery of globular plasma membrane associated complexes attached to cellulose microfibrils. These were eventually termed as the cellulose synthase complex (CSC) (Mueller 1980). These CSCs are believed to be responsible for cellulose production and were identified in maize. Further observations that one microfibril is made up of 36 glucan chains, prompted the idea that CSCs are organized in the form of a hexameric rosette where each subunit of the rosette consists of 6 cellulose biosynthesis units, thus producing six glucan chains (Doblin, et al., 2002). Zinc finger

motifs, moieties present in the N-terminal region, facilitate the interaction of these individual cellulose biosynthesis moieties (Joshi et al., 2007).

The main substrate for cellulose biosynthesis is UDP-glucose. Isolated tobacco membranes could synthesize cellulose in the presence of 1mM UDP-glucose (Cifuentes, et al., 2010). UDP-glucose is synthesized by two major enzymes, sucrose synthase and invertase, both of which catalyze sucrose to glucose and fructose.

The studies by (Coleman, et al., 2008) showed that upregulation of sucrose synthase (SUS) leads to higher cellulose production in hybrid poplar. However, another study showed quadruple mutants of *sus1/sus2/sus3/sus4* exhibited normal cellulose production and structure, reinforcing the importance of invertase in cellulose production. A contrasting study was published showing that 85% of the SUS activity in these mutants can be achieved by maintaining favorable pH conditions (Baroja-Fernández, et al., 2012). Evidence for direct interaction of SUS and CSCs was shown in bean epicotyls (Fujii, et al., 2010). The report showed that sucrose and UDP-glucose can form cellulose in the presence of a SUS-like protein *in vitro* emphasizing the role of sucrose synthase in cellulose production and structure.

Alternatively, active phosphorylation of glucose 1-phosphate by UDP glucose pyrophosphorylase also leads to UDP-glucose production. Occasionally, UDP-glucose is also provided by invertase. The exact mechanisms of how the precursor is made available for cellulose biosynthesis to the cellulase synthase complex however remains unknown.

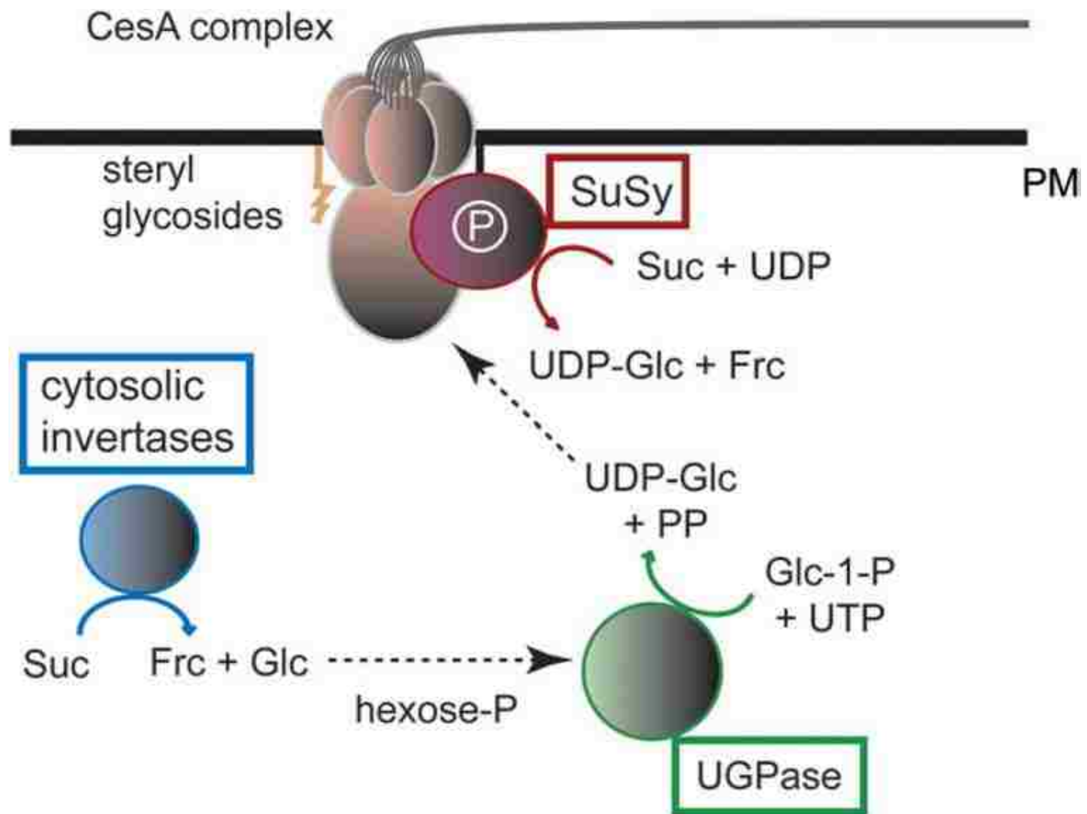


**Figure 1: Biosynthesis of cellulose at the plasma membrane** (Endler, et al., 2011)**.** The rosette structure represents the cellulose synthase complex which uses UDP-glucose (UDP-Glc) as the activated sugar for glucan chain synthesis. UDP-Glc is synthesized by Sucrose synthase or UDP-glucose pyrophosphorylase.

**Cellulose biosynthesis genes**

The first plant cellulose biosynthetic genes (*CesAs*) were identified from cotton based on similarities to the bacterial cellulose biosynthetic genes. These genes belong to the GT-2 family of genes. CESAs have a classic eight transmembrane topology, typical of the GT-2 family with one catalytic residue and one substrate binding residue. The residues are located between the second and the third loops of the transmembrane domain and are characterized by D,D,D,QXXRW motif. According to the latest model of cellulose biosynthesis, the first two aspartic acid residues function in UDP binding, the third is responsible for glucan catalytic extensions and QXXRW is involved

in providing the terminal glucan moiety. Plant CesAs also contain an additional N-terminal domain, primarily involved in dimerization. Experiments in cotton proved that the N-terminal domain of CesA1 could bind $Zn^{2+}$ to facilitate dimerization with CesA2 (Kurek, et al., 2002)

The number of plant CesA genes vary in different species. While Arabidopsis has 10 CesA genes (Holland, et al., 2000; Richmond, 2000), maize contains 12 (Appenzeller, et al., 2004), barley 8 (Burton, et al., 2004), and poplar possesses 7 CesAs ( Joshi et al., 2004). The genes in Arabidopsis are classified into two groups based on their function. The genes CesA1, -2, -3, -5, -6 and -9 are responsible for primary cell wall biosynthesis while the genes CesA4, -7, -8 function in secondary cell wall biosynthesis. Mutational analysis of these genes suggest that CesA1 and CesA3 are imperative to plant survival owing to the gamete lethal phenotypes of the knockout mutants. Leaky mutations in these genes resulted in mitigated symptoms such as reduced height and ectopic deposition of lignin (Cano-Delgado, et al., 2003). Single knockout mutational studies involving other primary cell wall biosynthetic genes exhibited milder phenotypes, but triple knockout mutants of *cesa2 cesa5 cesa6* were seedling lethal, while gamete lethality was seen in cesa2 cesa6 cesa9 triple mutants implying that at least one of these primary cell wall biosynthetic genes is required for proper plant growth and survival and there exists a partial redundancy between these CesA genes.

Secondary cell wall CesAs were identified from Arabidopsis using mutants exhibiting collapsed and irregular xylem phenotypes (*irx*) (Turner, et al., 1997). The defects in genes CesA4, -7 and -8 directly correlate to the mutations found in irx5, -3, and -1 mutants (Taylor, et al., 1999, 2000, 2003). The simultaneous expression pattern of these genes in the same tissue types confirms that there is no redundancy between these genes. Fragile mutants (*fra*) of Arabidopsis showing

altered mechanical strength in interfascicular fibers were also located to *CESA7* (fra5) and *CESA8* genes (fra6) ( Zhong et al., 2003)

As shown above, cellulose biosynthesis has been extensively studied in Arabidopsis, however the focus now is considerably shifting to monocots as majority of the lignocellulosic biomass feedstock belong to this group. Rice serves as a great model for grasses to understand and unravel the mechanisms responsible for cell wall biosynthesis.

The first genes and mutants involved in cell wall biosynthesis in rice were identified by transposon tagging and chemical mutagenesis. These studies led to the identification of many brittle culm (*bc*) mutants (Aohara et al., 2009; Hirano et al., 2010; Zhang et al., 2011). Tanaka et al. (2003) showed that three non-redundant genes viz., CesA4, -7 and -9 are responsible for secondary cell wall biosynthesis in rice. Mutations in these genes result in plants with phenotypes typically associated with altered cell wall biosynthesis which include reduction in secondary cell wall thickness, reduced mechanical strength and reduced plant height. CesA genes responsible for primary cell wall biosynthesis have not been identified in rice yet. However, it has been suggested that the same genes CesA4, -7, -9 are involved in primary cell wall formation in rice as primary cell wall defects will be much harder to isolate and characterize further. Rice cell wall genes have been classified into two clades based on phylogenic alignment. Clade I belonging to secondary cell wall biosynthesis and Clade II for primary cell wall (Wang et al., 2010).

Genes other than CesAs have been implicated in cell wall biosynthesis in rice. *OsBC1*, an ortholog of the Arabidopsis *COBRA* gene, expressed in vascular bundles and schlerenchyma cells when downregulated results in decrease in cellulose content and mechanical strength (Li, et al., 2003). Dai et al. (2011) reported on the role of *OsBC1l4*, a glycosylphosphatidylinositol (GPI)-

anchored protein in cellulose biosynthesis, showing that the expression of *OsBC1l4* was correlated with primary cellulose biosynthetic genes.

**Hemicellulose**

The hemicellulose polysaccharides of plant cell wall can be found as mannans (glucomannan, galactomannan and glucogalactomannan), xyloglycan, xylan (glucurunoxylan, arabinoxylan and glucuronoarabinoxylan), and arabinogalactan. In grasses, hemicellulose is represented by glucurono(arabino)xylans (GAXs), and β-(1,3/4) glucan also known as mixed-linkage glucans (MLG) (Ebringerova et al., 2005). Multiple functions have been attributed to these polymers such as playing a key role in tissue differentiation (Goubet, et al., 2009), and acting as storage polymers (Sánchez-Rodríguez, et al., 2012)

**Hemicellulose Biosynthesis**

Hemicelluloses can be grouped into xyloglucan, xylans, mannans, and mixed-linkage glucan (MLG). While xyloglucan acts as the major hemicellulose monomer in dicots, the monocot cell wall consists of glucuronoarabinoxylan (GAX), and MLG. MLGs are unbranched glucose polymer made up of β-(1→3, 1→4) linkages. MLGs have been found to be involved in the process of cell elongation in young tissue and imparting strength to the plant. The endosperm of rice consists of MLGs and xylans which cannot be digested by humans and hence are considered as dietary fiber. Studies have shown that regular uptake of xylans and MLGs reduce the potential risk of colorectal cancer, type II diabetes, and cardiovascular diseases and lowers blood cholesterol levels. However, little is known about the transcriptional regulation of hemicellulose biosynthesis.

## Regulation of hemicellulose biosynthesis

### MLG biosynthesis

Current knowledge on the regulation of MLG biosynthesis in rice is limiting. According to the proposed model of biosynthesis, these hemicelluloses are synthesized in the golgi complex followed by transport to the plasma membrane where the assembly occurs. A comparative genomics study in rice showed that MLG biosynthesis in grasses, is attributed to two cellulose synthase like gene families CslF and CslH (Fincher, 2009). Another study in barley, using a quantitative genetics approach showed MLG content in the endosperm of barley can be mapped to Csl genes (Houston, et al., 2014). Two rice genes *OsCsl2* and *OsCsl4* when overexpressed in Arabidopsis resulted in detectable levels of MLGs where they are not present inherently (Hazen, et al., 2002). Recently OsCslF6 was implicated in the process of MLG biosynthesis by its ability to produce MLG when overexpressed in tobacco leaves. Interestingly, the loss of function *cslf6* mutant shows reduced MLG biosynthesis along with dwarf phenotype and abnormal anthesis, opening roads to understand the role of MLG in plant development and architecture besides acting as the glue matrix for the plant cell wall (Vega-Sanchez, et al., 2013).

### GAX biosynthesis

Xylans represent the second most abundant class of biopolymer on earth next to cellulose. These are made up of linear β (1→4) linked xylose backbone which can be substituted depending upon the tissue type and abundance. Xylans exists as a structurally diverse group, and can be divided into three subgroups based on side-chain sugars and functional group substitutions: arabinoxylan (AX), (methyl) glucuronoarabinoxylan (M/GAX), and (methyl) glucuronoxylan (MGX). AX is found in the grains of most cereal and crop plants, and constitutes approximately 30% grain tissue in wheat bran and 13% of the grain flour from rye and barley. AX in grasses have

α-1, 2 and α-1,3 arabinosyl residue substitutions with a few glucuronosyl residues. A common feature of the grass xylans is the presence of the ferulate and coumarate esters linked to some of the α-1,3 arabinosyl residues at the O-5 position. This esterification of the xylans is believed to be the cause of interaction of xylans with the cellulose micro-fibril and structural proteins through covalent interactions contributing towards the strengthening of the cell wall. Dicot xylans are commonly made up of MGX. A common feature of the dicot xylan is the presence of the reducing end oligosaccharide β-D-Xylp-(1→4)- β-D-Xylp-(1→3)-α-L-Rhap-(1→2)-α- D-GalpA-(1→4)-D-Xylp. It exists as an essential component of the secondary cell wall, load bearing structures in the plant cell wall of dicots. Arabidopsis mutants deficient in xylan biosynthesis have impaired xylem leading to xylem vessel collapse and thus reduced growth and biomass.

Much of the knowledge about the regulation of xylan biosynthesis in rice stems from the studies conducted in the model plant Arabidopsis. Forward and reverse genetic screens coupled with numerous other studies have revealed the association of glycosyltransferases (GTs) with xylan biosynthesis. Three genes IRX9 (GT family 43), IRX14 (GT family 43), and IRX10 (GT family 47) have been implicated in the biosynthesis of xylans. The loss of function mutants of these genes show irregular xylem phenotypes, all of which exhibit dwarfism (Brown, et al., 2009; Chiniquy, et al., 2012; Hörnblad, et al., 2013). Paralogs from Arabidopsis have been identified in rice and their role has been discovered in xylan biosynthesis. It has been reported that *OsXAT2* and *OsXAT3* belonging to the GT 61 family in rice and wheat *TaXAT2* are responsible for arabinose substitutions unique to grasses (Anders, et al., 2012). *OsXAX1* was proven to be responsible for catalyzing the xylosyl transfer onto the arabinosyl residue attached to the xylan backbone. The *xax1* mutants display decreased content of xylose, p-coumaric acid, and ferulic acid and exhibit dwarfism (Chiniquy, et al., 2012), and also showed that the Arabidospsis irx9 and irx14 mutants

can be complemented by the rice *OsIRX9* and *OsIRX14* gene overexpression (Chiniquy, et al., 2013). Recently, *OsAT10* has been implicated in the transfer of p-coumaric acid onto the arabinosyl residue of AGX (Bartley, et al., 2013)

Although a vast degree of knowledge is available on hemicellulose biosynthesis in rice, a deeper insight into the regulatory mechanism is required. No transcription factor has yet been discovered to be responsible for the production of hemicellulosic cell walls of rice.

**Lignin**

Lignin, one of the major components of the plant cell wall, primarily functions as the supporting and conducting tissue. It provides mechanical strength to the plants making them able to withstand the negative pressure generated during water transport. Lignin also serves as the physical barrier to inhibit pathogen entry to the plants.

Lignin is a complex phenylpropanoid composed of monolignol G, S, and H units. Different proportions of these units are present in different plant species. The G unit derived from coniferyl alcohol is the characteristic of Gymnosperms, while a combination of G and S units are represented in the Angiosperms. This heterogeneity in the lignin content of plant species is a result of differential expression of lignin monomer specific enzymes in various tissues. The lignin biosynthesis pathway leading to formation of different monomers is outlined in Fig 2, showing the pathway initiates with phenylalanine which is produced by the shikimate biosynthetic pathway (Rippert, et al., 2009). All the genes from the pathway have been characterized in different species. The pathway has been genetically perturbed at various biochemical steps to decrease lignification. Downregulation of genes such as HCT (shikimatehydroxycinnamoyl transferase), 4CL (4-coumarate-CoA ligase), CAD (cinnamyl alcohol dehydrogenase), and CCR (cinnamoyl-CoA

reductase) reduce the overall amount of lignin present in various tissues depending on the species (Vanholme, et al., 2010) . It is also possible to specifically regulate the expression of G, S, and H units of lignin. It was found that silencing HCT or C3H induces H unit expression (Abdulrazzak, et al., 2006; Coleman, et al., 2008; Franke, et al., 2002) while down-regulating F5H or COMT decreases the S units (Stewart, et al., 2009).

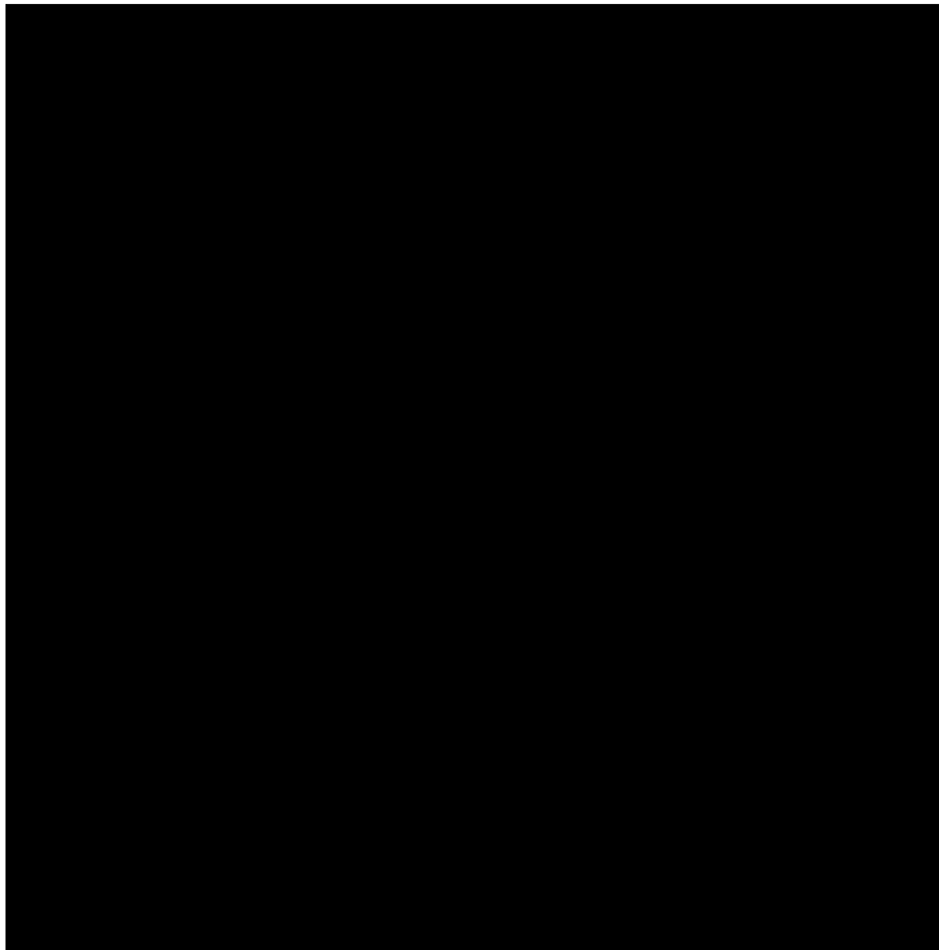

**Figure 2. Lignin Biosynthetic pathway:** The enzymes involved are: phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), cinnamoyl-CoA reductase (CCR), hydroxycinnamoyl-CoA: shikimatehydroxycinnamoyl transferase (HCT), coumarate 3-hydroxylase (C3H), caffeoyl CoA 3-O-methyltransferase (CCoAOMT), ferulate 5-hydroxylase (F5H), caffeic acid 3-O-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD), peroxidase (PER), and laccase (LAC). (Vanholme, et al., 2010)

## Section 1.2: Transcriptional machinery involved in cell wall biosynthesis

Secondary cell wall biosynthesis pathway is regulated by many cis-acting promoter elements and trans-acting transcription factors (Handakumbura, et al., 2012) . The secondary wall associated NAC domain1 (SND1) transcription factor acts as the primary signal for activation of the pathway (Zhong, et al., 2006). It has been observed that overexpression of SND1 leads to ectopic lignification of intrafascicular and xylary fibers, while dominant repression causes reduction in lignification of these tissues. SND1 interacts with various MYBs to regulate the activation of the cellulose, hemicellulose and lignin biosynthesis pathway (Ko, et al., 2009; Zhong, et al., 2007). MYBs can act as upstream and downstream components of secondary cell wall NACs. SND1/NST1 interact with MYB46 and MYB83 and directly activate secondary cell wall biosynthesis (McCarthy, et al., 2009; Yamaguchi, et al., 2010). NST1 and NST2 act downstream to MYB26 whose expression affects the secondary cell wall thickening in anthers (Wang, et al., 2012). Many other transcription factors such as WRKY12, MYB4, MYB2, MYB32, KNAT7, MYB103 and many more have been shown to be regulate the expression of secondary cell wall biosynthesis pathway at different levels (Zhong, et al., 2008). The array of transcription factors and their multiple interactions provide an insight to the amount of plasticity attained by the cell to survive under various environmental conditions.

### Cell wall regulation in Monocots:

Although a lot of overlap can be seen between the transcriptional factor activities between dicots and monocots, many TF families have likely gained diverged functions and hence the regulatory network for cell wall biosynthesis from eudicots is not fully transferable to monocots. One such example is the regulation of lignin biosynthesis by MYB58/63 TF. While *AtMYB58* and

*AtMYB63* are known to be lignin-specific TFs in Arabidopsis (Zhou, et al., 2009) however, *OsMYB58*/63 was shown to activate *OsCesA7* through AC elements in rice (Noda, et al., 2015).

Some TF and key biosynthetic genes have been isolated for their function in cell wall in monocots. In maize, MYB TF *ZmMYB31*, *ZmMYB42*, *ZmMYB2*, *ZmMYB8*, and *ZmMYB39* were identified as direct repressors for *ZmCOMT* (Fornale et al., 2006). TF *ZmMYB31* can directly bind to the AC-II elements present in *ZmCOMT* thus acting as a cell wall specific regulator (Fornale, et al., 2010). Recently, *PbMYB4*, ortholog of *AtMYB4* when over-expressed in switchgrass causes a reduction in lignin biosynthesis and lignin monomer ratio (Shen, et al., 2012)

In rice, *OsMPH1* has been characterized to regulate plant height and may have a role in cell wall biosynthesis (Zhang, et al., 2017). RNA-seq analysis showed that perturbaation of *OsMPH1* results in altered gene expression profiles of cell wall and cell elongation related pathways. *OsIDD2*, a zinc finger and indeterminate protein was implicated in the regulation of cell wall biosynthesis. Over-expression of *OsIDD2* results in short plants with reduced lignin while knockouts show an increase in lignin content. The report concludes that *OsIDD2* directly binds to the promoters of *OsCAD2* and *OsCAD3* to negatively regulate lignin biosynthsis in rice (Huang, et al., 2017). *OsMYB103L* can regulate the expression of secondary cell wall cellulose biosynthetic genes in rice through a gibberellic acid mediated regulation (Ye, et al., 2015). Recently, it was shown that *OsMYB61* and NAC 29/31 can interact to directly activate the expression of CesAs through a GA signaling pathway (Huang, et al., 2015). Thus a similar overlap of NAC-MYB transcriptional regulation might be involved in rice cell wall biosynthesis. However, exact characterization of many TFs and cell-wall related genes remains unknown.

## Section 1.3: Scope of the dissertation

The impacts of global warming have put pressure on the use of alternative energy sources for transportation, power generation and other energy uses. It is therefore the need of the time to understand the biology of biofuel synthesis and improve or tailor plants for sustainable feedstock production. Due to the increasing demand for food, non-food feedstocks such as switchgrass, Miscanthus, and alfalfa turn to be a great resource for lignocellulosic biomass. It has been reported that about 115 million metric tons of rice husk is being wasted annually (Kim, et al., 2004), and even burnt to release more $CO_2$. Rice therefore serves as a crop-waste feedstock and great model for grasses to understand and unravel the mechanisms responsible for biomass production. The key step in the production of biofuels is the breakdown of complex sugars into monomers and subsequently into ethanol, which however is hampered by the presence of lignin from secondary cell walls. The amount of lignin present in the secondary cell wall has been found to be inversely proportional to the amount of sugars produced by the feedstock. Hence, research needs to be focused on engineering plants that produce less lignin, and thereby enhancing the recovery of biofuels. (Mansfield, 2009; Sticklen, 2008; Weng, et al., 2008)

### Functions of the SHINE gene

The *AtSHN2* gene from Arabidopsis, an AP2/ ERF transcription factor regulates the cuticular wax/suberin content in Arabidopsis contributing to environmental protective functions of drought/salt tolerance (Aharoni et al., 2004), including enhancement of drought tolerance and water use efficiency in rice (Karaba et al., 2007).

Overexpression of *AtSHN2* in rice leads to coordinated regulation of lignin and cellulose biosynthesis pathway in rice (Ambavaram et al., 2011). Microarray data and qRT-PCR analysis of the *AtSHN2* rice lines suggest a pathway-wide repression of lignin biosynthesis genes and

associated transcription factors, with an up-regulation of cellulose biosynthetic genes. Phenotoypic characterization of the *AtSHN2* rice lines validated the compositional results. Phloroglucinol staining, calcoflour staining, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) experiments showed cellulose accumulation in the secondary cell wall of the transgenic lines and reduced accumulation of lignin especially in the sub-epidermal and vascular bundle cells. Lignin biosynthesis in rice is controlled by various transcription factors particularly MYBs and NACs (Figure 2). The inverse relation between cellulose and lignin correlates well with the gene-expression profiling data for these transcription factors.

Lignin, apart from being a major component of the cell wall has various biological functions such as host defense response, water transport and mechanical support. Reduction of lignin in secondary cell wall of plants has a considerable effect on plant fitness and survival (Pedersen et al. 2005). In maize, it has been seen that the *bm3* mutation resulting in reduced COMT activity leads to a 15-20 % reduced dry matter (Inoue and Kasuga 1989), a 20% reduction in yield (Cox and Cherney 2001), and 17-26% reduction in stalk strength (Zuber et al., 1977). *ATSHN2* rice lines exhibiting negative correlation between lignin and cellulose biosynthetic pathways and composition, however display normal plant phenotypes, seed yield and breaking force (Ambavaram, et al., 2011). This demonstrates that lignin down-regulation and reduction can be rescued by a relative compensating increase in the amount of cellulose in the secondary cell wall. A similar phenomenon was seen in *gh2* rice, where the plants exhibited long culm phenotype and improved lodging resistance despite decreased lignin accumulation. While decreased lignin deposition was attributed to a point mutation in *OsCAD2*, the authors speculate that higher densities of cellulose and hemicellulose polymers in these plants accounted for increased culm

thickness of the plants, giving rise to increased lodging resistance (Ookawa, et al., 2014). In transgenic aspen, it was seen that reduction in lignin by suppression of gene *Pt4CL1* can be compensated by an increase in cellulose suggesting an underlying regulatory mechanism in the accumulation of cell wall polymers in multiple plant species (Hu, et al., 1999).

In this work the goal was to understand the mechanism of cell wall biosynthesis in rice using *AtSHN2* as the genetic tool. The second chapter addresses to find out genes directly regulated by *AtSHN2* using Chromatin Immuno-Precipitation Sequencing (ChIP-Seq). To this end, a Tandem Affinity Purification (TAP-Tagged) *AtSHN2* overexpression line was generated and used further for ChIP-Seq to identify the putative regulated genes and the corresponding cis-regulatory elements. The regulation of putative targets was further confirmed by Dual Glo luciferase assay system and a steroid inducible system for confirming direct transcriptional regulation. The third chapter discourses the transcriptomic changes associated with the overexpression of *AtSHN2*-TAP in rice. The fourth chapter characterizes the role of OsSHN2, the ortholog of AtSHN2 in rice.. Overall, this dissertation seeks to answer questions associated with the complex interplay of transcription factors involved in cell wall biosynthesis in rice. The results of this dissertation can be potentially used to understand the regulation of lignocellulose biosynthesis in rice and develop genetically engineered rice as a model with high biofuel potential, providing information to improving other grasses for biofuel production.

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**CHAPTER 2: Systemic profiling of cis-elements bound by AtSHN2, an AP2/ERF transcription factor for cell wall biosynthesis in rice.**

## Section 2.1: Abstract

In the quest for alternative forms of renewable energy, the search is on to unravel and improve the productivity of biofuel crops using various functional genomics strategies. Cellulose, the largest biomass found on earth can be converted to fuels such as ethanol, methanol, dimethyl ether, or gasoline. Cellulose however is embedded in a tightly bound matrix of polymers such as lignin and hemicellulose. For the conversion to be possible, extensive cell wall modification must be performed so that cellulose can be disengaged from the highly tangled matrix. The results of various studies have shown that it is possible to de-lignify the plant secondary cell wall, but it is accompanied by negative effects on plant growth and stature. Approximately 115 million metric tons of rice husk and 730 million metric tons of rice straw are reported to being wasted annually. Hence rice is envisioned as a model for grasses to understand the biological pathways and economy of biofuel production. With this purpose in mind, a set of rice genotypes overexpressing the Arabidopsis transcription factor (TF) SHINE (*AtSHN2*) have been analyzed that showed a 34% increase in cellulose and 45% reduction in lignin content with no negative effects on plant performance. Using a detailed systems-level analysis of global gene expression in rice, a model of the SHN regulatory network is developed, with SHN as a master-regulator coordinating down-regulation of lignin biosynthesis and up-regulation of cellulose and other cell wall biosynthesis pathway genes. To validate the network, tandem affinity tagged *AtSHN2* protein (*AtSHN2*-TAP) was expressed in rice and used to identify promoters bound by the *AtSHN2* protein chromatin immunoprecipitation sequencing (ChIP-seq) assay. The GCC binding motif was highly enriched in the *AtSHN2*-TAP binding peaks. Genome wide expression analysis also revealed the role of

*AtSHN2* in diverse pathways. Further, the results show that besides activating cell wall biosynthetic genes directly, *AtSHN2* directly activates a downstream MYB gene LOC_Os02g49986. This MYB can further bind to secondary cell wall specific cellulose biosynthetic genes and directly activates CesA9 to influence cellulose deposition in rice under *AtSHN2* over-expression. Genome wide profiling of target genes of *AtSHN2* will help unravel the transcriptional machinery associated with cell wall deposition in rice thus supporting the feasibility and development of crop lignocellulosic wastes for utilization as biofuels.

**Section 2.2: Introduction**

Secondary cell wall biosynthesis has been studied extensively in Arabidopsis. It has been established that a complex hierarchal transcriptional machinery predominantly comprising of NAC and MYB TFs is responsible for orchestrating cell wall deposition (Handakumbura, et al., 2012; Hussey, et al., 2013). *MYB32* acts as a global repressor of secondary cell wall biosynthesis by downregulating the expression of *SECONDARY WALL-ASSOCIATED NAC-DOMAIN PROTEIN 1 (SND1)* (Wang, et al., 2011). *NAC SECONDARY WALL THICKENING FACTOR 1 (NST1), NST2, SND1* (also known as *NST3), VASCULAR-RELATED NAC-DOMAIN 6 (VND6),* and *VND7*, can activate secondary cell wall transcription (Zhong, et al., 2012). MYB58/63 can directly activate the expression of lignin biosynthetic genes (Zhou et al., 2009). Recent reports have suggested a similar framework for activation of cell wall machinery also exists in rice. *SECONDARY WALL NAC DOMAIN PROTEINs (SWNs)* have been shown to regulate secondary wall formation in rice (Chai, et al., 2015). Likewise, *OsMYB103L* was reported to directly activate the expression of secondary cell wall biosynthetic genes through gibberellin (Ye, et al., 2015). Many other transcription factors have been implicated in the process of secondary cell wall formation in rice (Hirano, et al., 2013), however finding the exact mechanism of these TFs

activating cell wall biosynthesis becomes difficult, as most of the TFs have redundant functions and overexpression or downregulation of these TFs leads to pleiotropic changes in plant development and architecture.

*AtSHN2* an AP2/ERF TF, originally described for its role in regulating epicuticular wax synthesis in Arabidopsis (Aharoni et al., 2004), has also been implicated in the process of cell wall biosynthesis in rice (Ambavaram et al., 2011). Overexpression of *AtSHN2* in rice led to an increase in the cellulose content by 34% with a concomitant decrease in lignin by 45% without affecting overall plant growth. Global gene expression analysis of transgenic *AtSHN2*-rice plants revealed that *AtSHN2* can regulate key TFs and biosynthetic genes related to secondary cell wall biosynthesis in rice. (Ambavaram et al., 2011).This observation led to the hypothesis that *AtSHN2* used as a transgenic TF perturbation of lignocellulose biosynthesis in rice, can be used as a tool to identify other key TFs associated downstream with the rice cell wall synthesis machinery.

Chromatin Immunoprecipitation (ChIP) followed by next generation sequencing (NGS) has become a powerful tool ChIP-Seq, for studying global TF-DNA interactions in vivo. This technology has enabled the profiling of genome-wide target regions in many plant species. In Arabidopsis, ChIP-Seq has been used to seek out genome wide positional distribution of TFs (Yu, et al., 2016), analyze histone modifications to maintain circadian rhythm (Malapeira, et al., 2014), find out direct targets of pivotal TFs such as *EIN2 (ETHYLENE INSENSITIVE PROTEIN 2)* (Zhang et al., 2017), *PHYTOCHROME INTERACTING FACTOR4 (PIF4)* (Hwang, et al., 2017) and *BRASSINAZOLE-RESISTANT1 (BZR1)* (Nolan, et al., 2017). In Populus, the technology has helped unravel the targets for *ARBORKNOX1 (ARK1)*, a TF regulating vascular cambium maintenance and cell differentiation (Liu, et al., 2015). Likewise in rice the identification of a complex regulatory network by *IDEAL PLANT ARCHITECTURE1 (IPA1)* was enabled by ChIP-

Seq analysis (Lu, et al., 2013). Similarly, the identification of genome wide binding targets for *AtSHN2* will help understand the complex transcriptional regulatory pathway underlying cell wall formation in rice.

In this study, a set of 1776 genes was identified as putative targets bound by *AtSHN2*-TAP by using Chromatin-tandem affinity purification. Motif [GA]CAACA[TG][AT], belonging to AP2/ERF class of TFs was enriched in the promoter regions of the downstream genes. Furthermore, it was found that *AtSHN2* can directly activate a suite of MYB TFs by binding to the GCC box motif present in the promoter sequence of these downstream genes. We also suggest the possible tole of LOC_Os02g49986 in regulating cell wall biosynthesis in rice. Thus, we show that genome-wide profiling of *AtSHN2* binding targets can be used as a tool to unravel genes associated with cell wall deposition, thus opening a novel path towards understanding the regulation of cell wall biosynthesis in rice.

## Section 2.3: Materials and Methods

### Construction of *AtSHN*-TAP rice transgenic lines:

The Arabidopsis SHN2 gene (LOC_At5g11190) was used for construction of *AtSHN2*-TAP lines. pCTAPa (CD3-679) (Rubio, et al., 2005) serves as a template to amplify coding sequence for six His repeat (6xHis), a 9x-myc peptide, a 3C protease cleavage site and two copies of the IgG binding domain (2xIgG-BD) collectively known as TAP-tag. The corresponding amplicon is flanked by unique *Kpn*I/*Sac*I sites. The full length *AtSHN* gene was amplified using Arabidopsis genomic DNA with *Bam*HI/*Kpn*I cloning sites at the 5' and 3' end. The above two fragments were ligated to generate the *AtSHN2*-TAP amplicon. Further, CaMV35S promoter and NOS terminator were assembled at the 5' and 3' sites of the *AtSHN2*-TAP amplicon flanked by *Xba*I and *Bam*HI sites and *Kpn*I/*Eco*RI sites respectively (Pietrzak, et al., 1986) . The entire

cassette was cloned into pUC19 using *Xba*I/*Eco*RI sites. This cassette was subcloned into the binary vector pMOG22 (Zeneca-Mogen), which contains a chimeric CaMV 35S-hygromycin phosphotransferase-tNos to serve as a selection marker during rice transformation. Agrobacterium-mediated transformation of *Oryza sativa* ssp. *japonica* cv. Nipponbare was performed using Agrobacterium strain LBA4404 (Nishimura, et al., 2006). Transgenic plantlets were further grown in environmentally controlled growth chambers maintained at 28ºC±1 day and 25ºC±1night temperature, 65% relative humidity (RH) with a daily photoperiodic cycle of 14 h light and 10 h dark. The plants were then transferred to the greenhouse where they were grown in soil till maturity.

**Plant Genotyping and Statistical Analysis:**

Out of the four transgenic rice *AtSHN2*-TAP lines generated (Nishimura, et al., 2006), T2 progeny was used to test for stable integration of the *AtSHN2* construct into the rice genome. The expression level of *AtSHN2*-TAP was quantified using qRT-PCR in all the lines. All inserts were also tested for stable expression pattern of CaMV35S promoter. Based on the results of qRT-PCR analysis of *AtSHN2*, three lines showed significant and stable expression, and were used for further experiments.

**Estimation of cell wall components:**

For compositional analysis of cell wall components, culm tissues of wild type and *AtSHN*-TAP rice lines were used. The analysis was carried out according to the National Renewable Energy Protocol. Briefly, 5g of culm tissue was ground using a Thomas Willey Mini mill (Swedesboro, NJ) and sieved with a 20-mesh screen, such that the particle size was uniformly reduced to 0.84 mm. 300 mg of the crushed samples were treated with 72% sulfuric acid at 30ºC for an hour. After hydrolysis, the acid was diluted to a 4% concentration. A set of sugar standards

were treated the same way. The samples and the standards were autoclaved at 121ºC for 1 h. The samples were then neutralized to pH 7.0, by adding calcium carbonate. The neutralized samples were filtered using 0.45 mm and 0.22 mm filter syringes (National Scientific Company, Rockwood, TN) to completely remove any particles. Protocols for HPLC detection were based on the NREL report #TP-510-42618. The analysis of monomers was conducted using Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) fitted with a SP-G pre-column and a SP0810 column (Shodex, Kawasaki, Japan).

**Microscopy:**

To perform scanning electron microscopy, fresh hand-cut sections from second internode of 45-day old rice were excised and dried. The dried samples were spur coated with gold particles using an E-100 ion sputter and viewed under a scanning electron microscope (Carl Zeiss EV040). For transmission electron microscopy, rice culm was sectioned using a microtome and the sections were fixed with2.5% (v/v) glutaraldehyde. The fixed samples were washed with 0.1 M sodium cacodylate buffer for 15 min. Post-fixation was performed in 1% $OsO_4$ for 1 h. The samples were critical point dehydrated through an ethanol gradient and embedded using Spurr's medium. Ultrathin sections were made using a microtome (MT-X; RMC) with a diamond knife. These ultrathin sections were well stained in aqueous 2% uranyl acetate for 10 min followed by lead citrate for 2 min. The sections were then viewed using an electron microscope.

**Quantitative Reverse Transcriptase PCR (qRT-PCR analysis):**

RNA was isolated using Trizol according to manufacturer's protocol (Life Technologies). Quantification of RNA was done using quantitative Reverse Trancriptase PCR (qRT-PCR). To this end, the comparative threshold cycle (Ct) method of quantitation was used with the rice Ubiquitin gene as the reference (Ambavaram, et al., 2011). Briefly, 2 ug of DNase treated RNA

was used with GoScript Reverse Transcription System (Promega) followed by qRT-PCR experiments using GoTaq qPCR Master Mix (Promega) with Ubiquitin as reference gene in a CFX-96 Bio-Rad thermocycler (Bio-Rad). Temperature ranges from 55ºC to 95ºC (0.5ºC/10s) were used to conduct melting curve analysis. Non-template reaction and no reverse trancriptase were used as negative controls for the analysis. For data analysis, the relative quantification was calculated by normalizing Ct value of candidate gene with the reference gene. The normalization was followed by quantification relative to the calibrator using $2^{-\delta \delta Ct}$ (Livak, et al., 2001).

**Chromatin Immuno-Precipitation:**

Four week old rice leaf samples from *AtSHN2*-TAP tagged lines were used to perform Chromatin Immuno-Precipitation (ChIP) (Haring, et al., 2007). In short, crosslinking of DNA with the proteins in rice leaf samples was done using 2 % formaldehyde with crosslinking buffer (3.4g of sucrose (0.1M), 1 ml sodium phosphate buffer (10 mM, pH 7), 1 ml NaCl (50 mM)), under vacuum for 25 minutes. The fixation was stopped using 2.5 M glycine. The leaf samples were freeze dried and nuclei isolated. The nuclei isolation procedure entailed homogenizing the freeze-dried leaf samples. Isolated nuclei were filtered and subsequently washed using buffer: 250 µl sodium phosphate buffer (10 mM, pH7), 500 µl NaCl (100 mM), 250 µl MgCl-2- (10mM), 625 µl Triton-X-100 (0.5%), 3.2 ml of 2-methyl 2.4 pentanediol, ½ tablet protease inhibitor and 17.7 µl of β-mercaptoethanol, and volume made upto 25 ml. The isolated nuclei were resuspended in sonic buffer (250 µl sodium phosphate buffer (10 mM, pH7), 500 µl NaCl (100 mM), 50 µl EDTA (10 mM), tablet protease inhibitor cocktail and 125 mg sarkosyl (0.5%) volume made upto 25 ml) and the chromatin was sheared to 200-600 bp size fragments using a sonication cycle of 30 seconds ON and 1 minute OFF for all cycles on full power. The above sonicated samples were mixed with equal volume of immunoprecipitation buffer (2.5 ml HEPES buffer (50 mM, pH 7.5), 750 µl NaCl

(150 mM), 125 µl $MgCl_2$ (5 mM), 2.5 µl $ZnSO_4$ (10 µM), 1.25 ml Triton-X-100 (1%) and 125 µl SDS (0.05%)) for pre-clearing. The precleared chromatin was incubated with 100 µl Protein A agarose for 1.5 hr, centrifuged and divided into three aliquots for subsequent immunoprecipitation into three fractions: with anti-His (R932-25, Invitrogen, 1.5–2 mg) and anti-MYC (AHO0062, Invitrogen, 1.5–2 mg) *AtSHN2*-bound chromatin, and anti-Histone H3 (AHO1432, Invitrogen, 1.5–2 mg) conjugate (nonspecific chromatin). The samples were incubated with appropriate antibodies for 10 h at 4ºC. Following centrifugation, the supernatant was collected and incubated with Protein A agarose for 2 h at 4ºC. The beads were washed with IP buffer and the DNA was eluted. Elution of chromatin bound DNA was performed by reverse crosslinking. Essentially, the samples were incubated with Proteinase K (20 mg/ml) at 37ºC for 24 h followed by another incubation at 65ºC. DNA was precipitated using sodium acetate (pH 5.2), ethanol and glycogen and purified using PCR purification columns. The precipitated DNA was sent to the NGS facility at Michigan State University for sequencing. Two replicates of 25 ng Single ChIP enriched DNA in 30ul nuclease-free H2O were shipped on dry ice. qPCR of the eluted DNA was done using CFX-96 thermalcycler (Bio-Rad) with the qPCR master mix (Promega) using the following cycle 95ºC for 3 min, 40 cycles of 95ºC for 15 s and 59ºC for 1min. The following equation was used to calculate relative fold enrichment of chromatin bound DNA with specific anti-His antibody and non-specific anti-Histone H3: Ct (target using DNA with specific anti-His antibody)- Ct (target using DNA eluted with anti-His antibody) = dCt (Kaufmann, et al., 2010). The resulting dCt value was normalized against the calibrator using the equation $2^{-\delta\ \delta Ct}$ (Livak, et al., 2001). The above experiment was performed using three biological replicates. Primers for putative targets regulated by *AtSHN*2 gene were designed using IDT and can be found in Appendix 1A.

**Electrophoretic Mobility Shift Assay (EMSA):**

For electrophoretic mobility shift assay, amplification of *AtSHN2* was performed using primers with attB1 and attB2 sites at 5' and 3' region respectively. The full fragment was subsequently cloned into pDEST42 (C-6X-His tag Gateway expression vector of choice, 12276-010) vector using the Gateway cloning strategy (BP and LR reaction system, Invitrogen). The clone was transformed by electroporation into E. *coli* strain BL-21. Induction of the recombinant protein in *E. coli* was done using 1mM isopropyl-b-D-thiogalactoside. The cells were lysed using sonication for 3 min (50% duty cycle) and the proteins were purified using Ni-NTA agarose (Invitrogen). Confirmation of the recombinant protein was done by immunoblotting using anti-His (R932-25, Invitrogen) and anti-MYC (AHO0062, Invitrogen).

To identify promoter regions bound by *AtSHN2*, 250 bp of the promoter segment (spanning the GCC core) of the putative targets were amplified using genomic DNA of Nipponbare rice as the template (Appendix 1A). The fragments were gel purified and labeled using the Biotin 3'End DNA Labelling Kit (Pierce). EMSA was performed according to the manufacturer's protocol (Light Shift Chemiluminescent EMSA kit). The protein and 100-fold excess of unlabeled competitor DNA were incubated in binding buffer (10mM Tris–HCl, pH 7.5, 50mM KCl, 1mM dithiothreitol), on ice for 10 min after adding 100-fold excess of unlabeled competitor DNA (gel-purified promoter DNA fragments). The reaction was further incubated on ice for 20 minutes after addition of labeled DNA. The complex was then loaded on to a 5% native polyacrylamide gel. Transfer of the resolved protein-DNA complex was done by electroblotting onto Nylon membrane (Biodyne) and the gel shift was detected using Chemidoc (Bio-Rad) according to chemiluminescent nucleic acid detection kit.

**Transactivation Dual Glo Luciferase Assay using rice protoplasts:**

**Isolation of protoplasts from Rice seedlings.**

Nipponbare rice seeds were dehulled and sterilized with 75% ethanol followed by washing in 75% bleach for 30 minutes. The sterilized dehulled seeds were incubated in half strength MS liquid medium for 15 days at 28ºC with a photoperiod of 12 h light (about 150 µmol m-2 s-1) and 12 h dark. Rice seedlings were harvested, cut into 0.5 mm strips and incubated in Buffer (0.6M mannitol, 10 mM MES [pH 5.7], 10 mM CaCl2) for 10 min at room temperature. This was followed by incubation of the rice seedlings for 6 h in Buffer (0.6 M mannitol, 10 mM MES (pH 5.7), 1.5% Cellulase (C1794, Sigma), 10mM CaCl2, 2.5 mM β-mercaptoethanol, 0.1 % BSA, 0.75% Pectinase). Undigested protoplasts were removed by washing the above material with buffer (154 mM NaCl, 125 mM CaCl2, 5 mM KCl and 2 mM MES (pH 5.7)). The enzyme/protoplast solution was filtered using cheese cloth. The isolated protoplasts were centrifuged and stored in MMG (0.4 M mannitol, 15 mM MgCl2 and 4 mM MES (pH 5.7)) at $2E^6$ cells per ml.

**Transfection of protoplast by electroporation:**

Electroporation of protoplasts with plasmid DNA was done using Multiporator (Eppendorf) with the conditions: 5 msec, 400 V and 200 µF and 2 pulses. The electroporated solution was incubated on ice for 10 min, diluted and resuspended in buffer (0.5 M mannitol, 20 mM KCl and 4 mM MES at pH 5.7) followed by incubation at 28ºC for 24 h. The protoplasts were harvested by centrifugation and stored at -80ºC until further use.

**Transactivation Assay:**

To prove activation of putative targets by *AtSHN2 in vivo*, co-transformation of three constructs was done in protoplasts. The first construct contained a) promoters of putative targets

cloned upstream of firefly luciferase b) a CaMV 35S Renilla luciferase construct control for transformation and (c) the coding regions of *AtSHN2* cloned in pUC19 between the CaMV 35S promoter and NOS terminator. Luminescence was measured by the Glomax 20/20 Luminometer (Promega). The relative luciferase activity was expressed normalized to Renilla Luciferase.

**Receptor based inducible system for direct activation of targets in vivo.**

To prove direct activation of downstream genes by *AtSHN2* coding sequences of the respective genes were amplified and ligated upstream of an estrogen inducible expression system (Human Estrogen receptor-HER) (Zuo, et al., 2000), and the entire cassette was cloned in between CaMV35S promoter and NOS terminator in pUC19. For the assay, protoplasts were transfected with *AtSHN2*-HER through electroporation. The reaction was incubated with 2 μM estradiol for 6 h to induce the activity of the respective genes. Where indicated, protoplasts were treated with cycloheximide (2 μM) to inhibit new protein synthesis. The protoplasts were resuspended (0.5 M mannitol, 20 mM KCl and 4 mM MES (pH 5.7)) and incubated for 6 h. Total RNA was isolated using the Trizol method (Invitrogen). cDNA was synthesized using 1ug RNA and analysis of expression was done using qPCR. To calculate the gene expression, the fold change after estradiol treatments over control (mock treatment with ethanol) was calculated. In case of cycloheximide and estradiol (CHX+EST) it was calculated as fold change over protoplasts treated with CHX.

**Bioinformatics analysis of *AtSHN2* ChIP-Seq dataset.**

The raw data was checked for quality using FastQC (Andrews, n.d.), and reads trimmed to remove adapter sequences. The reads were mapped to release 7 of the Michigan State University Rice Genome (Ouyang et al., 2007) using Bowtie 2 (Langmead, et al., 2012) with default parameters. The reads from the two replicates were merged using PICARD (Broad Institute). Singleton reads were removed, and uniquely mapped reads were retained. The uniquely mapped

reads from ChIP-Seq were used for peak calling using Model based analysis of ChIP-Seq (MACS) (Zhang, et al., 2008) with the following parameters: tag size = 86, band width = 500, model fold = 32 and p-value cutoff = 1.00e-03. The peak summits obtained from MACS were classified according to their presence in the promoter regions (5000 bp upstream of Transcription Start Site), or genic regions (intron-exon). The annotation of the peaks to the relevant genes was done using the PAVIS annotation tool (Huang, et al., 2013).

**Motif Discovery from ChIP-Seq data.**

For finding out cis-regulatory elements regulated by *AtSHN2*-TAP in rice, MEME-ChIP (Machanick, et al., 2011) was used. Fasta sequences of the first thousand highly enriched genes were obtained. An additional 1000 bp upstream and downstream of the genes' start and stop co-ordinates were also fetched. These sequences were used to find de-novo motifs regulated by *AtSHN2* in rice. For this, MEME-ChIP was run using default parameters except for the width parameter, with the maximum width being set to 15 bp.

**Section 2.4. Results**
**Rice *AtSHN2*-TAP lines have altered cellulose and lignin content**

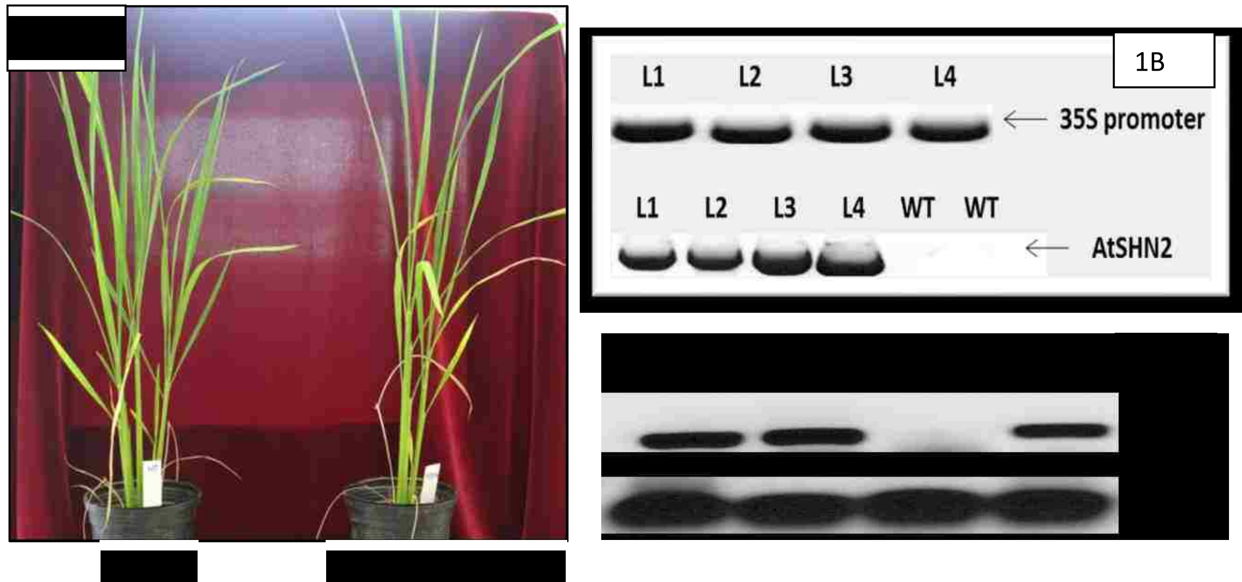



**Figure 1: Phenotypic characterization of *AtSHN2*-TAP line.** (1A) The plant stature and architecture of wild-type and *AtSHN2*-TAP rice lines were not significantly different, (1B) Confirmation of presence of *AtSHN2* transgene in *AtSHN2*-TAP rice lines as seen by PCR. No amplification of AtSHN2 in WT plants as compared to transgenic lines. (1C) RT-PCR confirming presence of *AtSHN2* in lines L1, L2 and L4

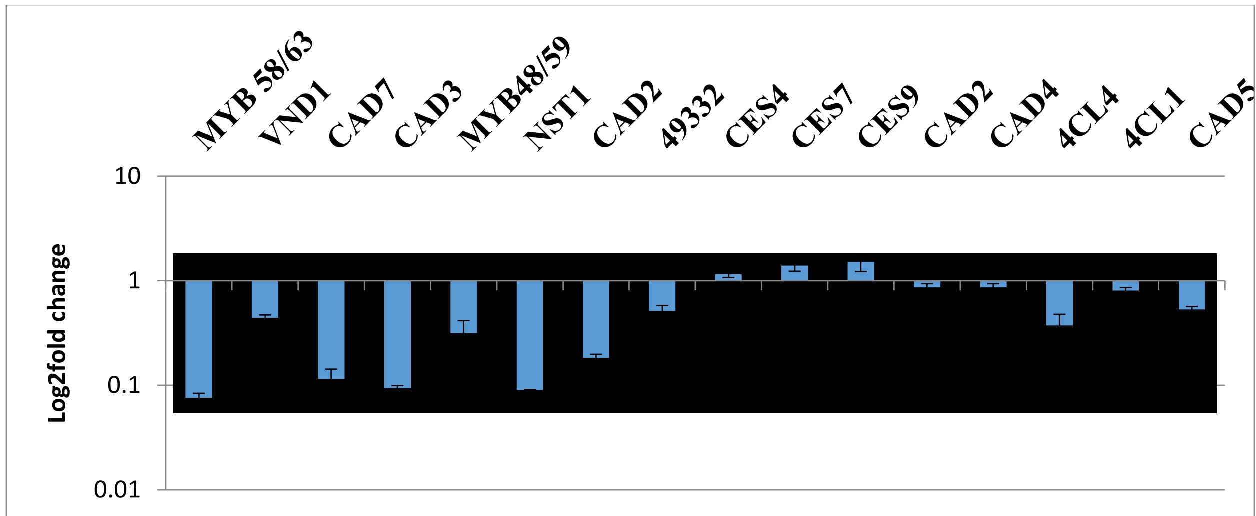



**Figure 2: Relative expression levels of cell wall related genes from *AtSHN2*-TAP lines.** Data are expressed as mean relative transcript levels in *AtSHN2*-TAP lines compared with the wild type (log2 ratio). Error bars represent SE (n = 3; three wild-type and three SHN lines).

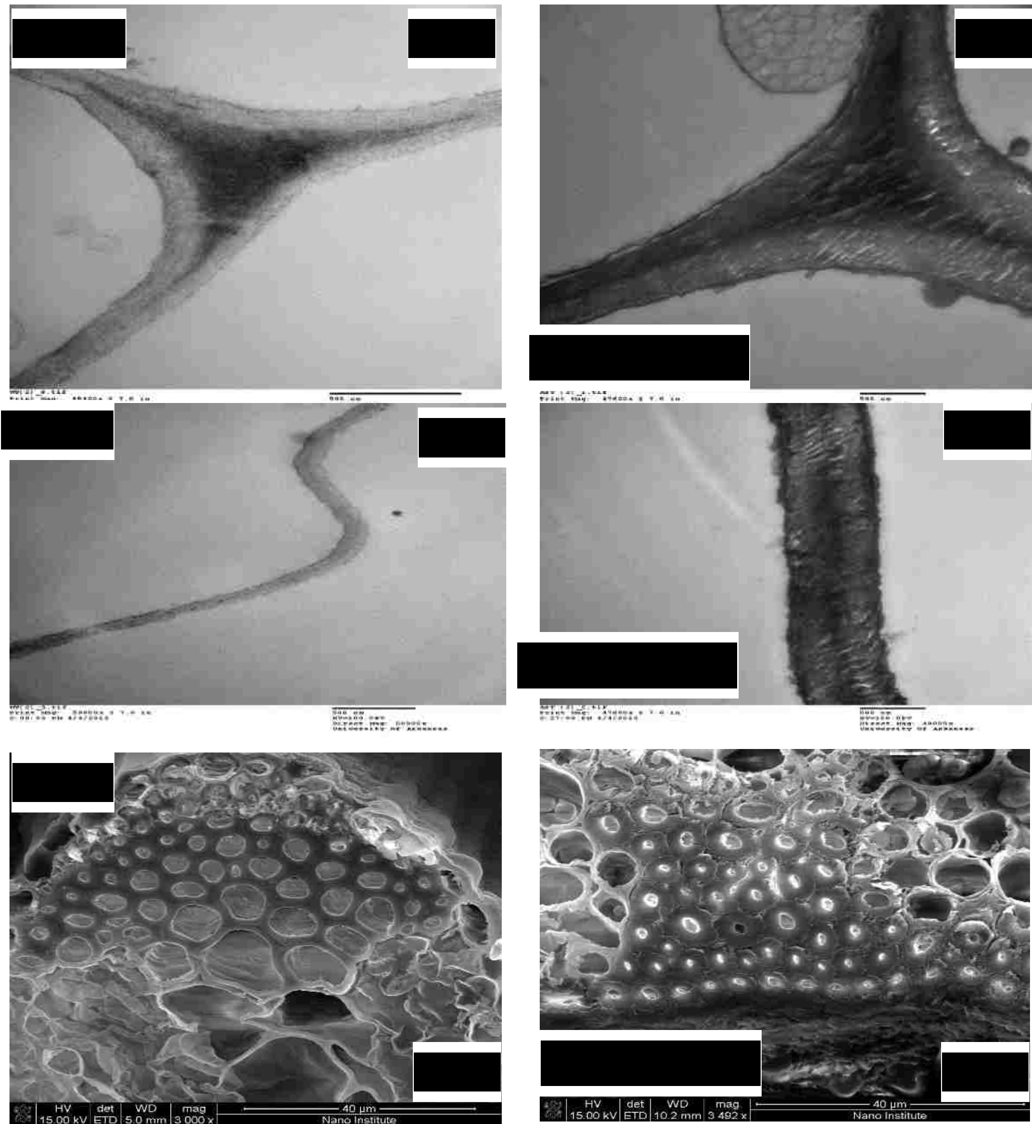
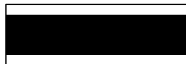



**Figure 3: Electron micrographs of culm sections from AtSHN2-TAP and WT.** Transmission electron micrographs (TEM) of wild-type (3A, 3B) and *AtSHN2*-TAP (3D, 3E) in parenchyma cell walls, 3C and 3F, Scanning electron micrographs of transverse sections of wild-type (3C) and *AtSHN*-TAP (3F) culms. Bars = 500 nm in 3A, 3B, 3D and 3E, 40 µm in 3C and 3F

**Table 1: Comparison of the contents of cell wall sugars between rice wild-type and *AtSHN2*-rice TAP culms.** (1A) Compositional analysis of WT plants and *AtSHN2*-TAP plants. The sugar contents of cell walls of wild-type and *AtSHN2*-TAP lines are represented as percentage composition of the total components isolated. (1B) Percentage change between WT and *AtSHN2*-TAP lines for glucan and lignin monomers. Asterisks indicate levels of significance compared with the wild type (t test; ** P ≤ 0.01).

| | **Composition of WT** | | **Composition of *AtSHN2*-TAP** | |
|---|---|---|---|---|
| **Components** | **Avg % (ODW basis)** | **Stdev** | **Avg % (ODW basis)** | **Stdev** |
| Glucan | 36.5 | 2.36 | 44.28** | 3.03 |
| Xylan | 17.81 | 0.47 | 15.62** | 0.4 |
| Arabinan | 2.06 | 0.4 | 2.25 | 0.53 |
| Ash | 7.05 | 0.94 | 6.11 | 0.33 |
| Total lignin | 13.63 | 1.61 | 8.18** | 0.42 |
| Total extractives | 22.55 | 1.38 | 23.55 | 1.13 |
| | | | | |
| Total | 99.61 | 3.37 | 99.99 | 3.34 |



| **Percentage change:** | **Wt and AtSHN2-TAP lines** |
|---|---|
| **Glucan** | 21.31% increase |
| **Lignin** | 39.98% decrease |

To study the phenotype of *AtSHN2*-TAP rice lines and *AtSHN2*-rice lines, the *AtSHN2*-TAP rice lines were characterized phenotypically and biochemically. Four independent transgenic lines were generated to this end. Presence of *AtSHN2* transgene in AtSHN2-TAP rice lines was confirmed by genomic PCR of *AtSHN2* in AtSHN2-TAP plants but not in WT (Figure 1B). Expression of the transgene in the transgenic lines was confirmed by RT-PCR. It was seen that AtSHN2 was expressed highly in transgenic lines L1, L2 and L4. Scanning Electron Microscopy (SEM) of transverse culm sections of *AtSHN2*-TAP rice lines L1, L2 and L4 was performed. It

was seen that the transverse sections of lines L1 and L2 had an increase in the thickness of the cell wall of the sclerenchyma cells (Figure 3C and 3F) These results were further confirmed by transmission electron microscopy where the deposition of cell wall components was also seen in parenchyma cells (Figure 3A and 3D) in addition to sclerenchyma cells (Figure 3B and 3E). These experiments revealed that the *AtSHN2*-TAP rice lines L1 and L2 have an altered cell wall composition as compared to WT plants.

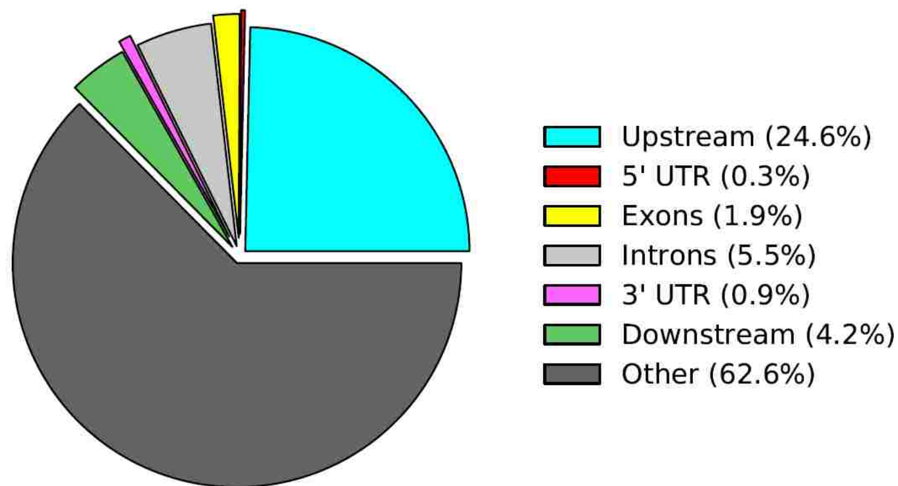
To examine if there was a change in the cellulose to lignin ratio, as exhibited by the *AtSHN2* rice lines, biochemical analysis of *AtSHN2*-TAP rice lines was performed as described (Ambavaram, et al., 2011) . *AtSHN2*-TAP rice transgenic lines were observed to have an increase in glucan monomers with a concomitant decrease in total lignin (Table 1A). An overall increase in cellulose content by 21.31 % with a concomitant decrease in lignin content by 39.98% was seen (Table 1B). These two results show that the *AtSHN2*-TAP rice lines exhibited similar phenotype as *AtSHN2*-rice lines with respect to changes in lignocellulose/cellulose i.e higher cellulose and lower lignin, and thus the *AtSHN2*-TAP lines in rice can be used as a tool to study the mechanism of cell wall biosynthetic machinery in rice.

**Genome-wide in-vivo promoter binding analysis of transcription factor *AtSHN2* through ChIP-Seq analysis.**

To further investigate the mechanism of cell wall regulation by *AtSHN2*, a systemic survey of global binding profiles of *AtSHN2* was done in vivo using ChIP-Seq analysis. To this end, *AtSHN2*-TAP transformed lines generated using the 35S:*AtSHN2*-TAP:Nos vector were characterized by chromatin immunoprecipitation (ChIP) using anti-His and anti-Myc antibodies.

The bound DNA was purified, precipitated, and sequenced using single end sequencing chemistry on the Illumina NGS Platform. The model-based algorithm for finding enriched regions in ChIP-Seq experiments (MACS) tool was subsequently used to find out targets regulated by *AtSHN2 in vivo*. Data from two biological replicates were used for the analysis.

A total of 4966 regions were enriched by MACS. Of these, 1775 regions were annotated to the genomic regions of Rice. Out of these annotated regions, 24.6% of the peaks were categorized to be bound to the promoter region 2 kb upstream of transcription start site (TSS) of putative downstream genes regulated by AtSHN-TAP (Figure 4A). About 4.2 % of all the peaks were annotated to the intronic region of the downstream genes. Profile plotting of the two BAM files used for ChIP-Seq analysis further revealed that the highest enrichment of peaks bound by AtSHN is +/- 2000 bp surrounding TSS of genes (Figure 4B). An in-depth calculation of scores after normalization of the BAM files for two replicates also showed that majority of the peaks seen in the BAM files surround 2000 bp upstream and downstream of TSS in rice. (Supplementary Figure 1A).



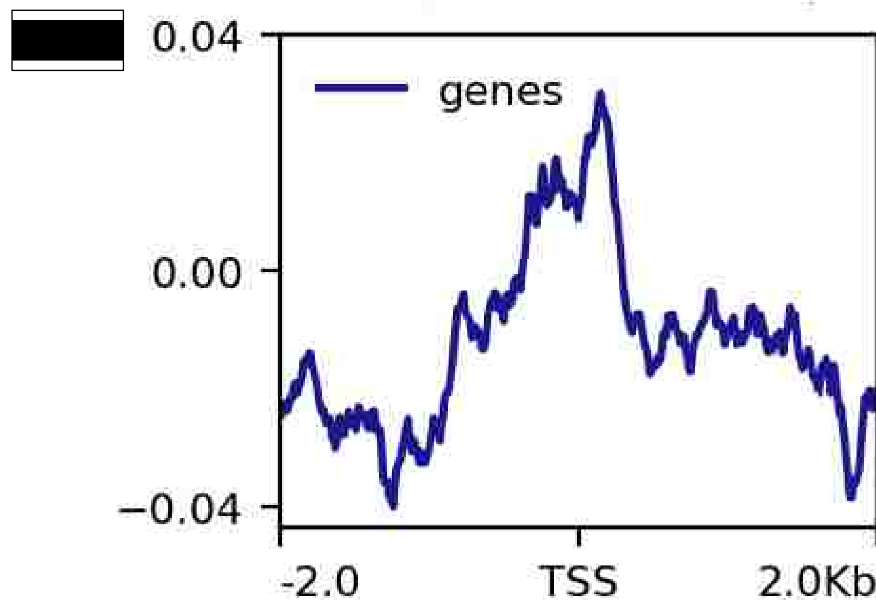




**Figure 4: Genome wide profiling of binding motifs of AtSHN2 in rice.** (4A) Distribution of *AtSHN2* binding peaks in rice genome: Out of the total annotated peaks, AtSHN2-TAP bound to upstream regions of 24.6% of the total genes followed by binding of 5% of the total genes in intronic regions. 4.2% of the total genes were bound at downstream regions and 1.9% of the genes were bound by AtSHN at exonic regions for their regulation. (4B) Profile plot of AtSHN2-TAP bound regions: Comparison of BAM files between *AtSHN2-* TAP and control datasets show that regions bound by AtSHN2-TAP are highly enriched around -500 bp to Transcription Start Site (TSS) of the downstream genes.

***AtSHN2* directly regulates genes involved in cell wall biosynthesis in Rice.**

Manual examination of the genes enriched by ChIP-Seq analysis revelead that putative targets regulated by *AtSHN2* include biosynthetic genes such as *Cellulose Synthase like Family gene 6 (CSLF6*), *and Cellulose Synthase like gene 7* (*CSL7*), and genes responsible for mixed linked glycan biosynthesis in rice (Vega-Sanchez, et al., 2013) (Table 2). *OsPAL2 (phenyl ammonia lyase*: LOC_Os02g41650), a prominent gene involved in lignin biosynthesis was also found among the genes predicted to be a potential target directly bound by *AtSHN2* (Table 2). Other relevant potential downstream targets identified were *4CL1* (*4-coumarate-CoA ligase:* LOC_Os03g05780), involved in phenylpropanoid metabolism; *OsWAK10 (wall associated receptor kinase*: LOC_Os01g49529) a wall associated kinase, paralogs of which are shown to play a role in cell expansion in Arabidopsis; *OsCOMT (Caffeic acid 3-O-methyltransferase*: LOC_Os09g30360), a key gene related to the lignin biosynthetic pathway; a putative *sucrose synthase gene* (LOC_Os06g09450); *OsGA2ox (gibberellin 2-oxidase*: LOC_Os01g22920); uncharacterized *YABBY domain containing genes* (LOC_Os03g44710, LOC_Os03g11600), and *glycosyl transferases* (LOC_Os01g33420, LOC_Os06g34020, LOC_Os06g28124). *AtSHN* also bound to promoter regions of a *Domain of Unknown function 260-containing gene* (LOC_Os03g41330) and a *B-box zinc finger gene* (LOC_Os04g45690) (Table 2). The promoters of these genes were shown to be bound by AtSHN2 in vitro using Electro Mobility Shift Assay (Figure 5). Thus, *AtSHN2* can activate biosynthetic genes and transcription factors related to myriad processes associated with cell wall biosynthesis in rice thereby leading to secondary cell wall deposition.

**Table 2**: ***AtSHN2* can bind potentially to the promoter regions of genes involved in cell wall biosynthesis in Rice.** Summary of ChIP-Seq regions bound by *AtSHN2* profiled, the locus ID of the gene bound, chromosomal position, location of binding region, binding specificity to the strand, distance from the transcriptional start site, Gene Symbol and the fold enrichment associated with each gene ($p<0.001$).

| Gene | chr | Start | end | Category | Strand | Distance to TSS | Gene Symbol | Fold Enrichment |
|---|---|---|---|---|---|---|---|---|
| **LOC_Os09g30130** | chr9 | 18323583 | 18324173 | Upstream | - | -2664 | OsCSLF6 | 8.68 |
| **LOC_Os10g20260** | chr10 | 10192126 | 10193120 | Upstream | - | -4984 | OsCSLF7 | 3.79 |
| **LOC_Os02g41650** | chr2 | 24987609 | 24988193 | Intron | - | 1487 | OsPAL2 | 6.91 |
| **LOC_Os01g49529** | chr1 | 28491948 | 28492668 | Intron | - | 3959 | OsWAK10 | 5.69 |
| **LOC_Os03g05780** | chr3 | 2888427 | 2889213 | Upstream | - | -898 | Os4CCL1 | 4.68 |
| **LOC_Os09g30360** | chr9 | 18478350 | 18481217 | Upstream | - | -3402 | OsCOMT | 4.47 |
| **LOC_Os01g22920** | chr1 | 12885798 | 12886313 | Upstream | - | -504 | OsGA2ox | 6.76 |
| **LOC_Os03g11600** | chr3 | 6042832 | 6043689 | Intron | + | 2015 | YABBY | 15.88 |
| **LOC_Os03g44710** | chr3 | 25196907 | 25197378 | 5'UTR | + | 85 | YABBY | 6.84 |
| **LOC_Os06g28124** | chr6 | 15976372 | 15976967 | Upstream | - | -2620 | GT | 7.79 |
| **LOC_Os06g34020** | chr6 | 19809481 | 19809994 | Upstream | + | -4341 | GT | 5.99 |
| **LOC_Os01g33420** | chr1 | 18401431 | 18401988 | Upstream | - | -10 | GT | 4.25 |
| **LOC_Os03g41330** | chr3 | 22978621 | 22979174 | Intron | - | 1039 | DUF | 9.01 |
| **LOC_Os04g45690** | chr4 | 27026872 | 27027342 | Upstream | + | -156 | B box Zn | 7.01 |

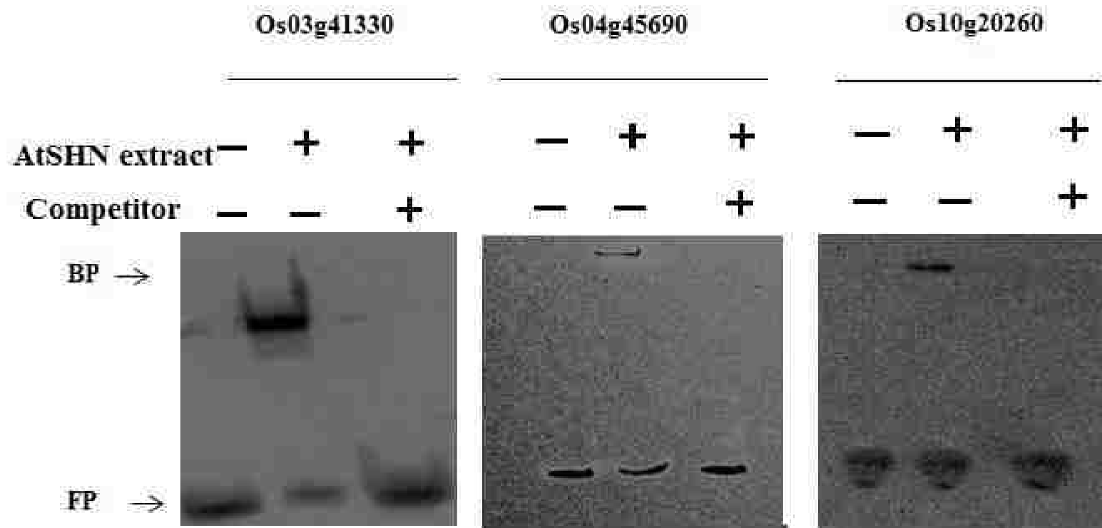
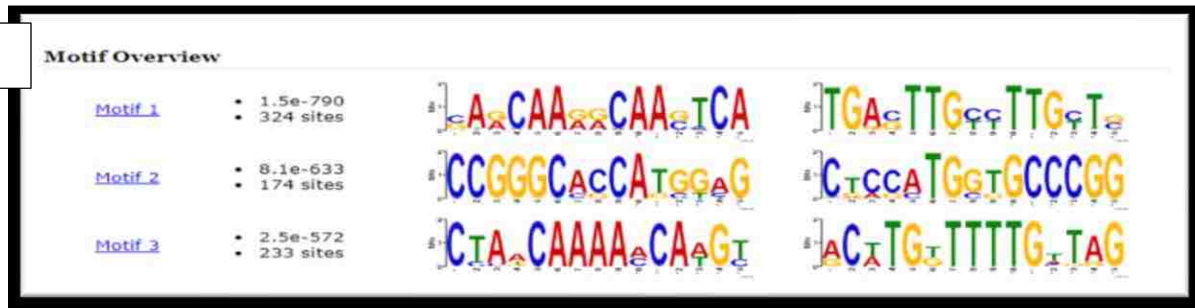



**Figure 5**: **Validation of ChIP-Seq results by in-vitro Gel Mobility Shift Assay.** *AtSHN2* binding to the promoters of downstream genes enriched in *AtSHN2* ChIP-Seq Assay. Each of the end-labeled probes was competed with 100-fold excess of the competing unlabeled probe. + and − indicate the presence and absence of the respective component in the assay. Bound probe (BP) and free probe (FP) positions are indicated in Figure.

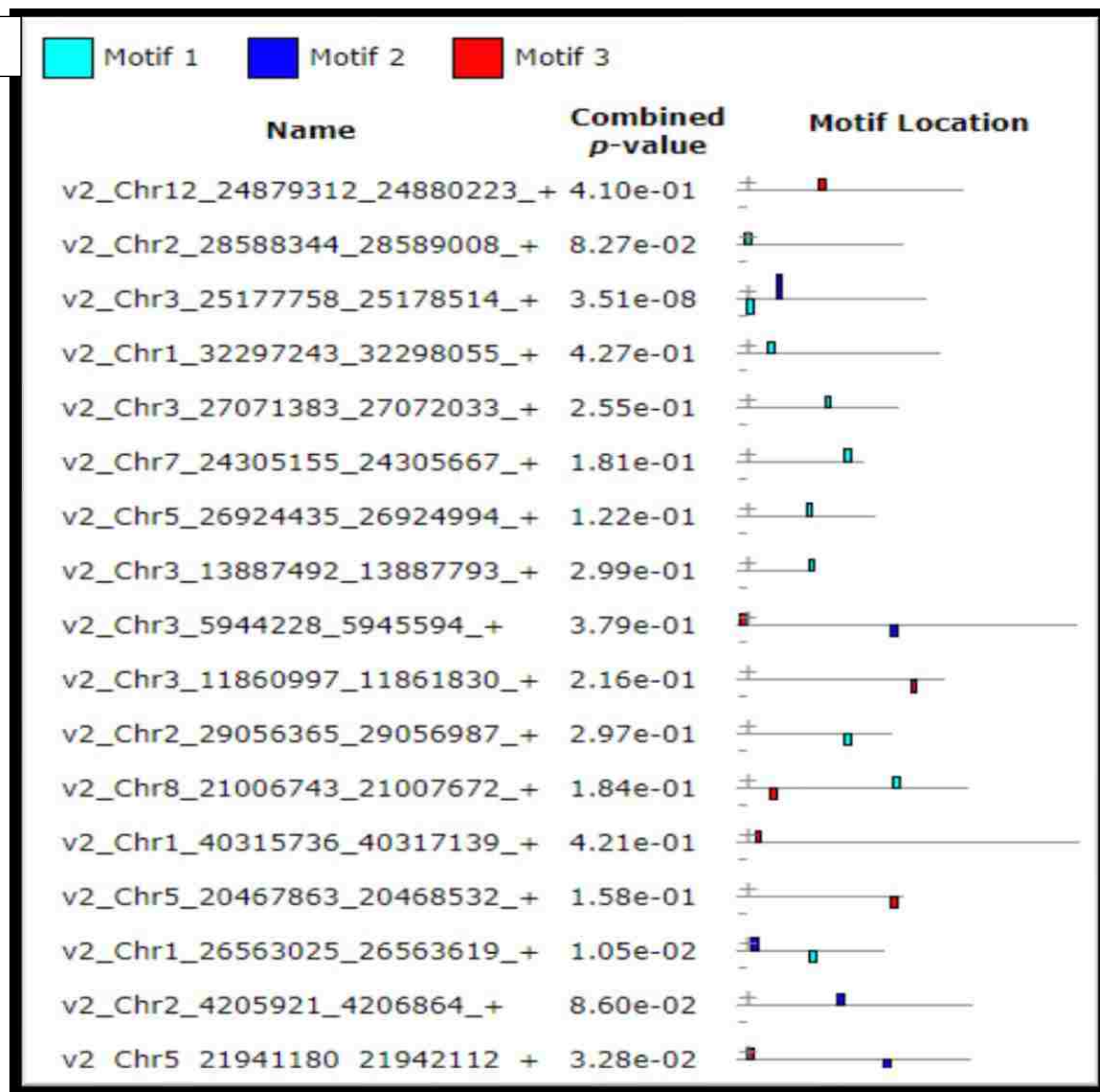
**Identification of *AtSHN2* binding motifs**

To investigate the binding sites for *AtSHN2*, 1000 bp +/- flanking sequence of the promoter regions of the highly enriched genes were downloaded, and these sequences were queried using MEME-ChIP to find out highly overrepresented binding motifs. Three different motifs with $p<0.0001$ were obtained using a de-novo approach (Figure 6A). These motifs can be represented as [CG]A[GA]CAA[GA][GA]CAA[GC]TCA, CCGGGCACCATGGAG and CTA[AT]CAAAA[AC]CA[AT]G[TC]. These three motifs were further queried against the plant cistrome database (representing cis-acting targets of a trans-acting factor on a genome-wide scale) using TOMTOM to search for similar motifs. The analysis showed that CTA[AT]CAAAA[AC]CA[AT]G[TC] can be reduced to [GA]CAACA[TG][AT] (Figure 7A) while CCGGGCACCATGGAG could be reduced to the GCC box motif (Figure 7B) bound by the AP2/ERF class of TF. Interestingly, it was also seen that both motifs CTA[AT]CAAAA[AC]CA[AT]G[TC] and CCGGGCACCATGGAG had WRKY associated motifs. (Supplementary Figure 2A and 2B).

6A




6B



6C

| Name | Strand | Start | p-value | Sites |
|---|---|---|---|---|
| v2_Chr10_3611433_3611835_+ | + | 375 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr8_5102369_5103010_+ | + | 359 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr6_5350543_5351007_+ | + | 424 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr3_34009924_34010403_+ | + | 353 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr4_22514760_22515396_+ | - | 39 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr12_24017775_24018184_+ | + | 370 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr6_28115531_28115944_+ | + | 369 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr7_19604827_19605108_+ | + | 259 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAA |
| v2_Chr5_14324253_14325247_+ | - | 166 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr4_31444911_31445316_+ | - | 36 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr10_18676836_18677469_+ | + | 358 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr12_22569951_22570561_+ | - | 43 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr6_1537724_1538118_+ | - | 11 | 1.18e-10 | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr8_3500941_3501381_+ | + | 392 | 1.18e-10 | ACAATAGTGC CCGGGCACCATGGAG CACCAAACAA |
| v2_Chr11_7567380_7567789_+ | - | 23 | 1.18e- | ACAATGGTGC CCGGGCACCATGGAG CACCAAACAA |

**Figure 6**: **Motif discovery from *AtSHN2*-TAP bound regions.** De novo motif discovery was performed using MEM-ChIP to seek statistically overrepresented sequences in the ChIP-Seq binding loci. (6A) Logos for the three significant motifs found, (6B) Non-overlapping binding sites with a p-value better than 0.0001. The height of the motif "block" is proportional to -log(p-value), truncated at the height for a motif with a p-value of 1e-10. (6C) Occurrence of sites in the promoter regions of the downstream genes.

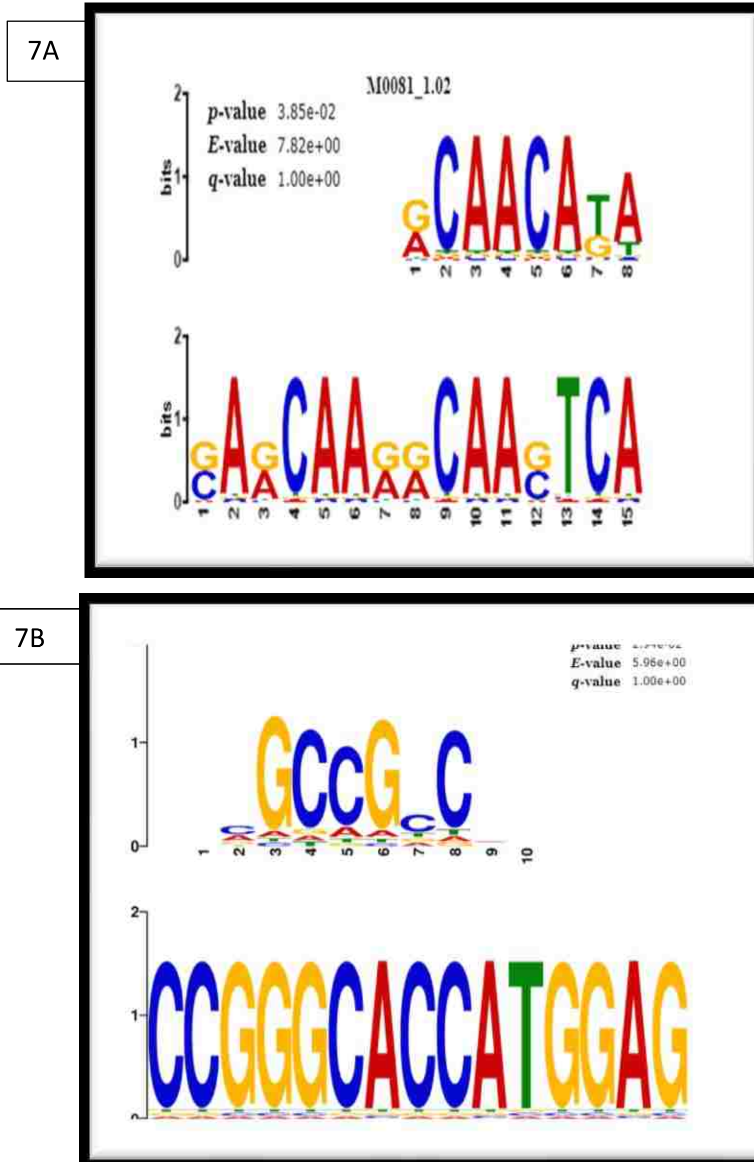



**Figure 7**: **Comparison of *AtSHN2*-TAP binding motif to known motifs.** The first two motifs obtained using MEME-ChIP were probed again the known rice motif database using TOMTOM. The motifs show the presence of CAA box (7A) and GCC box (7B), domains recognized by AP2/ERF class of TFs.

**Biological Process Enrichment of genes associated with *AtSHN2* binding sites.**

Cell wall biosynthesis is a complex process and serves myriad functions through the plants developmental cycle. Besides playing a central role in maintaining overall architecture of the plant, cell wall acts as first line of defense against abiotic and biotic stress and enables cell differentiation (Hamann, et al., 2004; Trafford, et al., 2013). To further investigate if changes in cell wall biosynthesis caused due to *AtSHN2*-TAP overexpression led to changes in the developmental pathways, biological process (BP) enrichment of genes associated with *AtSHN2* binding motifs was performed. The Gene Ontology terms associated with generation of carbohydrate metabolites was shown to be strongly enriched (Figure 8). Other pathways which were seen to be enriched by BP enrichment were abiotic stimulus response and flower development suggesting the role of *AtSHN2* in modulating the expression of genes involved in the aforementioned processes. Overexpression of *AtSHN2* in arabidopsis results in drought tolerance by mediating the expression of wax biosynthetic genes and alteration of cuticle properties (Ambavaram, et al., 2011). SHN Family of TFs have also known to be involved in floral organ morphology and underlying cell wall structure (Shi, et al., 2011). Based on these observations, it is proposed that multiple aspects of plant development are affected by *AtSHN2* overexpression in rice.

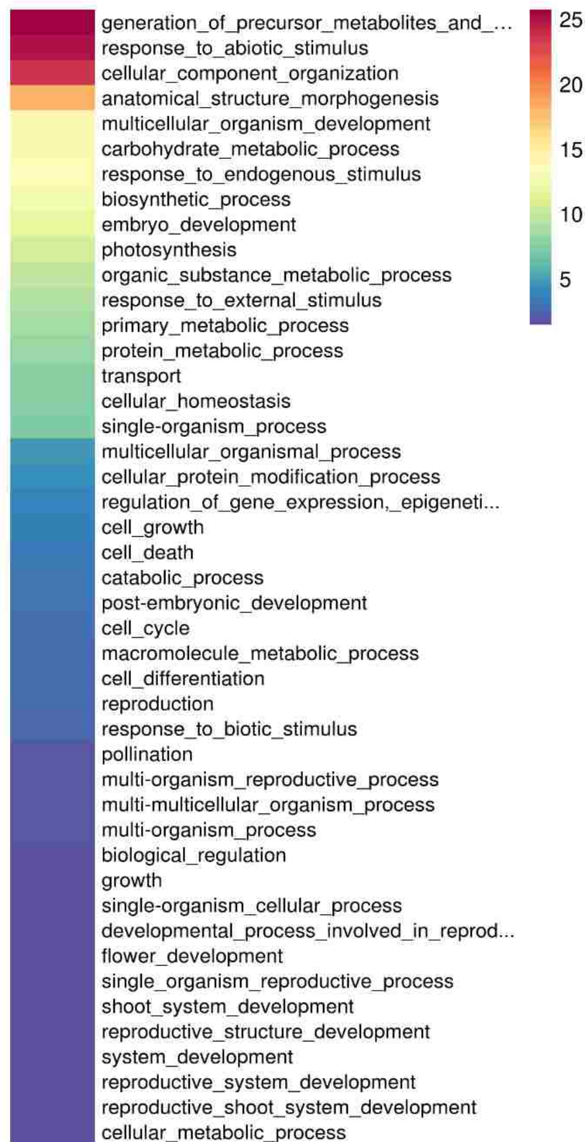



**Figure 8: Biological Process Enrichment of genes associated with *AtSHN2* peaks.** The scale bar represents -log (p value), red to blue indicating statistically overrepresented annotations for a process in a descending order.

***AtSHN2* leads to cellulose deposition predominantly through MYB TFs.**

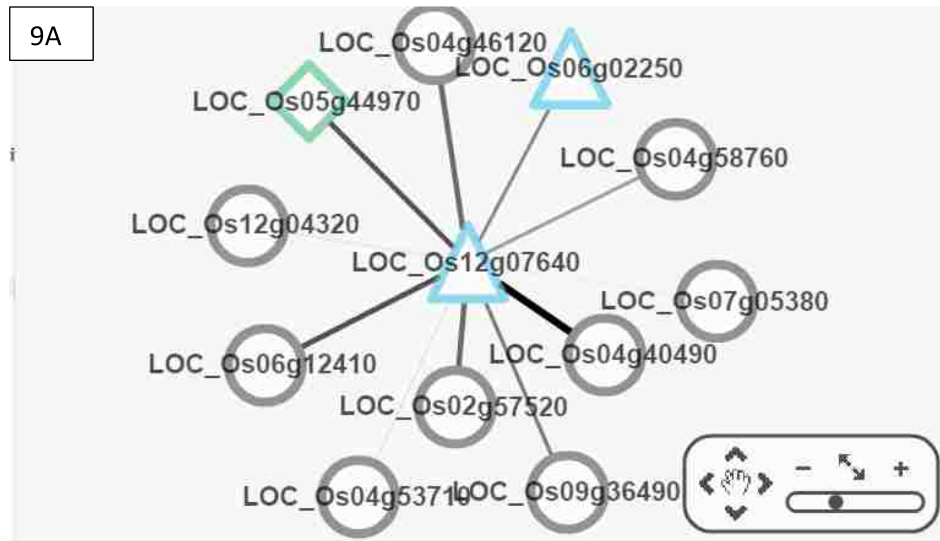
Cell wall machinery is regulated by a hierarchal network containing TFs predominantly belonging to NAC and MYB families. NAC TFs can directly activate MYB TFs; activation of MYBs leads to the activation of secondary cell wall biosynthetic genes (Chai, et al., 2015; Huang, et al., 2015). To further elucidate the mechanism of cell wall regulation by *AtSHN2*, transcriptional regulation of the MYB TFs by *AtSHN2* was studied. 15 different MYB gene promoters were predicted to be bound by *AtSHN2* (Table 2). These MYBs were probed onto a genome-scale coexpression network, RECoN (Krishnan, et al., 2017) (https://plantstress-pereira.uark.edu/RECoN/) to identify clusters of genes tightly co-expressed with these TFs. Of these, two uncharacterized MYBs, LOC_Os12g07640 and LOC_Os06g02250 were found to be closely associated with cell wall biosynthetic process (Figure 9A and 9B). While the ortholog of LOC_Os12g07640 in Arabidopsis, AtMYB4 is a known repressor of cinnamate 4-hydroxylase (Zhou, et al., 2017), the ortholog of LOC_Os06g02250 in Arabidopsis, *AtMYB9* is involved in suberin deposition (Lashbrooke, et al., 2016). Among the many TFs and kinases associated with these MYBs were LOC_Os04g50770 (MYB58/63), a characterized MYB known to regulate cellulose deposition through *OsCesA7* (Noda, et al., 2015); VND4/5/6, vessel specific secondary cell wall TF; LOC_Os01g18110, cinnamyl CoA Reductase (CCR) and LOC_Os08g38920, caffeoyl-CoA O-methyltransferase (CCOMT) both involved in lignin biosynthesis.

Another MYB, LOC_Os02g49986 was shown to be bound by *AtSHN2* through GCC box present in the promoter region (Ambavaram, et al., 2011). This gene was the only candidate to be upregulated during the initial studies with *AtSHN2*. A recent report also suggests that LOC_Os02g49986 was one of the genes to be upregulated during cell wall deposition (Chai, et al.,

2015). Furthermore, the binding of LOC_Os02g49986 by *AtSHN2* was confirmed by ChIP-qPCR. (Figure 9B). In lieu of the above information, the role of LOC_Os02g49986 was studied further.

**Table 3**: ***AtSHN2* binds to the promoter regions of MYB TFs.** Summary of promoter regions bound by *AtSHN2* annotated to MYB TFs, the locus ID of the gene bound, chromosomal position, location of binding region, binding specificity to the strand, distance from the transcriptional start site, the fold enrichment associated with each gene (p<0.001).

| **Gene** | **Chr** | **Start** | **end** | **Category** | **Strand** | **Distance to tss** | **fold_ enrichment** |
|---|---|---|---|---|---|---|---|
| **LOC_Os06g45410** | chr6 | 27452034 | 27452663 | Upstream | + | -4562 | 8.01 |
| **LOC_Os08g33800** | chr8 | 21153655 | 21154080 | Upstream | + | -1657 | 7.53 |
| **LOC_Os01g74410** | chr1 | 43091251 | 43091866 | Upstream | + | -4834 | 6.45 |
| **LOC_Os01g09590** | chr1 | 4921656 | 4922169 | Upstream | - | -2688 | 6.14 |
| **LOC_Os12g03150** | chr12 | 1202730 | 1204359 | Upstream | - | -1342 | 6.13 |
| **LOC_Os03g55760** | chr3 | 31752961 | 31753309 | Intron | + | 3666 | 5.76 |
| **LOC_Os01g06320** | chr1 | 3008795 | 3009339 | Upstream | - | -3339 | 5.32 |
| **LOC_Os03g19120** | chr3 | 10716872 | 10717419 | Upstream | - | -4130 | 5.23 |
| **LOC_Os06g02250** | chr6 | 712742 | 713604 | Upstream | + | -3646 | 4.85 |
| **LOC_Os06g07660** | chr6 | 3711750 | 3712277 | Upstream | - | -195 | 4.72 |
| **LOC_Os04g56990** | chr4 | 33973618 | 33974195 | Intron | - | 2346 | 4.46 |
| **LOC_Os01g19970** | chr1 | 11348942 | 11349494 | Upstream | + | -1289 | 4.43 |
| **LOC_Os01g63160** | chr1 | 36606352 | 36608203 | Exon/CD | - | 858 | 4.2 |
| **LOC_Os02g49986** | chr2 | 30540019 | 30541267 | Exon/CD | - | 470 | 3.64 |
| **LOC_Os12g07640** | chr12 | 3835606 | 3837246 | Upstream | + | -4311 | 3.31 |



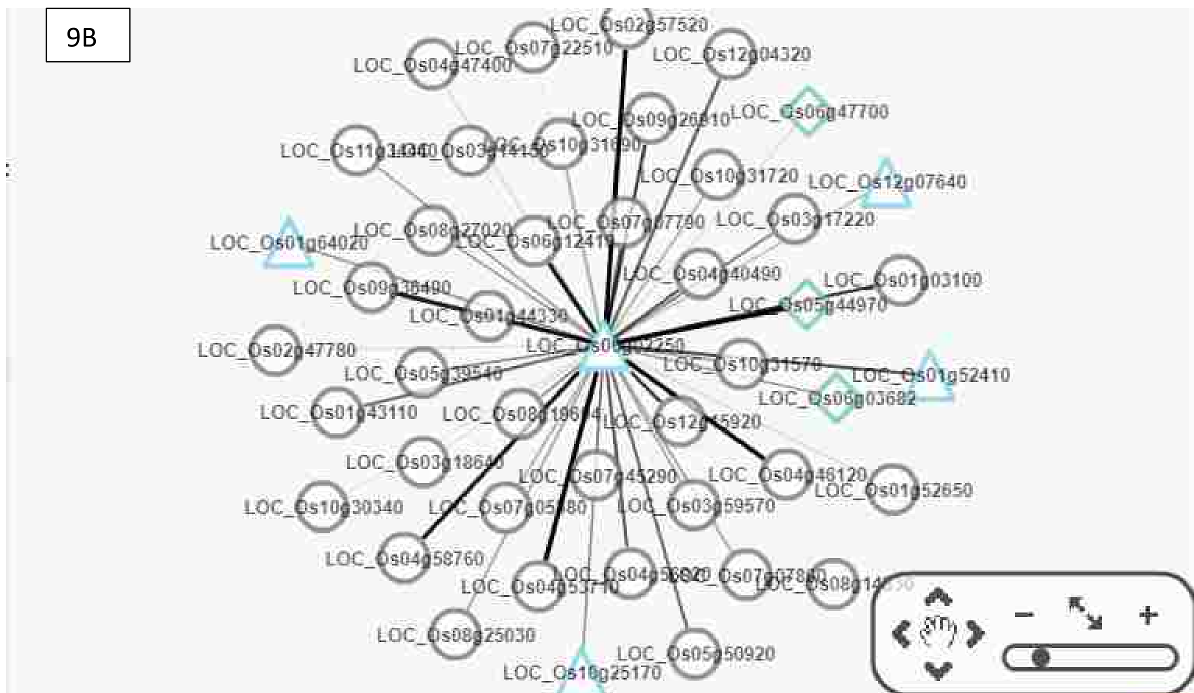
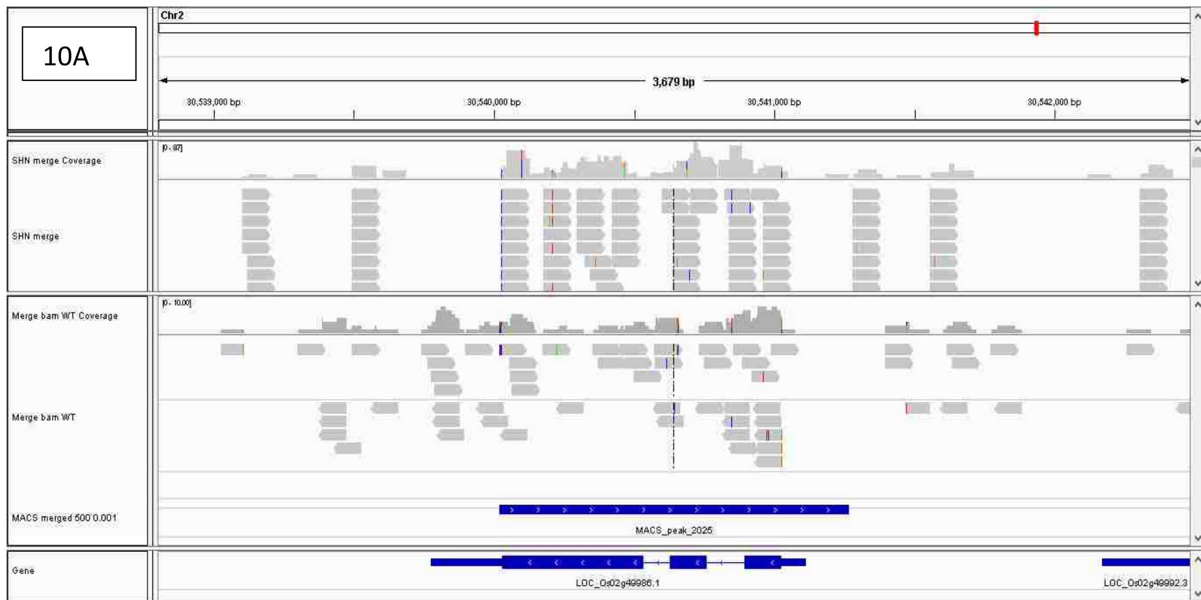




**Figure 9**: **Regulation of MYBs by AtSHN2.** Graphical visualization of (9A) LOC_Os12g07640 and B) LOC_Os06g02250 as probed by ReCoN. LOC_Os12g07640 is connected to 2 TFs, a kinase and 9 genes related to cell wall biosynthesis with an edge weight of 0.95. (9B) LOC_Os06g02250 is connected to 5 TFs, 3 kinases and 37 Genes with an edge weight of 0.95. TFs are represented by triangles, diamonds represent kinases whereas circles represent other genes



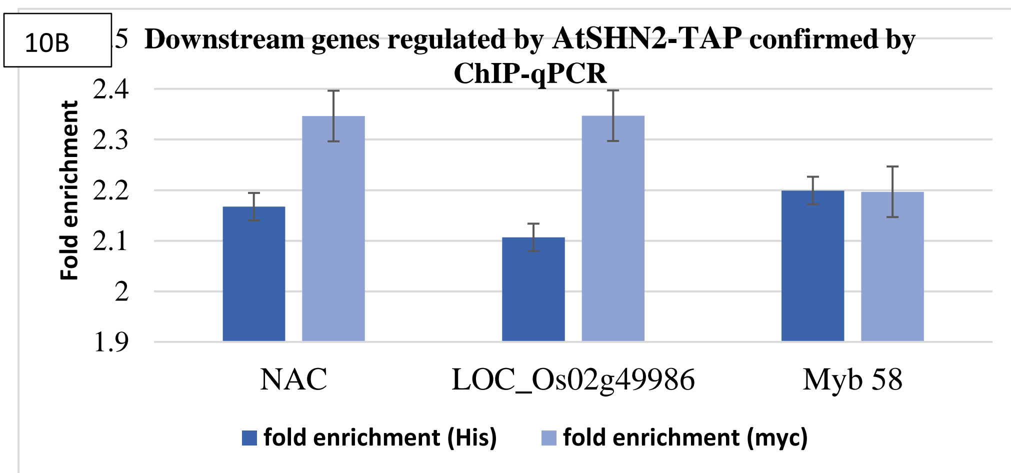




**Figure 10**: **Regulation of LOC_Os2g49986 by AtSHN2.** (10A) IGV snapshot of ChIP-Seq reads representing peak location at LOC_Os02g49986. (10B) Examples of AtSHN2 bound downstream genes by ChIP-qPCR. Data are means ± s.e. (n=3).

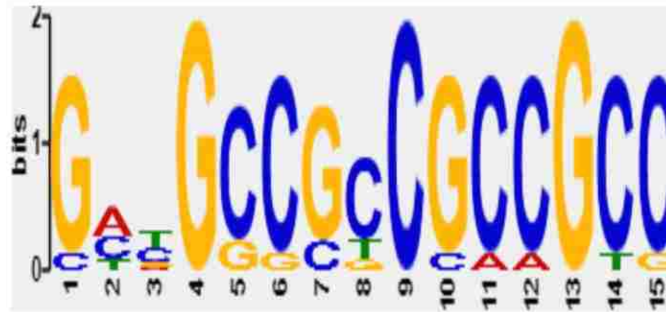
**Binding profiles in MYBs.**

To further characterize the transcriptional regulation of MYB TFs by *AtSHN2*, the promoter regions of all MYBs enriched from ChIP-Seq assay were obtained and analyzed to find binding motifs using MEME-ChIP. Three binding motifs with $p<0.001$ were highly overrepresented in the MYB promoter regions (Figure 11A). Out of the three motifs, the first motif G[AC][TC]GCCGCCGCCGCC (Figure 11B) was found to be over-represented in the promoter regions of 11 MYBs out of the 15 MYBs bound by *AtSHN2* (Figure 11C and Figure 11 D). Motif 1 contains the GCC box ([AG]CCGNC) known to be bound by AP2-ERF TFs (Figure 11 E), suggesting that *AtSHN2* can regulate directly regulate the expression of these MYB TFs by binding to the GCC box present in the promoter region of these MYBs.

These results were further validated using the gel mobility shift assay. 250bp fragment from the promoters of *OsMYB58*/63 (LOC_Os04g50770) and LOC_Os02g49986 containing the GCC box region were amplified and incubated with 6His-*AtSHN2* to study protein-DNA interaction. *AtSHN2* binding to the GCC box motif was evident as incubation of *AtSHN2* with the probe containing GCC motif, lead to gel retardation (Figure 11F).

Motif Overview

| | | | |
|---|---|---|---|
| Motif 1 | • 1.7e-012<br>• 11 sites | | |
| Motif 2 | • 8.9e-007<br>• 14 sites | | |
| Motif 3 | • 2.5e-006<br>• 14 sites | | |




| Name | Strand | Start | *p*-value | Sites |
|---|---|---|---|---|
| _Chr1_36605352_36609203_- | + | 1133 | 5.90e-11 | CCGCCGCTTC GCCGCCGCCGCCGCC GCCGAATCCA |
| _Chr4_33972618_33975195_- | - | 37 | 5.90e-11 | CGCCTTGGCC GCCGCCGCCGCCGCC GTACCGAGAG |
| _Chr1_4920656_4923169_- | + | 747 | 3.41e-10 | GTCCCCCGTC GATGCCGCCGCCGCC GCGCCCATCC |
| _Chr1_11347942_11350494_+ | - | 1866 | 1.13e-09 | CGCCAGCATG GCTGGCGCCGCCGCC TCTATTCCTG |
| _Chr1_3007795_3010339_- | + | 262 | 1.88e-09 | ATGTCCCGGT GAAGCCGCCGCCGCC AGCCGTTCGT |
| _Chr6_3710750_3713277_- | + | 1560 | 3.83e-09 | ACTTCCTCGG CATGCCGCCGCCGCC GGTGCAACAA |
| _Chr6_711742_714604_+ | + | 1013 | 6.97e-09 | CGACAGCATG GCTGGCCCCGCCGCC TCCATTCCTG |
| _Chr3_10715872_10718419_- | + | 1389 | 4.23e-08 | CCTAAAATAA GTTGCGGTCGCCGCC CTTTTCAATG |
| _Chr2_30539019_30542267_- | + | 3026 | 4.90e-08 | TAGGTGCCCG GTCGCCGGCGCCGCG CGGCCCAGGT |
| _Chr12_1201730_1205359_- | - | 2571 | 9.75e-08 | GAGCCCGGCC GAGGCCGCCCCAGCC AGTTATTCCT |
| _Chr12_3834606_3838246_+ | + | 1248 | 3.51e-07 | ATACCAGCTG GACGCCCTCGACGTC TCTATTATGA |

**Figure 11**. **Motif discovery in AtSHN2 bound MYB TFs.** De novo motif discovery was performed using MEME-ChIP to seek statistically overrepresented sequences from *AtSHN2*-bound MYBs. (11A) Logos for the three significant motifs found, (11B) Logo for the most overrepresented motif found in MYB TFs bound by *AtSHN2*. (11C) Occurrence of binding motif in the promoter regions of the downstream genes (11D) Motif location in MYB TFs. The height of the motif "block" is proportional to -log(p-value), truncated at the height for a motif with a p-value of 1e-10. (11E) Comparison of the motif to known motifs reveal *AtSHN2* potentially binds to promoter regions of MYBs using GCC box. (11F) Electrophoretic mobility shift assay indicating binding of “GCC” box present in promoter regions of MYBs by *AtSHN2*. Each of the end-labeled probes was competed with 100-fold excess of the competing unlabeled probe. + and − indicate the presence and absence of the respective component in the assay. Bound probe (BP) and free probe (FP) positions are indicated in Figure.

***AtSHN2* directly activates the expression of LOC_Os02g49986.**

To further investigate the hypotheses that regulation of cell wall biosynthesis by *AtSHN2* is potentially mediated by a MYB TF, direct activation of LOC_Os02g49986 by *AtSHN*2 was studied. For this purpose, a steroid receptor based inducible system was used in rice protoplast assay. The regulatory region of human estrogen receptor (HER) was fused with *AtSHN2* driven by a 35S promoter. Fusion of *AtSHN2* with HER renders the TF to be inactive by sequestering it into the cytoplasm through binding to a cytoplasmic complex. Addition of estradiol causes the receptor to bind to the ligand thus freeing the TF from the cytoplasmic complex, and subsequently allowing the movement of the TF into nucleus, to facilitate the transcription of direct downstream genes.

To test direct activation, transfection of rice protoplast with 35S: *AtSHN2*: HER, followed by addition of estradiol and cycloheximide where indicated was done. While addition of estradiol can induce translocation of TF to the nucleus, addition of cycloheximide will stop the activation of downstream genes due to inhibition of new protein synthesis. It was observed that the addition of estradiol led to an increase in the transcripts of LOC_Os02g49986. In addition, LOC_Os02g49986 was induced even after addition of cycloheximide. Since addition of cycloheximide inhibits new protein synthesis, increase in transcripts of LOC_OsO2g49986 suggests that *AtSHN2* is directly capable of activating the expression of LOC_Os02g99986 in rice protoplasts.

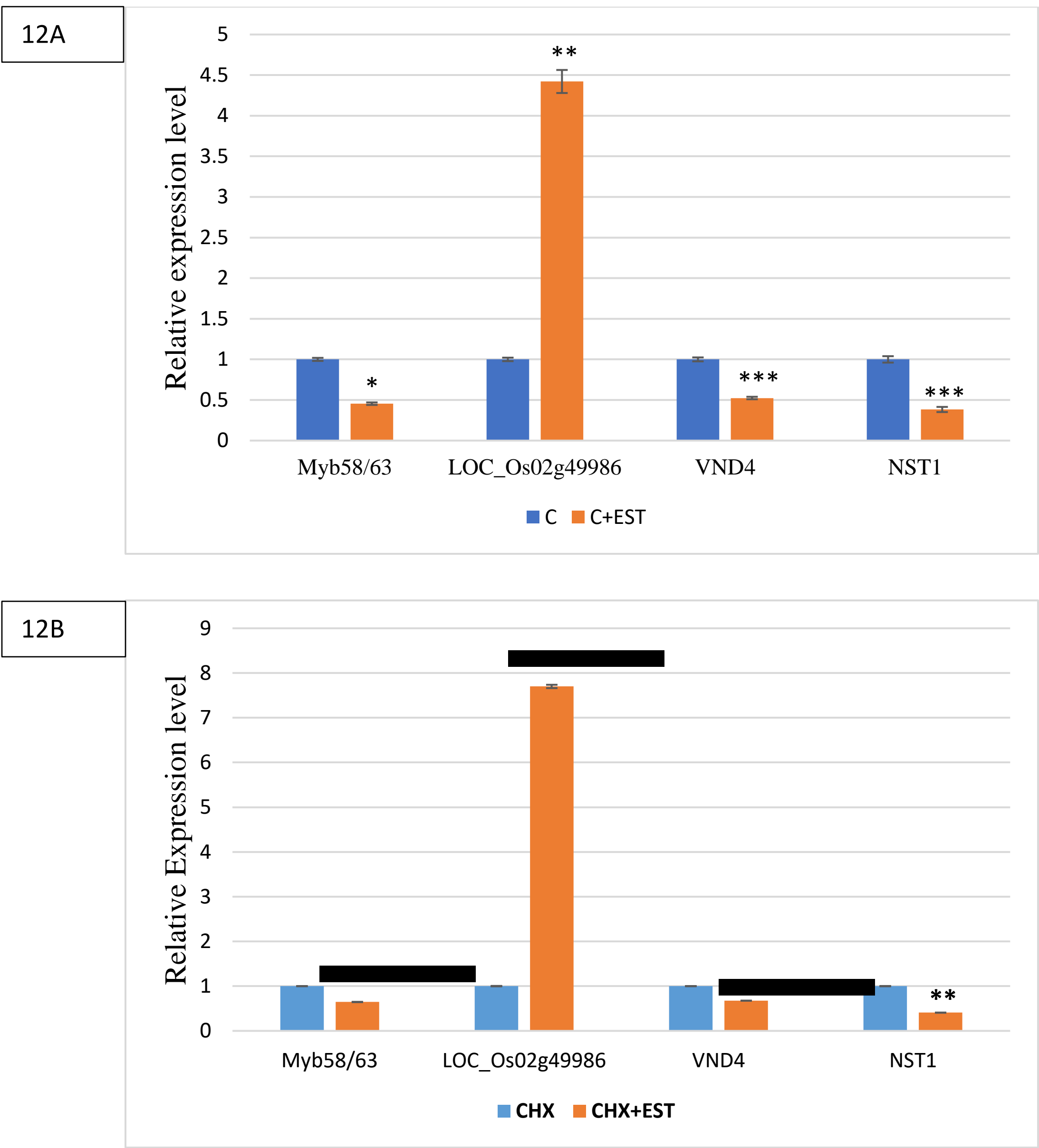
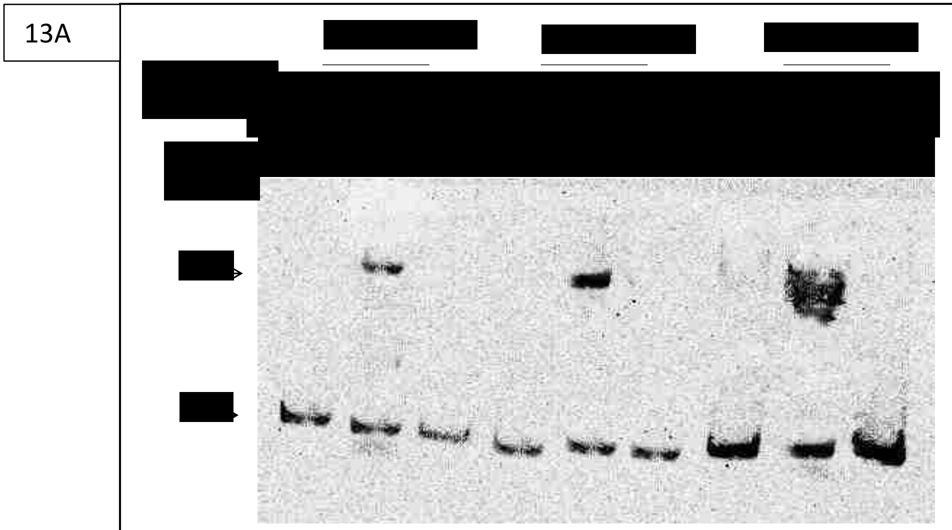



**Figure 12: Direct Activation of cell wall biosynthetic genes by *AtSHN2*.** Rice leaf protoplasts were transfected with *AtSHN2*-HER under control of the 35S promoter. Post transfection, the protoplasts were treated with estradiol (EST) (12A), cycloheximide (CHX) (12B), cycloheximide + estradiol (CHX+EST (B). qRT-PCR quantified the expression of the individual transcripts. The quantitative differences in the expression of the tested genes between the control and treated samples are statistically significant (* represents p-value < 0.05, ** indicates p-value<0.01, and *** indicates p-value < 0.001. The expression level of each gene in the mock-treated (control) or cycloheximide-treated protoplasts (CHX) is set to 1. Error bars represent SE of three biological replicates.

**LOC_Os02g49986 can directly activate cellulose biosynthetic genes.**

Based on the studies that MYBs have the capacity to bind to cell wall biosynthetic genes (Ye et al, 2015), it was tested if LOC_Os02g49986 gene can activate the expression of CesA4, CesA7 and CesA9 using gel retardation experiments. Promoter fragments of CesA4, 7, 9 were amplified and a recombinant 6His-MYB was used for protein-DNA interaction study. Gel mobility shift assay showed binding between MYB and promoter regions of *OsCesA4*, *OsCesA7* and *OsCesA9*. The recombinant MYB protein could bind to the promoters of all three genes with almost equal affinity, while the extent of binding could be reduced using 100-fold excess unlabeled probe suggesting that LOC_Os02g49986 can bind to the promoters of cellulose biosynthetic genes and thereby potentially activate the expression of these genes (Figure 13A).

To further investigate if direct activation of these CesAs can be achieved by LOC_Os02g49986, a steroid receptor inducible system in rice protoplast was used. Although the expression of all three CesAs was induced after addition of estradiol, transcripts of only *OsCesA9* were quantified after addition of cycloheximide. This led to the conclusion that LOC_Os02g49986 can activate the expression of *OsCesA9* in vivo leading to cellulose deposition in rice (Figure 13B).



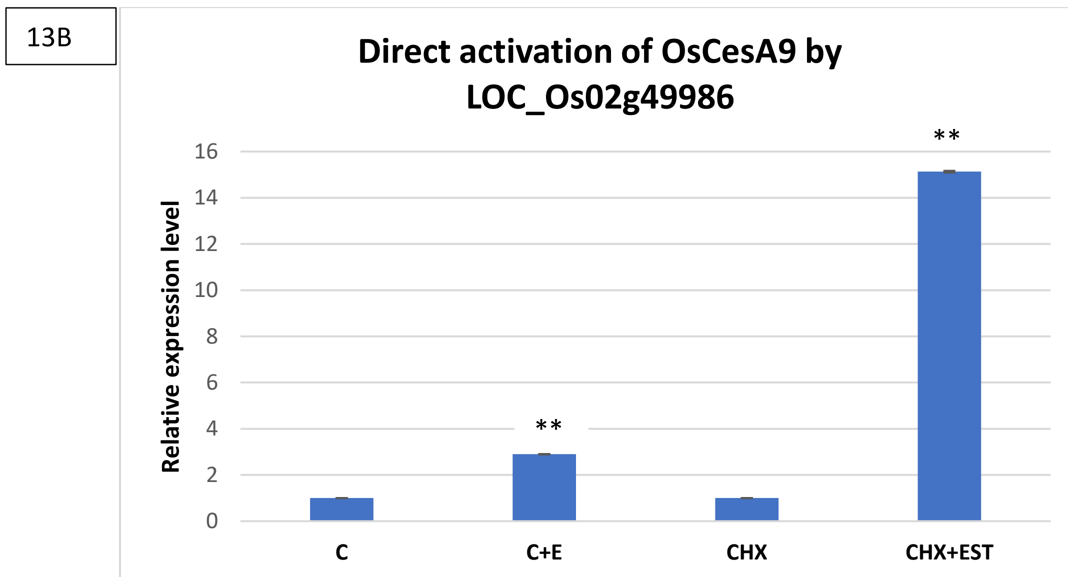




**Figure 13: Regulation of OsCesAs by LOC_OsO2g49986.** (13A) Electromobility shift assay representing binding of secondary cell wall biosynthetic genes by LOC_Os02g49986. Each of the end-labeled probe was competed with 100-fold excess of the competing unlabeled probe. + and − indicate the presence and absence of the respective component in the assay. Bound probe and free probe are indicated (13B) Direct Activation of *OsCesA9* by LOC_Os02g49986(* represents p value < 0.05, ** indicates pvalue<0.01 whereas *** indicates pvalue < 0.001. The expression level of each gene in the mock-treated (control) or cycloheximide-treated protoplasts (CHX) is set to 1. Error bars represent se of two biological replicates.

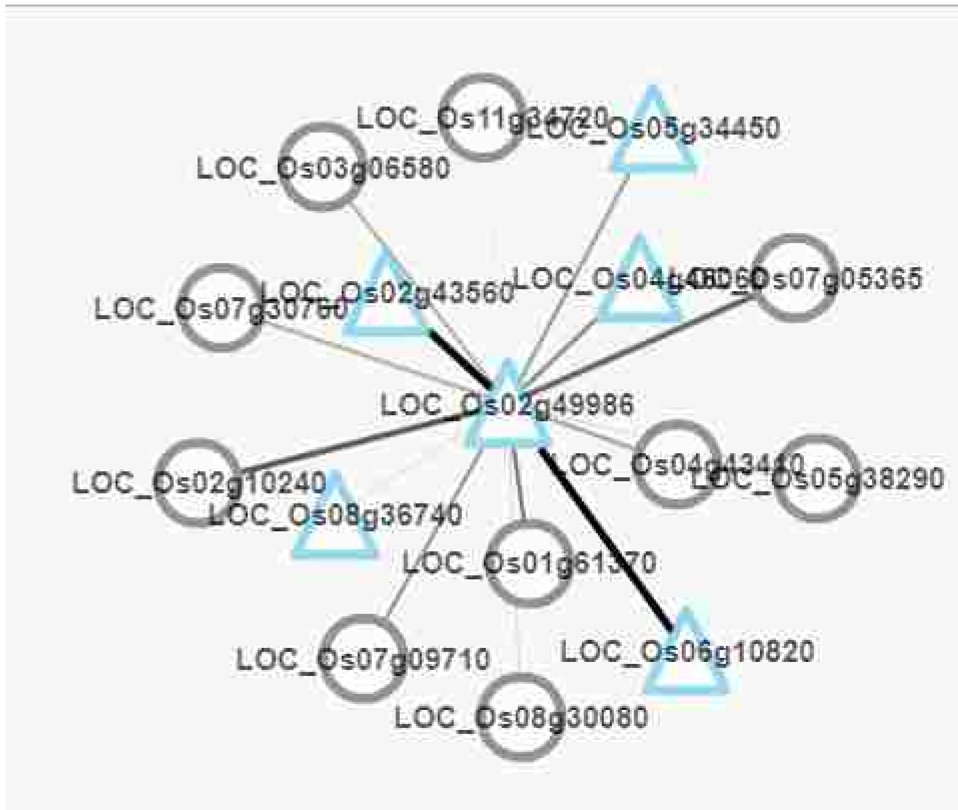



**Figure 14**: **Co-expression network for LOC_Os02g49986.** LOC_Os02g48886 is co-expressed with eight genes with weight of 0.75 for the module. Triangles represent TF while circles denote other genes. The thickness of the edges connecting different genes represent the degree of co-expression

## Table 5: List of genes co-expressed with LOC_Os02g49986

| Gene | Annotation | Arabidopsis homolog | MF |
|---|---|---|---|
| **LOC_Os01g61370** | expressed protein | AT3G51760 | Gene |
| **LOC_Os02g10240** | ZOS2-05 - C2H2 zinc finger protein, expressed | - | Gene |
| **LOC_Os02g43560** | WRKY34, expressed | AT2G44745 | TF |
| **LOC_Os02g49986** | MYB family transcription factor, putative, expressed | AT1G66230 | TF |
| **LOC_Os03g06580** | MTN26L2 - MtN26 family protein precursor, expressed | AT4G14746 | Gene |
| **LOC_Os04g43410** | Os4bglu18 - monolignol beta-glucoside homologue, expressed | AT4G21760 | Gene |
| **LOC_Os04g46060** | WRKY36, expressed | AT2G44745 | TF |
| **LOC_Os05g34450** | DUF260 domain containing protein, putative, expressed | AT1G65620 | TF |
| **LOC_Os05g38290** | protein phosphatase 2C, putative, expressed | AT1G07430 | Gene |
| **LOC_Os06g10820** | helix-loop-helix DNA-binding domain containing protein, expressed | AT4G00120 | TF |
| **LOC_Os07g05365** | photosystem II 10 kDa polypeptide, chloroplast precursor, putative, expressed | AT1G79040 | Gene |
| **LOC_Os07g09710** | OsFBX220 - F-box domain containing protein, expressed | AT3G17490 | Gene |
| **LOC_Os07g30760** | UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein, expressed | AT1G22360 | Gene |
| **LOC_Os08g30080** | 1-aminocyclopropane-1-carboxylate oxidase homolog 4, putative, expressed | AT1G06650 | Gene |
| **LOC_Os08g36740** | Helix-loop-helix transcription factor, putative, expressed | AT3G50330 | TF |
| **LOC_Os11g34720** | Ser/Thr protein phosphatase family protein, putative, expressed | AT1G25230 | Gene |

To further understand the role of LOC_Os02g49986 in cell wall regulation in rice, the gene was probed against ReCON to find out genes co-expressed genes. Gene interaction network of LOC_Os02g49986 showed strong co-expression with 16 genes with an edge score of 0.75 (Figure 14). Of the TFs co-expressed (Table 5), ortholog of LOC_Os02g43560 (*OsWRKY34*) and LOC_Os04g46060 (*OsWRKY36*) from Arabidopsis *AtWRKY12*, functions in negative regulation of secondary cell wall formation by inhibiting the expression of NST2 master switch (Wang, et al., 2010). *PtrWRKY19*, WRKY12 ortholog from Poplar also functions in the regulation of secondary cell wall biosynthesis and causes a reduction in the biosynthesis of lignin biosynthetic genes (Yang, et al., 2016). Ortholog of the same gene from Miscanthus when overexpressed in *atwrky12* mutant background causes early flowering and changes in pith secondary cell wall formation (Yu, et al., 2013). Interestingly, ortholog of LOC_Os11g34720 from Arabidopsis is also involved in flowering (Wang, et al., 2016). Moreover, the gene interaction network included cell wall related genes such as monolignol beta-glucoside gene and UDP-glucoronosyl and UDP-glucosyl transferase further demonstrating the role of LOC_Os02g49986 in cell wall biosynthesis in rice.

**Section 2.5. Discussion**

Cellulose forms the most abundant polymer on earth. Recent studies have advanced our understanding towards regulation of cellulose biosynthesis, but a lot remains unknown. In this study, we have used a systems-level cis-regulatory element analysis to understand the complex regulation of cell wall formation in rice using an innovative ChIP-TAP technique.

TFs regulate gene expression by binding to the cis-regulatory elements present in the promoter regions of their target genes. Many approaches have been used in probing binding sites for TFs. Most of these involve immunoprecipitation of DNA-protein complex using TF-specific

antibody followed by quantification of known target genes using qPCR or sequencing of the eluted DNA (ChIP-Seq). These techniques are however limited by the lack of specificity of antibodies for the required TF. The results get more convoluted due to the presence of several isoforms of the same TF and the difference in the binding affinities of these antibodies to the TF. Recently, a modified version of this technique requiring fusion of TF of interest with Tandem Affinity Purification (TAP)-tag was successfully used to map genome wide targets in muscle cells and fibroblasts of humans (Soleimani, et al., 2012). A modified version of the technique has also been used to identify downstream targets of E2Fa in Arabidopsis (Verkest, et al., 2014). In this study, we have used a chromatin tandem affinity purification tag containing his and myc tag with IgG domain with high-throughput sequencing to identify genome-wide cis-regulatory elements bound by *AtSHN2*.

The study showed that *AtSHN2*-TAP lines exhibit a significant >20% increase in cellulose with highly decreased lignin content (>1/3$^{rd}$) with no effects on plant growth and status. These results are collateral with earlier studies (Ambavaram et al., 2011) where overexpression of AtSHN2 in rice caused a concomitant cellulose increase by 34% and reduction of lignin by 45%. The discrepancy in the percent changes related to cellulose and lignin content from the earlier studies can be attributed to many factors such as transcription factor titer, the difference in the two transgenic lines or interference from the TAP tag. Nevertheless, we conclude that *AtSHN2*-TAP can be used as a tool to fish out novel genes and understand the process of cell wall modulation in rice from outside in.

ChIP-Seq analysis of *AtSHN2*-TAP lines reveal that *ATSHN2* can bind to cell wall related genes such as *OsCSLF7* and *OsDUF* (Table 2). These observations were confirmed by invitro binding of *AtSHN2* to the promoter regions of these genes using EMSA (Figure 5). The role of

DUF proteins in cell wall biosynthesis was first reported through protein interaction studies (Irshad, et al., 2008). Recently interaction of Arabidopsis DUF642 with cellulose and hemicellulose was shown invitro, thus proposing the idea that these proteins might be involved in strengthening of cell walls by interacting with cellulosic polysaccharides (Vázquez-Lobo, et al., 2012). Furthermore in populus, overexpression of DUF266 resulted in high cellulose with decreased recalcitrance (Yang, et al., 2017). We propose that some rice DUF genes might have a similar function in cell wall modification in rice plants and LOC_Os03g41330 can serve as the candidate gene for studying the role of DUF domain genes in rice cell wall biosynthesis.

BBX proteins are known to regulate complex processes in plant growth and development (Gangappa, et al., 2014). Their role in flowering has been extensively studied. In Arabidopsis, bbx4 mutants flower early irrespective of the day length (Datta, et al., 2006) while repression of flowering was seen in BBX7 repression lines. In rice, *OsBBX27* and *OsBBX5* are implicated in the process of flowering (Kim, et al., 2008; Lee, et al., 2010). Ectopic expression of TFs in rice have been known to cause secondary effects such as changes in flowering time and plant height. However, it has been shown that SHN TF act redundantly to orchestrate floral organ morphology, surface formation and transition by modulating the expression of associated lipids and cell wall components (Shi, et al., 2011) . In this study, evidences such as enrichment of flower development process from ChIP-Seq dataset (Figure 8) and binding of AtSHN2 to the promoter region of Bbox protein in rice by EMSA (Figure 5) makes us conclude that SHN family of TF in rice might have an important role in mediating flowering biosynthesis in rice by virtue of Bbox genes.

To further understand the mechanism of *AtSHN2* regulation, *de novo* motif discovery on upstream regions of peaks enriched from the ChIP-Seq dataset was performed. Three significant motifs were enriched in this analysis (Figure 5A), of which the most significant could be reduced

to [GA]CAACA[TG][AT], a motif specific to AP2 class of TFs. Interestingly, two significant motifs contained sequences which can be bound by WRKY of TFs. Since many WRKY TFs have been implicated in cell wall biosynthesis in rice (Wang, et al., 2010; Yang, et al., 2016), it is possible that the promoters of these downstream biosynthetic genes may contain binding sites for multiple TFs in turn the demonstrating the complex regulatory mechanism that exists in cell wall formation in plants.

The role of MYB TFs in cell wall regulation is very well known (Zhong, et al., 2008). Earlier studies from the lab showed that *AtSHN2* can bind to promoter regions of MYB TFs (Ambavaram, et al., 2011). Several lines of evidences show that *AtSHN2* can regulate MYB TFs to modulate cell wall regulation. It was seen that promoter regions of *AtSHN2* bound MYBs contain a conserved ([AG]CCGNC) motif (Figure 11E) for recognition. Further confirmation of these results was done by invitro binding of AtSHN2 to these motifs. We further demonstrate the role of LOC_Os02g49986 in cell wall biosynthesis. Direct activation of LOC_OsO2g49986 by *AtSHN2* (Figure 12) followed by direct activation of *OsCesA9* by LOC_Os02g49986 (Figure 13) exhibit a two-layered mechanism in which *AtSHN2* can act as a master regulator for cell wall formation in rice.

**Summary**

Lignocellulose forms the major biomass for alternative source of energy on earth. The role of *AtSHN2* in the regulation of cell wall biosynthesis in rice shows its potential use to engineer and modify cell walls for biofuel purposes. In this study, we unravel potential pathways by which *AtSHN2* causes increased cellulose and decreased lignin phenotype. We also unravel uncharacterized genes with a putative role in cell wall biosynthesis in rice. Information from this

study can be used to advance our understanding the regulation of cellulose and lignin pathway in many plant species.

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**Section 2.6: Supplementary Tables and Figures:**

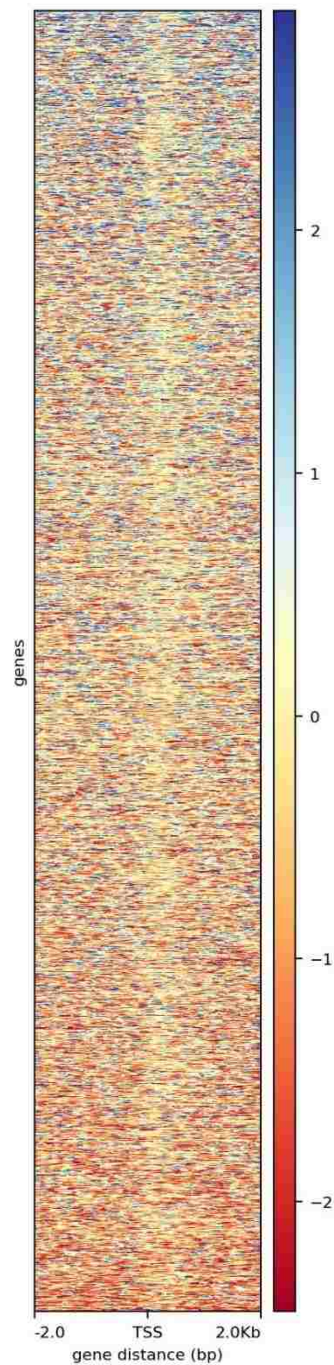


**Figure 1A**: **Heatmap representing binding profiles of *AtSHN2* in AtSHN-TAP rice lines across known regions in the genome.** The scores are computed using bamCompare which normalizes and compares the BAM files to obtain log2ratio between control and ChIP-Seq samples.

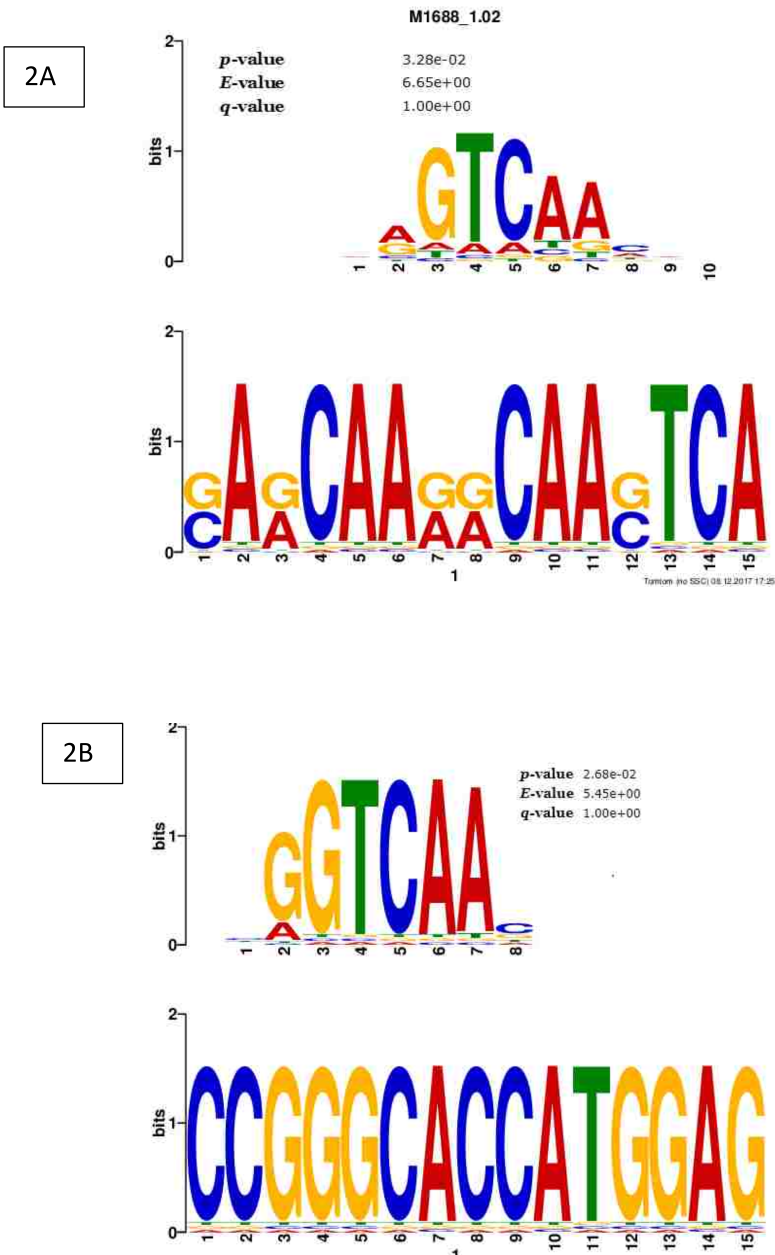


**Figure 2: Binding motifs present in *AtSHN2* bound downstream sequence.** Comparison of the motif to known motifs reveal *AtSHN2* enriched genes also contain motifs enriched for WRKY binding TFs. (2A) Motif 3 enriched for WRKY binding site. (2B) Motif 1 enriched for WRKY binding site

## CHAPTER 3: RNA-Seq transcriptome analysis of Arabidopsis *AtSHN2*-TAP gene overexpression lines in rice

### Section 3.1: Abstract

Rice, the monocot model plant has also become the model species to study cell wall regulation in grasses. Ectopic overexpression of the Arabidopsis *AtSHN2* gene in rice was shown to coordinately regulate the expression of cellulose and lignin (Ambavaram et al., 2011). To understand the global changes associated with *AtSHN2* expression in rice, differential gene expression analysis of *AtSHN2*-TAP (Tandem Affinity-tagged) transgenic lines was carried out to to the perturbed transcriptome. RNA-sequence analysis of *AtSHN2*-TAP lines revealed 11,627 differentially expressed genes at FDR<0.05. Functional annotations of the *AtSHN2* regulated genes show changes in carbohydrate metabolism pathway, phenylpropanoid metabolism, increased photosynthesis, and higher wax biosynthesis among many others. Functional annotation of the genes led to the association of *OsSHN2*, the true ortholog of *AtSHN2* from rice to carbohydrate biosynthesis pathway. In addition, integration of RNA-Seq data with direct targets obtained through ChIP-Seq analysis of *AtSHN2*-TAP lines (Chapter 2), reveal direct and indirect targets of *AtSHN2* in rice. Thus, RNA-seq coupled with ChIP-Seq analysis provides a systems level analysis of the regulation of cellulose biosynthesis in rice.

### Section 3.2: Introduction

The plant cell wall is an intricate complex made of polysaccharides such as cellulose, hemicelluloses embedded in a mesh of glycoproteins and lignin. The plant cell wall is an abundant source of renewable energy. The two types of cell wall, albeit primary cell wall and secondary cell wall, provide the cell the functionalities of flexibility, structural support, and defense amongst

many others. Although the mechanism of cell wall formation has been studied primarily in Arabidopsis, many energy crops have cell walls distinct from other species. There is therefore the need to understand the mechanism of regulation of cell wall in grasses such as rice to engineer crops with higher biofuel potential.

Recent work on rice have revealed some TFs associated with cell wall biosynthesis pathway. A recent study implicated *SECONDARY WALL NAC DOMAIN PROTEINs (SWNs)* for their role in secondary cell wall biosynthesis in rice (Chai et al., 2015). Another approach used cco-expression networks have been used to find genes and TFs associated with the pathway (Hirano et al., 2013). This approach was supplemented by RNAi analysis, where the roles of different MYB and NAC TFs were studied (Hirano, et al., 2013).

While a lot of studies focus on the role of NAC and MYB TFs, the role of AP2 TF in rice cell wall biosynthesis remains unclear. The only study to implicate an AP2 TF came from Ambavaram et al., 2011 where it was shown that overexpression of *AtSHN2*, a heterologous AP2 TF in rice showed perturbation of the rice cell wall biosynthesis pathway. The APETALA2/ethylene responsive factor (AP2/ERF) family, is a large group of TFs that control a myriad of processes such as growth and development, and response to environmental stimuli. These TFs are largely characterized by the presence of one or two 60-70 amino acid long AP2 DNA binding domains. *AtSHN2*,has been shown to be involved in epicuticular wax deposition in Arabidopsis and tolerance to drought (Aharoni, et al., 2004). Rice transgenic plants overexpressing the *AtSHN2* gene were shown to have increased deposition of cellulose with simultaneous decrease in lignin. In Chapter 2, it was shown that *AtSHN2*-TAP transgenic lines, with the TAP-tag fusion (coding sequence for six His repeat (6xHis), a 9x-myc peptide, a 3C protease cleavage site and two copies of the IgG binding domain 2xIgG-BD, collectively known as TAP-tag), can be used as

a novel tool to isolate and identify DNA sequences bound by the *AtSHN2*-TAP. Systemic analysis of transcriptional binding sites for *AtSHN2*-TAP also revealed its capacity to directly regulate key TFs and genes involved in cell wall biosynthesis by binding to the GCC and CAA motifs in the promoter regions of these genes.

To gain further insight into the mechanism of cell wall regulation by *AtSHN2*, a transcriptomic approach was followed to identify genes that are differentially expressed under *AtSHN2*-TAP overexpression. The list of differentially expressed genes was further integrated with ChIP-Seq results from Chapter 2 to identify direct and indirect targets of transcriptional regulation by *AtSHN2*. Further, to annotate *AtSHN2* gene functions and their role in biological processes in rice, the transcriptome data was queried against rice regulatory networks to identify clusters of genes involved in biological processes and the more specific clusters that perform a biological function in an autonomous manner. The results show that *AtSHN2* orchestrates the phenotype of perturbed cell wall by directly regulating the expression of TFs, biosynthetic genes and kinases involved in various steps of the cell wall biosynthesis pathway. *AtSHN2*-TAP also causes potentially changes in wax deposition in rice plants by increasing the expression of *OsGL1-1*, a key gene involved in wax biosynthesis.

## Section 3.3: Materials and Methods

### Construction of AtSHN2-TAP rice transgenic lines

The Arabidopsis SHN2 gene (LOC_At5g11190) was amplified and ligated to a DNA fragment containing six His repeat (6xHis), a 9x-myc peptide, a 3C protease cleavage site and two copies of the IgG binding domain (2xIgG-BD) collectively known as TAP-tag obtained from pcTAPa (Rubio, et al., 2005). Further, the *AtSHN2*-TAP amplicon was ligated to the CaMV35S promoter and NOS terminator and cloned into pUC19 using *Xba*I/*Eco*RI sites. The cassette was

subcloned in the binary vector pMOG22 (Zeneca-Mogen), for efficient hygromycin based selection of rice. Transformation of *Oryza sativa* ssp. japonica cv.Nipponbare was performed using Agrobacterium strain LBA4404 (Nishimura, et al., 2006). Plants were grown in growth chambers under following conditions: 28±1ºC day and 25±1ºC night temperature, 65% relative humidity (RH), photoperiodic cycle of 14 h light and 10 h dark. Plants were later transferred to the greenhouse and grown till maturity.

**Plant Genotyping and Statistical Analysis**

Four transgenic lines were generated for AtSHN2-TAP lines and the expression of *AtSHN2* in the *AtSHN2*-TAP lines was confirmed by qRTPCR in T2 progeny. All plants were also checked for the presence of CAMV35S promoter by PCR. Based on positive expression of *AtSHN2* in the above lines, three T2 lines were chosen for further experiments.

**RNA extraction and sequencing**

Total RNA (n=2; biological replicates) was extracted from 35-day old *AtSHN2*-TAP transgenic plants using RNeasy Mini Kit (Qiagen, USA). The quality of the RNA samples was checked using 2100-Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA samples with RNA integrity number greater than 6.5 were sent for sequencing at Genomics Core Facility at Michigan State University.

**Mapping reads to the *Oryza sativa* Genome**

The raw data was quality checked using FastQC (Andrews, n.d.) followed by trimming of reads at the ends to remove potential adapter sequences. Single end reads were mapped to release

7 of the Michigan State University Rice Genome (Ouyang, et al., 2007) using Tophat (Trapnell, et al., 2009) with default parameters using the reference guided mode.

**Differential Expression analysis for RNA-Seq datasets**

HTSeq with union count mode was used to obtain raw read counts of each known or predicted gene for the two biological replicates (Anders, et al., 2015). Differential expression analysis was performed by using DESeq2, R package using a model based on negative binomial distribution (Anders, et al., 2010). The counts were normalized by estimating dispersion factors. The normalized count table was used to perform statistical significance tests. Differentially expressed genes with Benjamini and Hochberg-adjusted P-values (FDR) $\leq 0.05$ were selected for functional enrichment studies.

**Functional enrichment tests using rice regulatory network**

Differentially expressed genes (P values $< 0.05$) obtained using DESeq2 were probed against the Rice Regulatory Network (https://plantstress-pereira.uark.edu/RRN/) to obtain co-regulated clusters in the RNA-seq dataset. The regulators predicted using RRN were further probed against RECoN (Krishnan, et al., 2017) to find clusters of highly coexpressed genes and the hierarchical regulatory cascade of biochemical processes involved in the observed phenotypes.

**Quantitative Reverse Transcriptase PCR (qRT-PCR analysis)**

RNA was isolated using Trizol according to the manufacturer's protocol (Life Technologies). A comparative threshold cycle (Ct) method of quantitation was used with the rice Ubiquitin gene as the reference in all cases for quantification of genes through qRT-PCR (Ambavaram, et al., 2011).

**Section 3.4: Results**
**Alignment statistics and Quality metrics of RNA Seq dataset**

To study the global transcriptomic changes associated with overexpression of the *AtSHN2*-TAP construct in rice plants, RNA-seq analysis of wild-type and *AtSHN2*-TAP overexpressing rice transgenic lines was performed. An average of ~24 million reads were obtained for the RNA-Seq dataset per sample. The raw reads were quality checked, filtered and subsequently aligned to release 7 of the *Oryza sativa* reference genome from Michigan State University using TopHat (Trapnell et al., 2009). Among the mapped reads, about 89% of the reads showed unique alignments to the reference genome and the remaining 11% exhibited multi-mapping (Table 1). Concordance between the two biological replicates used for expression analysis for each condition was determined by calculating Pearson correlation coefficient using normalized counts obtained from HTSeq. High reproducibility among biological replicates were observed with a Pearson correlation coefficient of 0.9995 between *AtSHN2*-TAP rice lines replicates and 0.9936 between the wild-type dataset (Figure 1).

**Table 1: Alignment Statistics for RNA-Seq data.** The table represents the total reads obtained for each sample sequenced. The table also enlists the percentage of mapped, multimapped reads and the overall mapping rate as obtained by Tophat alignments.

| | **Total Reads** | **Mapped Reads** | **Multi-mapped Reads** | **Overall Mapping Rate** |
|---|---|---|---|---|
| **WT1** | 24451523 | 22831108 | 10.30% | 89.10% |
| **WT2** | 25775032 | 22938090 | 9.50% | 89.00% |
| ***AtSHN2*-TAP1** | 24042823 | 21662701 | 9.30% | 90.10% |
| ***AtSHN2*-TAP2** | 23117104 | 20521403 | 11.90% | 88.80% |

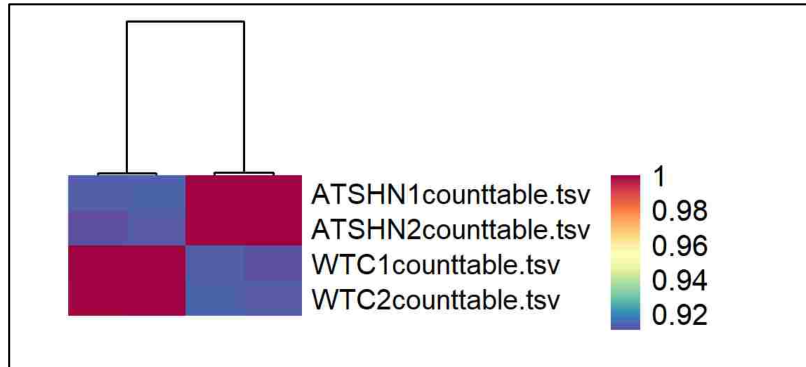


**Figure 1: Heatmap representing Pearson correlation coefficients between biological replicates.** A matrix for counts obtained using HTSeq was computed and the correlation between the counts for each replicate was calculated using Pearson's correlation method in R. High correlation coefficients can be seen between biological replicates belonging to the same condition.

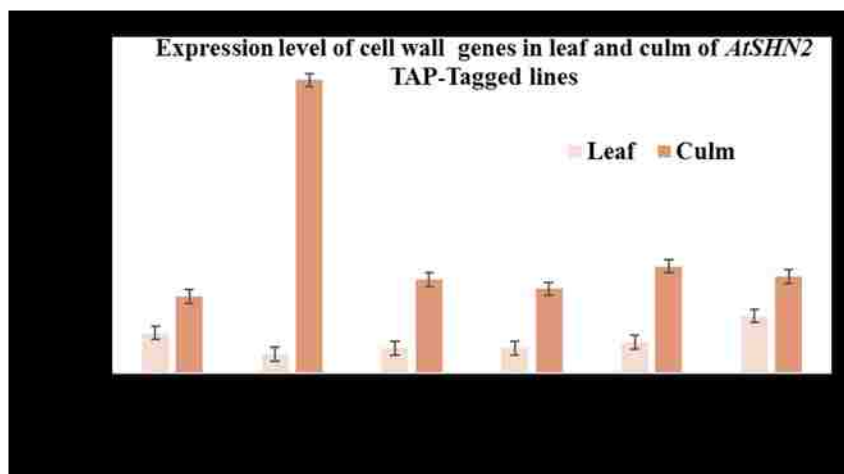


**Figure 2:** Validation of RNA-Seq data by real-time qRT-PCR: Validation of several genes by qRT-PCR enriched in the RNA-Seq dataset from *AtSHN2*-TAP lines by qRT-PCR. Data are expressed as mean relative transcript levels in transgenic lines compared with the wild type (log2 ratio). Error bars represent SE (n = 3; three wild-type and three SHN lines).

**Identification of Differentially Expressed Genes (DEGs)**

DESeq2 was used to call DEGs between wild-type and *AtSHN2*-TAP overexpression lines using a model with a combination of fitted versus per-gene estimates (Supplementary figure 1). False Discovery Rate (FDR) was set to 0.05 to call for DEGs. A total of 11,627 DEGs were

identified at FDR< 0.05 (Supplementary Figure 2). Of these, 812 showed upregulation with a log2fold change (FC) of > 2 while 625 genes exhibited downregulation with a FC of < -2. Of these, 272 genes were expressed only in *AtSHN2*-TAP overexpression lines as evident by zero counts for reads in the wild-type samples for the same genes. Genes belonging to this set included genes such as glycosyl hydrolases (LOC_Os01g71380 FC 2.6, LOC_Os04g51460 FC 1.3); genes involved in the cell wall polysaccharide metabolism (Minic, 2008), DUF domain containing genes (LOC_Os07g42390 (FC 3), LOC_Os02g41840 (FC 2.0), LOC_Os01g60810 (FC 1.96), LOC_Os02g45620 (FC 1.5), LOC_Os01g52730 (FC 1.5); Arabidopsis mutants of which show a marked change in secondary cell wall lignin content (Mewalal, et al., 2016), *OsNAC025* (LOC_Os11g31330 (FC 1.6); orthologous gene from Arabidopsis has been shown to interact with VND7 and regulate xylem vessel formation (Yamaguchi, et al., 2010). Two MYB transcription factors; LOC_Os01g47370 (FC 4.0) and LOC_Os01g16810 (FC 1.8) showed expression only under *AtSHN2*-TAP overexpression. While LOC_Os01g47370 showed no co-expression with genes of known function, it has been reported that LOC_Os01g16810 regulates sugar partitioning in rice for effective pollen development (Zhang, H et al., 2010). *AtSHN2*-TAP overexpression also caused repression of 188 genes which are expressed under the wild-type conditions. Genes completely shut down by *AtSHN2*-overexpression include genes belonging to wall associated kinase (WAK) family (LOC_Os04g29790 (FC -6.1), LOC_Os04g21790 (FC -4.3) and LOC_Os10g09700 (FC -1.9)). WAKs have been implicated in cell expansion by acting as pectin receptors in Arabidopsis (Kohorn, et al., 2012). In rice, WAKs have been shown to regulate defense response against fungal and bacterial pathogens (Delteil, et al., 2016; Harkenrider, et al., 2016). Genes such as cytokinin-o-glycosyl transferase (LOC_Os11g47310 (FC -5.2)), UDP-glycosyl transferase (LOC_Os06g16000 (FC -3.4) and LOC_Os06g39080 (FC -3.4)) also

belonged to this set. Thus, *AtSHN2*-TAP overexpression causes a significant change in the regulation of genes involved in cell wall biosynthesis is rice.

Interestingly, LOC_Os03g54170, MADS-box TF 34, involved in spikelet and inflorescence development in rice exhibited 5-fold upregulation in the *ATSHN2*-TAP transgenic plants. Loss of function mutants of *OsMADs34* have been shown to exhibit an altered inflorescence morphology with lower spikelets and higher number of primary branches (Gao, et al., 2010).

**Table 2: List of genes differentially expressed under AtSHN2-overexpression in rice transgenic lines.** The table represents the Locus ID for the genes, chromosome number, Log2FoldChange as calculated by DESeq2 and the gene symbol the gene.

| Gene | Chr | log2FoldChange | Gene Symbol |
|---|---|---|---|
| **LOC_Os01g71380** | Chr1 | 2.67 | Glycosyl Hydrolase |
| **LOC_Os04g51460** | Chr4 | 1.28 | Glycosyl Hydrolase |
| **LOC_Os07g42390** | Chr7 | 3.07 | DUF581 |
| **LOC_Os02g41840** | Chr2 | 2.02 | DUF584 |
| **LOC_Os01g60810** | Chr1 | 1.96 | DUF623 |
| **LOC_Os01g52730** | Chr1 | 1.50 | DUF584 |
| **LOC_Os02g45620** | Chr2 | 1.49 | DUF623 |
| **LOC_Os01g47370** | Chr1 | 4.08 | MYB |
| **LOC_Os01g16810** | Chr1 | 1.79 | MYB |
| **LOC_Os11g31330** | Chr11 | 1.65 | OsNAC05 |
| **LOC_Os04g29790** | Chr4 | -6.16 | OsWAK40 |
| **LOC_Os04g21790** | Chr4 | -4.29 | OsWAK34 |
| **LOC_Os10g09700** | Chr10 | -1.87 | OsWAK110 |
| **LOC_Os07g30330** | Chr7 | -4.95 | Cytokinin-O-glycosyl transferase |
| **LOC_Os06g16000** | Chr6 | -3.43 | UDP-glycosyl transferase |
| **LOC_Os06g39080** | Chr6 | -3.02 | UDP-glycosyl transferase |

## Functional classification of DEGs

To identify pathways and processes altered by *AtSHN2*-TAP overexpression in rice, DEGs with FDR<0.05 were probed against the Rice Regulatory Network (RRN) (https://plantstress-pereira.uark.edu/RRN/). RRN facilitates functional annotation of high-throughput screening data by finding co-regulated clusters in the expression dataset, that suggest a similar function. At a Q-value threshold of 0.01, enrichment of 21 clusters was seen (Table 3). Of these, 12 clusters had

functional annotations associated with several annotated biological processes and KEG pathways. Among the highly expressed clusters were cl_161 associated with maintaining plant circadian rhythm regulated by LOC_Os01g74020, cl_11 pertaining to pentose and glucose interconversions regulated by LOC_Os11g32100 and cl_114 related to fatty acid metabolism with LOC_Os03g58250 as the central regulator. The downregulated clusters were represented by clusters cl_7 associated with phenylpropanoid metabolism regulated by LOC_Os02g46780 and sulfur metabolism with LOC_Os03g47740 as the regulator and identified by cl_42. (Table 3).

**Table 3: Functional enrichment of DEGs.** Cluster enrichment using RRN reveals significant pathways and predicted regulators. The table represents significant clusters shown by the log2 Fold Change, the biological processes, KEGG pathways and the regulators for the pathway. The overlap column represents the overlap of the regulator with the genes in the cluster.

| Cluster | log2 Fold Change | Biological Processes | KEGG Pathway | Regulator | Overlap |
|---|---|---|---|---|---|
| **cl_5** | 13.17 | Translation | RIBOSOME | LOC_Os02g03730 | 0.98 |
| **cl_161** | 4.899 | signal transmission | CIRCADIAN RHYTHM-PLANT | LOC_Os01g74020 | 1.00 |
| **cl_11** | 4.729 | lipid localization | PENTOSE AND GLUCURONATE INTERCONVERSIONS | LOC_Os11g32100 | 0.95 |
| **cl_2** | 4.543 | photosynthesis | PHOTOSYNTHESIS | LOC_Os03g24590 | 1.00 |
| **cl_6** | 4.395 | microtubule-based movement | DNA REPLICATION | LOC_Os12g25120 | 0.98 |
| **cl_114** | 3.863 | carboxylic acid metabolic process | FATTY ACID METABOLISM | LOC_Os03g58250 | 0.81 |
| **cl_1** | 3.402 | Death | NA | LOC_Os12g03660 | 1.00 |
| **cl_157** | 3.066 | NA | NA | LOC_Os07g40000 | 0.93 |
| **cl_24** | 3.036 | Translation | SPLICEOSOME | LOC_Os09g04720 | 0.99 |
| **cl_8** | 2.845 | ion homeostasis | NA | LOC_Os02g51090 | 0.87 |
| **cl_196** | 2.642 | cellular nitrog compound metabolic process | NA | LOC_Os08g40430 | 0.92 |
| **cl_12** | 2.589 | tRNA metabolic process | NA | LOC_Os01g33370 | 0.87 |
| **cl_89** | -2.609 | NA | NA | LOC_Os02g51300 | 0.96 |
| **cl_30** | -2.763 | regulation of proteinmetabolic process | UBIQUITIN MEDIATED PROTEOLYSIS | LOC_Os05g43950 | 0.94 |
| **cl_199** | -2.889 | NA | NA | LOC_Os03g42820 | 0.96 |

| Table 3 Continued | | | | | |
|---|---|---|---|---|---|
| **Cluster** | **log2 Fold Change** | **Biological Processes** | **KEGG Pathway** | **Regulator** | **Overlap** |
| **cl_141** | -2.945 | NA | NA | LOC_Os10g41260 | 1.00 |
| **cl_42** | -3.182 | NA | SULFUR METABOLISM | LOC_Os03g47740 | 0.98 |
| **cl_13** | -4.221 | cellular component organization | SPLICEOSOME | LOC_Os02g02290 | 0.82 |
| **cl_9** | -4.701 | apoptotic process | HOMOLOGOUS RECOMBINATION | LOC_Os08g32440 | 0.98 |
| **cl_7** | -7.523 | carbohydrate metabolic process | PHENYLPROPANOID BIOSYNTHESIS | LOC_Os02g46780 | 1.00 |
| **cl_135** | -13.224 | NA | NA | LOC_Os06g07350 | 1.00 |

**_AtSHN2_-TAP overexpression leads to altered phenylpropanoid metabolism.**

Functional enrichment of DEGs showed that cluster 7 (cl_7) associated with phenylpropanoid biosynthesis was downregulated with a log2foldchange value of -7.5. cl_7 consisted of 187 genes with KEGG pathways belonging to phenylpropanoid metabolism, phenylalanine metabolism, sugar, and starch metabolism among others (Table 4). Some of the biological processes associated with the cluster include carbohydrate metabolic process, cellulose metabolic process, cellular polysaccharide metabolic process (Supplementary Table 1), suggesting an overall change in cell wall metabolism under *AtSHN2*-TAP overexpression. Manual examination of genes belonging to cl_7 showed that LOC_Os02g41630 (phenyl ammonia lyase), LOC_Os08g34280 (cinnamyl coA-reductase), LOC_Os01g61160 (laccase), LOC_Os06g16350

(peroxidase) exhibited repression in *AtSHN2*-TAP overexpression lines. Furthermore, it was seen that OsCesA4, OsCesA7 and OsCesA9 belonged to the same cluster.

TFs belonging to NAC family namely LOC_Os01g48130, LOC_Os04g43560 and LOC_Os06g0409 showing downregulation in the expression analysis, were enlisted in cluster 7. The ortholog of LOC_Os01g48130 in Arabidopsis is SND2 *(SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 2),* a TF responsible for secondary cell wall development in Arabidopsis fibers (Hussey, et al., 2011) . Ortholog of LOC_Os04g43560 from Arabidopsis, ANAC012 acts as a negative regulator of secondary cell wall biosynthesis. Ectopic overexpression of ANAC012 causes suppression of secondary cell wall formation in xylary fibers (Ko, et al., 2007). It was also seen that ortholog of LOC_Os06g0409 from Arabidopsis, NST1 *(NAC SECONDARY WALL THICKENING PROMOTING FACTOR1),* has been known to interact with SND1 and NST2 for orchestrating secondary cell wall formation in fibers of inflorescence stems (Zhong, et al., 2007).

Another prominent class of TF enriched in the cluster were MYB TFs. It has been known that a NAC-MYB hierarchal TF machinery exists in Arabidopsis for secondary cell wall formation. Eight different MYB TFs were associated with this cluster with LOC_Os02g46780 as the master regulator for the cluster (Table 4). The ortholog of LOC_Os02g46780 in Arabidopsis, *AtMYB58/63* act as transcriptional activators for lignin deposition (Zhou, et al., 2009). Two other MYBs which showed downregulation were LOC_Os01g50720 and LOC_Os05g51160, while LOC_Os01g50720 is a jasmonate responsive MYB responsible for flavonoid synthesis in rice (Ogawa, et al., 2017), LOC_Os05g51160 has a single MYB domain and belongs to MYBS family previously shown to be responsive to sugar sensing in rice (Lu, et al., 2002). Cytoscape display of the cluster exhibits an interconnected co-expressed complex network of genes associated with cluster (Figure 3)

**Table 3: KEGG Pathways associated with cluster 7.** The table presents genes associated with the respective listed pathways and the score (-log q-value) for each pathway

| Cluster | Pathway | # of Genes | Score (-log qvalue) |
|---|---|---|---|
| **cl_7** | PHENYLPROPANOID BIOSYNTHESIS | 68 | 15.25 |
| **cl_7** | PHENYLALANINE METABOLISM | 60 | 9.923 |
| **cl_7** | STARCH AND SUCROSE METABOLISM | 88 | 3.427 |
| **cl_7** | PHENYLALANINE | 31 | 1.42 |

**Table 4: MYB TFs associated with cluster 7 and the corresponding Arabidopsis orthologs.**

| Rice Locus ID | Arabidopsis Ortholog |
|---|---|
| **LOC_Os01g18240** | AT1G09540 |
| **LOC_Os07g44090** | AT1G09540 |
| **LOC_Os08g05520** | AT1G63910 |
| **LOC_Os01g36460** | AT5G26660 |
| **LOC_Os05g04820** | AT1G09540 |
| **LOC_Os01g50720** | AT5G26660 |
| **LOC_Os05g51160** | AT5G47390 |
| **LOC_Os04g50770** | AT1G79180 |

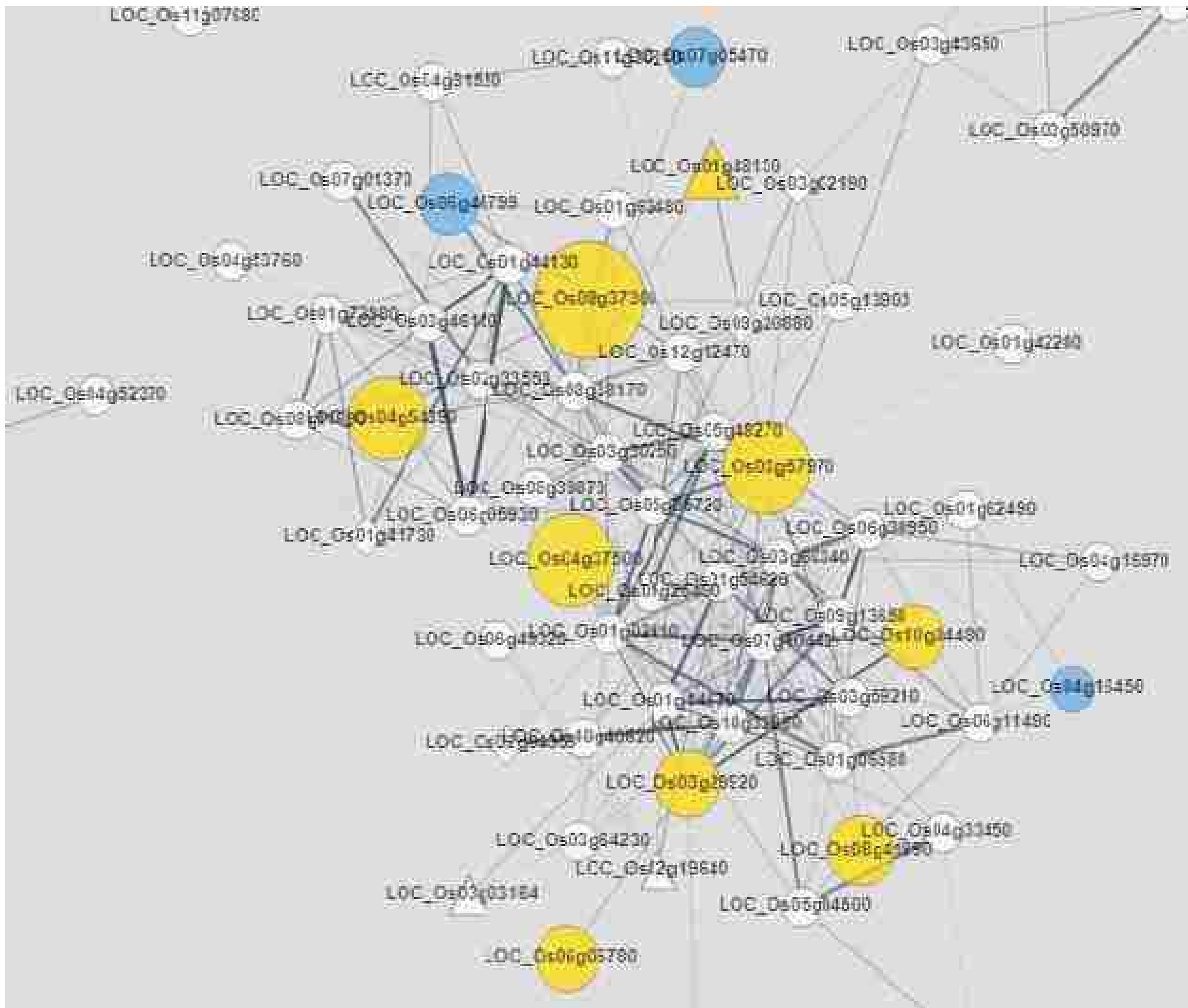

**Figure 3: Cluster 7 associated with phenylpropanoid biosynthesis regulated by *AtSHN2* in rice.** The network displayed in Cytoscape shows genes in blue that are upregulated and genes in yellow are downregulated. The size of the shape exhibits the extent of upregulation/downregulation. Genes co-regulated together relate to each other by edges. The thickness of the edges connecting different genes represent the degree of co-regulation

***AtSHN2*-TAP overexpression positively regulates carbohydrate metabolism.**

Functional classification of DEGs also showed enrichment of genes associated with pentose and glucoronate interconversions represented by cluster 11 with a size of 94 genes and FC value of 4.729. Some other pathways associated with the cluster were Glycolysis/Gluconeogenesis pathway with a FC value of 1.5 and pyruvate metabolism with 1.3 FC indicating a potential change

in t carbon metabolism under *AtSHN2*-TAP overexpression (Table 5). The biological processes enriched in the cluster included lipid localization and metabolic process, macromolecule localization and cell wall organization.

**Table 5: KEGG Pathways associated with cluster 11.** The table presents the number of genes associated with the respective pathways and the score (-log q value) for each pathway

| Cluster | Pathway | # of Genes | Score (-log q-value) □ |
|---|---|---|---|
| **cl_11** | PENTOSE AND GLUCURONATE INTERCONVERSIONS | 20 | 5.735 |
| **cl_11** | TRYPTOPHAN METABOLISM | 13 | 2.596 |
| **cl_11** | PLANT HORMONE SIGNAL TRANSDUCTION | 110 | 2.047 |
| **cl_11** | GLYCOLYSIS / GLUCONEOGENESIS | 74 | 1.550 |
| **cl_11** | PYRUVATE METABOLISM | 49 | 1.370 |

**Table 6: Gene Ontology (GO) enrichments associated with cluster 11.** The table presents the Cluster number, ID for the GO process, number of genes associated with the process, the name of the process and the score (-log q value ) for each pathway.

| Cluster | GO ID | # of Genes | GO Description | Score (-log qvalue) □ |
|---|---|---|---|---|
| **cl_11** | GO:0006629 | 320 | lipid localization and metabolic process | 6.264 |
| **cl_11** | GO:0033036 | 290 | macromolecule localization | 3.576 |
| **cl_11** | GO:0071555 | 46 | cell wall organization | 1.548 |

Functional enrichment of DEGs enlisted TFs potentially acting as central regulator for the pathway. LOC_Os11g32100, a bHlH domain containing protein, with an overlap of 0.95 with all genes in the cluster was predicted as one of the regulators. The TF has been proven to interact with *OsMYBS*, a single DNA-binding repeat MYB transcription factor essential for cold tolerance in transgenic Arabidopsis ((Deng, et al., 2017). Another TF, LOC_Os07g06620, a YABBY

domain containing protein, implicated in feedback regulation of gibberellin metabolism in rice with an overlap of 0.93 was also predicted to be the regulator(Dai, et al., 2007). The list also included LOC_Os09g33580, OsBUL1 COMPLEX1 (*OsBUL1*) with an overlap of 0.8841, encoding a bHLH domain TF activator in rice and responsible for regulating grain size and cell elongation in rice by interaction with *OsBUL1* (*Oryza sativa* BRASSINOSTEROID UPREGULATED 1-LIKE1) (Jang, et al., 2017). Among other significant candidates as central regulators of the pathway are LOC_Os01g66590, a DUF domain containing protein with an overlap of 0.85 and LOC_Os08g33940, an uncharacterized MYB TF, overlap (0.8429) and LOC_Os06g40150, an AP2/ERF TF with an overlap of 0.81 with all the genes in the pathway. LOC_Os06g40150 belongs to the SHINE clade of TF in rice, representing the orthologous function of *AtSHN2* TF in rice cell wall biosynthesis. The expression profiles of these regulators showed upregulation of LOC_Os09g33580 by 3.89-fold and downregulation of LOC_Os07g06620 by 2.1-fold. Interestingly, LOC_Os01g66590, LOC_Os08g33940 and LOC_Os06g40150 are not expressed in *AtSHN2*-TAP overexpression lines, indicating that these genes might be associated with the cluster inherently and not because of *AtSHN2*-TAP overexpression. Biosynthetic gene LOC_Os02g05830 *(OsRBCS1)*, RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 1, was also associated with the cluster with an increase in expression by 1.9-fold.

**Table 7: TF regulators for cluster 11.** The table presents the Cluster number, MSU Locus ID for potential TF regulator, overlap of TF with the pathway, Annotation for TF, RAP ID and the corresponding ortholog from Arabidopsis.

| Cluster | Transcription Factor | overlap | Annotation | RAP ID | Arabidopsis |
|---|---|---|---|---|---|
| **cl_11** | LOC_Os11g32100 | 0.9504 | inducer of CBF expression 1, putative, expressed | Os11g0523700 | AT3G26744 |
| **cl_11** | LOC_Os07g06620 | 0.9281 | YABBY domain containing protein, putative, expressed | Os07g0160100 | AT1G08465 |
| **cl_11** | LOC_Os08g42470 | 0.8889 | BEE 1, putative, expressed | Os08g0536800 | AT5G50915 |
| **cl_11** | LOC_Os09g33580 | 0.8841 | BEE 1, putative, expressed | Os09g0510500 | AT5G50915 |
| **cl_11** | LOC_Os11g04600 | 0.8785 | BTBA5 - Bric-a-Brac, Tramtrack, Broad Complex BTB domain with Ankyrin repeat region | Os11g0141900 | AT2G41370 |
| **cl_11** | LOC_Os01g66590 | 0.8500 | DUF260 domain containing protein, putative, expressed | Os01g0889400 | AT1G65620 |
| **cl_11** | LOC_Os08g33940 | 0.8429 | MYB family transcription factor, putative, expressed | Os08g0437300 | AT1G74650 |

**Figure 4: Cluster 11 associated with carbohydrate metabolism regulated by AtSHN2 in rice.** The genes in blue are upregulated while the yellow genes are downregulated. The node is proportional to fold change while the edge thickness is proportional to co-expression score

***AtSHN2*-TAP overexpression leads to expression of genes related to carboxylic acid organic acid metabolic process**

Cluster 114 associated with fatty acid metabolism with an overall increase in fold change by 3.8 and central regulator LOC_Os03g58250 was also enriched. The cluster consists of 26 genes with GO processes such as carboxylic acid metabolic process and organic acid metabolic process connected with the cluster (Table 8). LOC_Os03g58250 *(OsbZIP33/ REB rice endosperm basic leucine zipper)* belongs to bZIP transcription factor family and has been characterized to function in starch biosynthesis in rice by interacting with the promoter elements of *Wx (Waxy)* and *SBE1 (Starch branching enzyme I)* (Cai, et al., 2002). While the *Wx* gene, also known as *OsGBSSI* encodes ADP-glucose starch glycosyl transferase is responsible for synthesis of amylose in rice (Sano, et al., 1985), and *OsSBE1* catalyzes the formation of amylopectin (Kawasaki, et al., 1993). Interestingly the cluster also contains TFs such as LOC_Os11g03300 *(OsNAC10)*, a NAC TF and LOC_Os01g64730 *(OsABF1)*, a bZIP TF both increased in expression by 1.2-fold under *AtSHN2*-TAP overexpression. *OsNAC10* has been shown to be involved in stress tolerance in rice. *OsNAC10* is expressed in both roots and panicles and the gene is inducible by stresses such as drought, high salt and ABA (Jeong, et al., 2010)**.** *OsABF1* is known to mediate drought-derived signal for delayed flowering time in rice. Drought induces the expression of *OsABF1* and *OsWRKY104*, which in turn causes the activation of a repressor of *Ehd1* thus causing delay of heading date in rice (Zhang, et al., 2016). It has also been reported that *OsABF1* can be induced by D-allose, a C-3 epimer of glucose primarily through the enzyme hexokinase (Fukumoto, et al., 2013). LOC_Os02g44990, F-box and DUF domain gene (*MAF1)*, is a sucrose responsive gene and also induced by plant hormones such as cytokinin and auxin, thus manifesting a role in multiple plant signaling pathways (Yan, et al., 2017). Biosynthetic genes such as malate synthase

(LOC_Os04g40990) and acyl-coenzyme A dehydrogenase (LOC_Os05g03480) also belong to the same cluster and were found upregulated in the RNA-expression analysis. Thus *AtSHN2*-TAP overexpression causes perturbation in expression of many key genes related to carbon biosynthesis and sugar signaling in rice.

**Table 8: Gene Ontology (GO) enrichment associated with cluster 114.** The table presents the Cluster number, ID for the GO process, number of genes associated with the process, the name of the process and the score (-log q value) for each pathway.

| Cluster | GO ID | # of Genes | GO Description | Score (-log qvalue) |
|---|---|---|---|---|
| **cl_114** | GO:0019752 | 296 | carboxylic acid metabolic process | 1.555 |
| **cl_114** | GO:0006082 | 296 | organic acid metabolic process | 1.555 |
| **cl_114** | GO:0043436 | 296 | oxoacid metabolic process | 1.553 |
| **cl_114** | GO:0042180 | 299 | cellular ketone metabolic process | 1.548 |

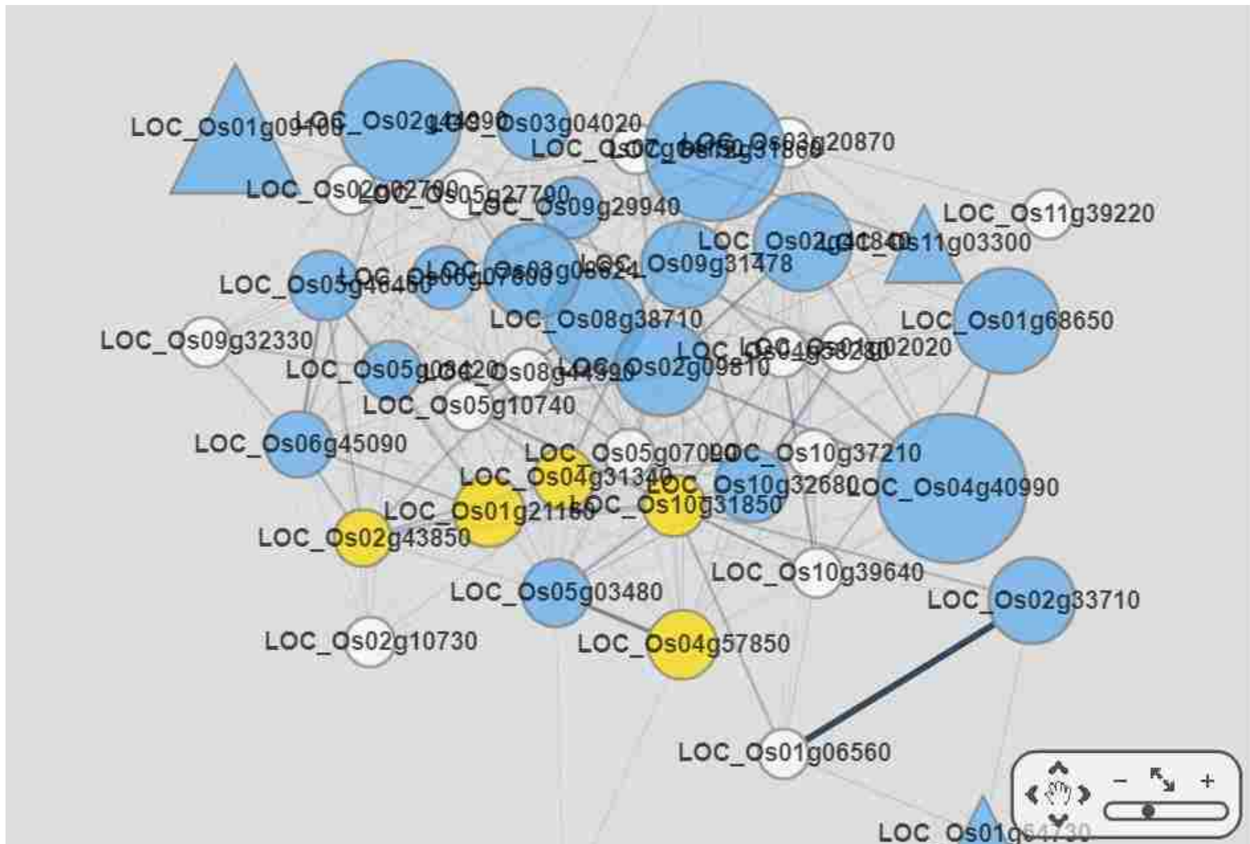


**Figure 5: Cluster 114 associated with carboxylic acid metabolism regulated by *AtSHN2* in rice.** The genes in blue are upregulated while the yellow genes are downregulated. The node size is proportional to fold change while the edge thickness is proportional to co-expression score

**Gene expression profiling of genes directly regulated by *AtSHN2*.**

To determine the direction of regulation of genes under direct regulation by *AtSHN2*, RNA-expression profile of genes enriched in ChIP-Seq experiment was examined (Refer to Chapter 2). To this end, the genes enriched from the ChIP-Seq dataset were classified based on their functions and divided into three categories namely Transcription factors, Kinases, and Biosynthetic genes. This dataset was intersected with DEGs obtained through DESeq2 analysis (Figure 4) to obtain expression profile of these genes. It was found that 476 genes that were directly and differentially expressed by *AtSHN2*. The genes can be further sub-categorized into 40 TFs, 21 kinases and 416 biosynthetic genes.

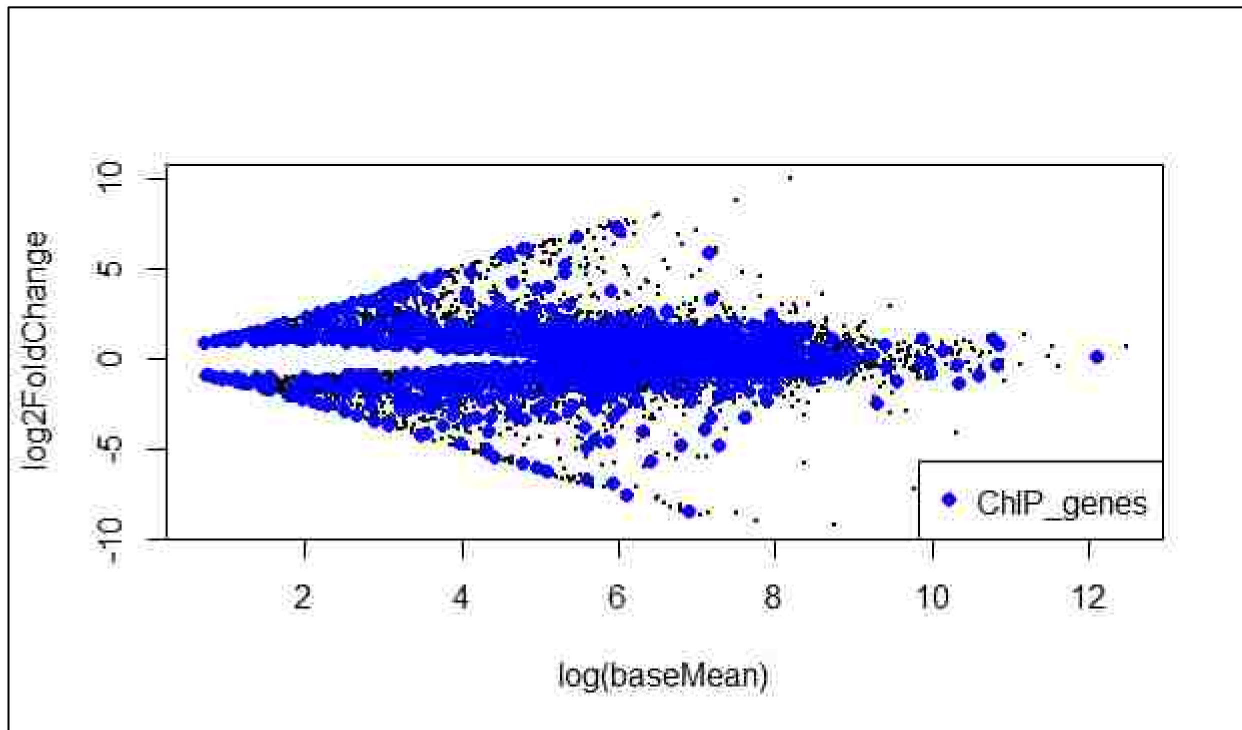


**Figure 6. Integration of ChIP-Seq with DEGs**. The graph represents the overlay of genes enriched by ChIP-Seq analysis with DEGs. The plot represents all the DEGs log(baseMean) vs log2Fold Change. The black dots represent all the DEGs with p value < 0.05 and the blue circles represent the genes enriched by ChIP-Seq analysis

**Direct differential expression of a suite of TFs mediated by *AtSHN2* results in altered cell wall deposition in rice.**

Hierarchical transcriptional regulation exists in plants to regulate the deposition of cell wall components. A NAC-MYB machinery is involved in the orchestration of the process. Integration of *AtSHN2*-TAP ChIP-Seq results with RNA-Seq dataset revealed 40 TFs directly and differentially regulated by *AtSHN2*, although only 11 of them had fold change >1 or fold change < -1. (Supplementary Table 1). Among these, MYB TFs namely LOC_Os01g63160 and LOC_Os12g07640 were observed. While the expression of LOC_Os01g63160 was increased by 2-fold, LOC_Os12g07640 was downregulated by 1.2-fold (Supplementary Table 1). While LOC_Os01g63160 has not been characterized yet, the ortholog of LOC_Os12g07640, *AtMYB4* from Arabidopsis has been shown to involved in phenylpropanoid biosynthesis. *AtMYB4* targets the expression of cinnamate 4-hydroxylase by repressing the repressor of the gene (Jin, et al., 2000). Interestingly, *AtSHN2* also caused direct repression of LOC_Os09g25060, *OsWRKY76*. Double knock downs of *OsWRKY76* and *OsWRKY62 s*how increased accretion of defense compounds and upregulation of genes involved in phenylpropanoid biosynthesis namely 4-coumarate:CoA ligase and cinnamate CoA ligase (Liang, et al., 2017). 4-fold direct repression of LOC_Os06g01890, a MADS-box TF was also seen. The function of LOC_Os06g01890 however remains unknown.

Other TFs directly and differentially regulated by *AtSHN2* include LOC_Os12g07640, uncharacterized MYB TF and LOC_Os03g58350 (*OsIAA14*). Moreover, LOC_Os03g44710 (*OsSh1*), a seed shattering gene was also directly downregulated by *AtSHN2*. (Supplementary Table 1)

**A complex network of kinases and biosynthetic genes regulated by *AtSHN2* causes altered cell wall biosynthesis.**

*AtSHN2* directly causes upregulation of LOC_Os08g34240 and LOC_Os05g16430 both kinases by more than 1-fold. Although functions of both remain unknown in rice, LOC_Os08g34240 showed strong association with LOC_Os07g05370, a CrRLK1L receptor-like kinase, a cell wall associated kinase, orthologs of which act as signal transmitters for biotic and abiotic stress in Arabidopsis (Lindner, et al., 2012). More than 4-fold repression of LOC_Os11g46950 was observed. LOC_Os11g46950 while uncharacterized showed strong association with LOC_Os03g03540, a NAC TF; orthologs of LOC_Os03g03540 have also been implicated in secondary cell wall biosynthesis (Zhong, et al., 2015).

Other genes in the dataset include LOC_Os06g36560 *(OsMIOX)*, a myo-inositol oxygenase upregulated by 4-fold. Although *OsMIOX* has been shown to be drought responsive, predominantly in scavenging reactive oxygen species (Duan, et al., 2012), myo-inositol oxygenases are also involved in the biosynthesis of UDP-Glucuronic acid, substrates for biosynthesis of cell wall polymers (Kanter, et al., 2005). LOC_Os04g43410, a beta-glucosidase gene manifested 2-fold direct upregulation, members of the gene family shown to be involved in cell wall metabolism in rice (Opassiri, et al., 2006). Interestingly, LOC_Os04g43410 also showed strong association with LOC_Os12g17600 *(OsRBSC2)*, RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 2, the photosynthetic enzyme involved in carbon fixation. LOC_Os11g47570, a glycosyl hydrolase was completely shut down under *AtSHN2*-TAP overexpression. Downregulation of LOC_Os02g41650, *OsPAL-4*, phenylalanine ammonia-lyase gene (PAL) was also seen. PAL catalyzes the breakdown of cinnamic acid thus facilitating the first and committed step towards the phenylpropanoid pathway (Yoon, et al., 2015). These examples

of known genes show that *AtSHN2* modulates the expression of various kinases and biosynthetic genes directly thus mediating a complex network for regulating cell wall biosynthesis in rice.

**_AtSHN2_-TAP causes direct expression of genes involved in cuticular wax biosynthesis in rice**

*AtSHN2* has been implicated in the biosynthesis of wax and cutin in Arabidopsis (Aharoni et al., 2004). Recently, reports have surfaced showing that orthologs of *AtSHN2* can induce cuticle and wax production in tomato (Shi, et al., 2013), apple (Lashbrooke, et al., 2015) , wheat (Jäger, et al., 2015) and soybean (Xu, et al., 2016). To explore if *AtSHN2* overexpression causes any significant changes in the genes related to wax biosynthesis in rice, the expression profiles of genes in the pathway were also examined. This revealed that LOC_Os09g25850 (*OsGL1)*, a biosynthetic gene, containing domains homologous to sterol desaturases and short chain dehydrogenases was a putative direct target of *AtSHN2*. The gene was upregulated by 2-fold as seen by RNA-expression analysis. Mutants of *OsGL1* exhibit decreased wax deposition and increased sensitivity to drought (Qin, et al., 2011). Also, LOC_Os01g48874, an uncharacterized putative wax biosynthetic gene was also one of the direct targets of *AtSHN2*.

## Section 3.5: Discussion

RNA-seq mediated profiling of gene expression is a powerful tool to mine gene expression and functions. In this study, the technique is used in combination with ChIP-Seq results obtained from Chapter 2 to study genome wide changes observed due to *AtSHN2*-TAP overexpression in rice. DESeq2 enabled identification of differentially expressed genes in association with functional annotation by rice regulatory network enabled the identification of co-regulated clusters regulated by putative TFs.

*AtSHN2*-TAP rice lines have reduced lignin (Chapter 2). These results were corroborated by expression patterns of DEGs identified from *AtSHN2*-TAP lines as many lignin biosynthetic genes such as *OsPAL*, *CCR*, and laccases exhibited down-regulation in *AtSHN2*-TAP rice lines. This result was further substantiated with functional annotation of the DEGs since KEGG pathways and biological processes associated with the phenylpropanoid pathway exhibited downregulation (Table 3).

Pentose-glucuronate interconversion pathway is a subset of central carbon metabolism in plants. Interestingly, all the carbon required for cell wall biosynthesis comes from photosynthetically fixed carbon. UDP-glucose phosphorylase or sucrose synthase can synthesize UDP-glucose which acts a substrate for cellulose biosynthesis in plants. Since an increase in the carbohydrate metabolism and photosynthesis is seen in *AtSHN2*-TAP rice transgenic lines, it is hypothesized that a continuous supply of carbon is created by the plant, initiating a feed forward loop for glucose biosynthesis in rice. It was seen that sugar sensing genes such as LOC_Os01g16810 and LOC_Os05g51160 were differentially expressed in *AtSHN2*-TAP rice lines. We propose that *AtSHN2* overexpression results in overexpression of sugar sensing genes, which help in sugar partitioning in rice thus preventing the plant from high sugar induced feedback inhibition. This is further corroborated by functional association of the *AtSHN2* ortholog from rice, LOC_Os06g40150 (*OsSHN2*) as one of the potential regulators of the pentose-glucoronate interconversion pathway associated with carbohydrate metabolic processes in rice.

In addition, integration of *AtSHN2*-TAP ChIP-Seq with RNA-Seq results reveal about 41 TFs and 146 biosynthetic genes are directly and differentially expressed by *AtSHN2*. *AtSHN2* also directly regulates the expression of many key TFs such as LOC_Os01g63160 and *OsWRKY76*, both known to be involved in phenylpropanoid biosynthesis in rice. Moreover, the results show

that *AtSHN2* potentially binds to *OsPAL2 t*hereby inhibiting its expression. Thus, *AtSHN2* directly causes the suppression of key TFs and biosynthetic genes responsible for phenylpropanoid biosynthesis thereby achieving the phenotype of decrease in lignin. Two MYB TFs, LOC_Os01g63160 and LOC_Os12g07640 (Supplementary Table 1) were identified in this study. It is noteworthy that the ortholog of LOC_Os12g07640 is involved in repression of phenylpropanoid biosynthesis in rice (Jin, et al., 2000). Gene interaction network analysis of LOC_Os01g63160 showed that the gene is co-expressed with auxin-signaling F-box proteins indicating a potential role of hormonal interplay of *AtSHN2* induced cellulose biosynthesis (Supplementary Figure 3). These genes can serve as potential candidates to further our knowledge of cell wall biosynthesis in rice.

*AtSHN2*, an AP2/ERF TF has been involved to enhance performance of plants to drought tolerance by increasing wax and cutin biosynthesis in multiple plant species. Direct upregulation of key wax biosynthetic genes such as *OsGL1*-1 and LOC_Os01g48874 may be one of the mechanisms for higher wax modulation of *AtSHN2* rice lines. However, the plants were not phenotypically assessed for wax content. *AtSHN2*-overexpression also caused upregulation of TFs such as *OsNAC10* and *OsABF1*, both involved in drought response in rice (Jeong, et al., 2010; Zhang, et al., 2017) .

**Summary**

Cell wall biosynthesis is one of the most complex processes in rice as it involves a wide and diverse interplay between TFs, hormones, proteins etc. *AtSHN2* is known for its role in modulating cell wall biogenesis in rice. In this study, we present an integrative approach to identify direct and indirect targets, shown by differentially regulated genes in response to *AtSHN2*

overexpression in rice, thus providing a top down view of cell wall formation in rice. We also reveal ~30 uncharacterized TFs which might have a putative role in rice cell wall biogenesis.

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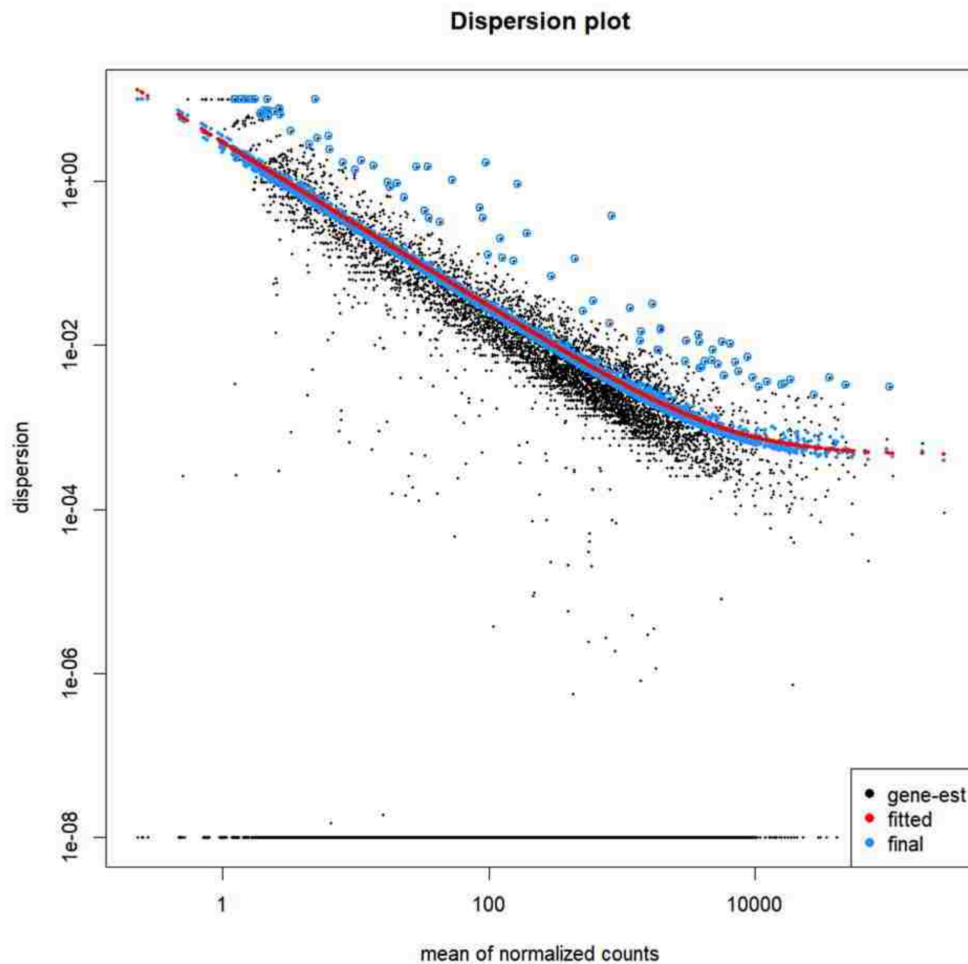
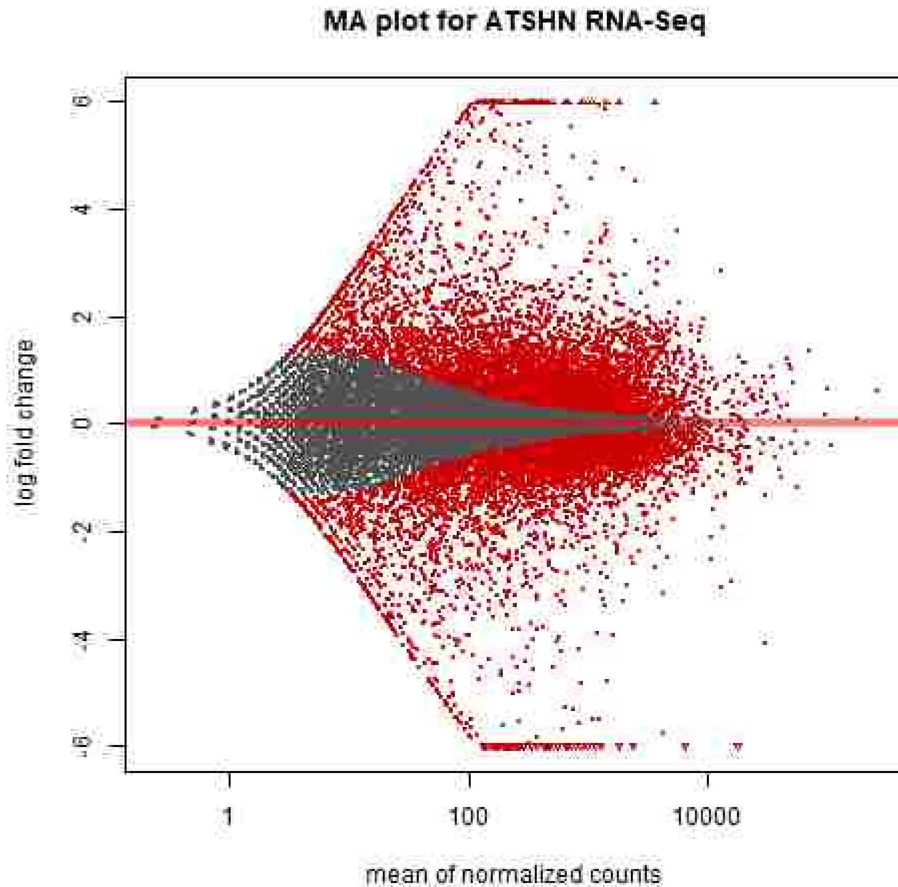
**Section 3.6: Supplementary Figures and Tables.**




**Supplementary Figure 1: DESeq2 dispersion values plotted against mean of normalized counts for RNA-Seq dataset**. The black dots represent the dispersion values for gene-wise

estimates, the red line represents the fitted estimates while the blue dots represent the final estimates shrunk from the gene-wise estimates towards the fitted estimates.




**Supplementary Figure 2: MA plot between *AtSHN2*-TAP overexpression lines and wild-type samples.** The y axis represents the log-fold change (The M values are the log of the ratio between the counts for *AtSHN2*-TAP vs wild-type). The x-axis represents the mean of normalized counts (A-values) for each gene across the two genotypes.

## CHAPTER 4: Investigating the role of *OsSHN2* in cell wall biosynthesis in rice

### Section 4.1: Abstract

Cell wall biosynthesis is a highly coordinated process involving multiple transcription factors and genes interacting at different levels. The regulation of these transcription factors and biosynthetic genes at different times, tissues, and response to internal and external signals results in the changing and adaptive nature of the growing cell wall. Plant cell wall biosynthesis has been studied at the genetic level extensively in Arabidopsis with recent advancements in rice. In this study, we show the regulation of cell wall biosynthesis in rice (Oryza sativa) by a AP2 (APETALA 2) transcription factor, *OsSHN2*. To discover the multiple roles of *OsSHN2* in rice, *OsSHN2* co-suppressed transgenic lines were developed and characterized. Phenotypic analysis was correlated with global gene expression studies, showing induction of lignin and down regulation of cellulose biosynthetic genes. To study the intrinsic role of *OsSHN2* in rice, RNA was isolated from diverse rice germplasm and the expression of *OsSHN2* and its putative targets were quantified. Results from the study demonstrate the role of *OsSHN2* in regulating cell wall biosynthesis and potentially stalk strength in rice germplasm. The results will help understand the complex nature of the cell wall biosynthesis process and the details by which it adapts to changing developmental and environmental cues.

### Section 4.2: Introduction

The plant cell wall is primarily made of polysaccharides, comprising varying amounts of cellulose, hemicellulose, pectin, and the polyphenolic compound lignin that is characteristic of the secondary cell wall. The deposition of cell wall imparts the cell with properties such as thickness, rigidity and hydrophobicity and hence envisioned as an important layer involved in the transition of plants from aquatic to terrestrial habitat. Cell wall biosynthesis is a very complex process

involving many transcription factors (TFs) and proteins interacting at various cellular and molecular levels. In Arabidopsis, approximately 1000 genes have been predicted to be involved in biosynthesis of cellulose, lignin, and hemicellulose. These genes include a suite of transcription factors, biosynthetic enzymes, transporters, and/or accessory proteins. A recent study using protein-DNA interaction analysis revealed an elaborate network of genes involved in cell wall biosynthesis in Arabidopsis under abiotic stress (Taylor-Teeples et al., 2015).

Rice is one of the major staple food crops grown in the world with a huge biomass potential. Extraction of fermentable sugars from the cell wall is hampered by the presence of lignin. Studies towards reducing lignin formation have been attempted but these plants typically met with negative effects on plant growth and stature. Previous studies from our research group have shown that *AtSHN2* when overexpressed in rice causes coordinated regulation of cellulose and lignin, with increased cellulose and reduced lignin and no negative effects on plant growth and stature. This model of compensatory behavior between cellulose and lignin was therefore examined in different ways within the research presented here, to characterize the regulation of lignocellulose changes at a systems level.

In this study, we investigated the role of *OsSHN2*, the ortholog of *AtSHN2* from Arabidopsis (Aharoni et al., 2004) in cell wall formation in rice. Although members of the family regulate epicuticular wax biosynthesis and impart properties of drought tolerance in multiple plant species, their role in cell wall formation has not been characterized yet. We demonstrate here that suppression of *OsSHN2* causes an increase in phenylpropanoid metabolism, with a decrease in glycan formation and carbohydrate metabolism in rice lines, thus causing poor growth with *OsSHN2* as the putative central regulator for pentose-glucuronate conversion. The results provide

insights into a more central role of *OsSHN2* and family members, and provide a new model for cell wall regulatory mechanism in rice.

## Section 4.3: Materials and Methods

### Plant growth conditions and phenotyping

The *OsSHN2* construct for overexpression of LOC_Os06g40150 under control of the CaMV35S promoter was transformed into O. sativa ssp japonica cv Nipponbare. $T_1$ seeds for two different transgenic lines (OsSHN1 and OsSHN21) were obtained from Dr. Niranjan Baisakh's laboratory (LSU-AgCenter). *OsSHN2* T2 progeny lines were grown at University of Arkansas and the expression checked by qRTPCR. All plants were also checked for the presence of CAMV35S promoter. Based on expression analysis of the above two transgenic lines, the progeny of two T2 lines were chosen for further experiments. The *OsSHN2* plants were first grown in growth chambers 28ºC±1 day and 25ºC±1night temperature, 65% relative humidity (RH) and 14 h light and 10 h dark, and then transferred to the greenhouse. 30-day old culm sections were used for scanning electron microscopy (SEM) as detailed in Chapter 2.

### Isolation of RNA and sequencing

The RNeasy Mini Kit (Qiagen, USA) was used to isolate total RNA from 35S:*OsSHN2* lines. Following quality check of the isolated RNA by the 2100-Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), the samples were sent to the Genomics Core Facility at Michigan State University for RNA-sequencing.

**Differential gene expression profiling by DESeq2 and functional enrichment analysis of genes**

Raw data from the different RNA-seq data sets were quality checked by FastQC (Andrews, n.d.). The reads were mapped to release 7 of the Michigan State University Rice Genome (Ouyang et al., 2007), using Tophat (Trapnell, Pachter, & Salzberg, 2009) with the reference guided mode. Reads were converted to counts using HTSeq (Anders, Pyl, & Huber, 2015). DESeq2 was used to identify differentially expressed genes (Anders & Huber, 2010). Normalization of the counts was performed by estimating dispersion factors. Differentially expressed genes with Benjamini and Hochberg-adjusted P-values (FDR) $\leq 0.05$ were selected for functional enrichment studies. Functional enrichment of differentially expressed genes was performed using the Rice regulatory network (https://plantstress-pereira.uark.edu/RRN/), developed by Dr. Chirag Gupta, to identify co-regulated clusters in the RNA-seq dataset.

**Osmotic stress studies on MS media**

The T3 generation of plants were used for osmotic stress experiments. The seedlings were transferred to half-strength MS media and supplemented with 3 µM ABA, 100 mM Nacl, 200 mM NaCl, PEG 1000 and 4ºC for cold stress. After a week, the plants were removed from the media, and RNA was isolated from shoot samples of WT and transgenic lines. Students t-test was used to perform the statistical significance of the differences in the means for WT and transgenic lines under control and stress conditions.

**Quantitative Reverse Transcriptase PCR (qRT-PCR) analysis**

Trizol mediated isolation of RNA was done according to manufacturer's protocol (Life Technologies). A comparative threshold cycle (Ct) method of quantitation was used with Ubiquitin

gene as the reference in all cases for quantification of genes through qRT-PCR (Ambavaram & Pereira, 2011).

**Section 4.4: Results**

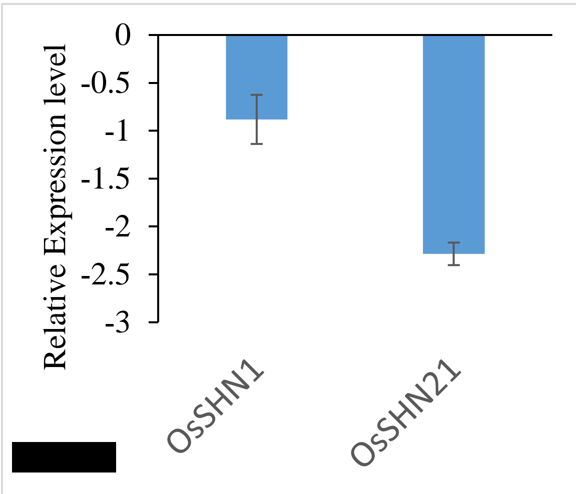
**Phenotypic Characterization of 35S:*OsSHN2* lines**

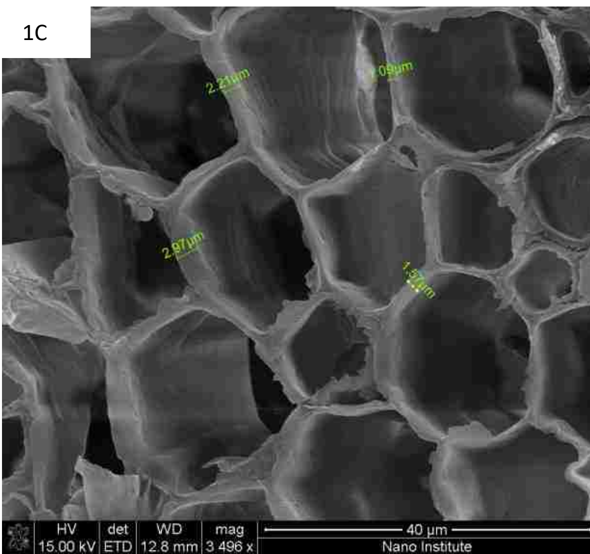
To characterize the role of *OsSHN2* in rice, 35S:*OsSHN2* overexpression lines were generated, and two independent lines characterized. Transcript level of *OsSHN2* were quantified by qPCR. Surprisingly, it was found that *OsSHN2* was downregulated in both these lines (Figure 1A) indicating that supraoptimal expression of *OsSHN2* leads to negative regulation of *OsSHN2*.

Phenotypically *OsSHN2* 'co-suppressed' lines exhibited reduced plant height during the entire plant lifecycle as comparted to WT (Figure 1B). These plants also showed thin rolled leaves, a slight increase in tiller numbers, and overall reduced biomass (Supplementary Figure 1-3). Further, the plants exhibited thinner culms. Scanning electron microscopy of culm sections from transgenics revealed that *OsSHN2* exhibited reduction in cell wall thickness (Figure 1C). Taken together these data indicated a potential role of *OsSHN2* in overall plant growth and development in *OsSHN2* transgenic lines.

*AtSHN2*, an ortholog of *OsSHN2*, causes increase in cellulose biosynthesis with a concomitant decrease in lignin biosynthesis when overexpressed in rice (Ambavaram et al., 2011). Since 35S:*OsSHN2* lines had thinner cell walls, we speculated that perturbation in OsSHN2 expression causes change in expression of cell wall biosynthesis genes. To this end, qRT-PCR mediated quantification of genes related to cell wall biosynthesis was performed. It was observed that the expression of secondary cell wall cellulose biosynthesis genes such as OsCesA7 and OsCESA9 was repressed in 35S:*OsSHN2* lines with an expression of lignin biosynthetic genes such as *Os4CL*-3, *OsCAD2* and *Os4CL*-4 (Figure 1D), suggesting that *OsSHN2* repression

exhibited in 35S:OsSHN2 co-suppressed lines causes a change in the expression of cell wall related genes in rice.





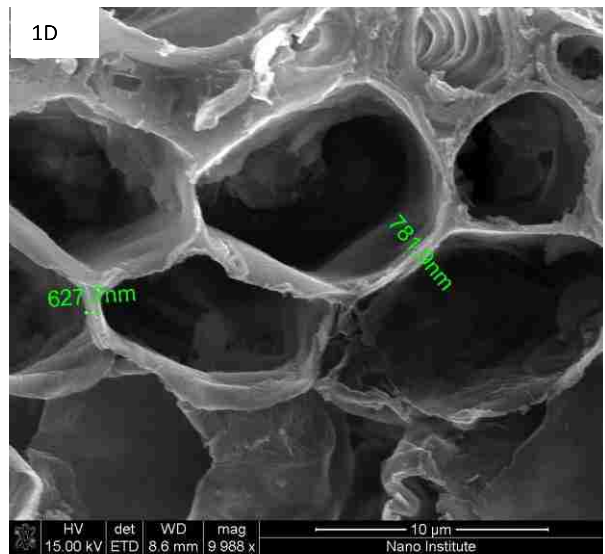




**Figure 2: Phenotypic characterization of 35S:OsSHN2 lines:** (1A) Quantification of *OsSHN2* transcripts through qRT-PCR reveal downregulation of *OsSHN2* in *OsSHN2* lines. (1B) *OsSHN2* lines exhibit stunted size and reduced plant growth at mature stage as compared to WT. (1C) and (1D) Scanning electron microscopy of transverse sections of WT (scale bar: 40 µm) and *OsSHN2* (scale bar: 10 µm) respectively. SEM analysis reveal *OsSHN2* has reduced cell wall thickness as compared to WT plants.

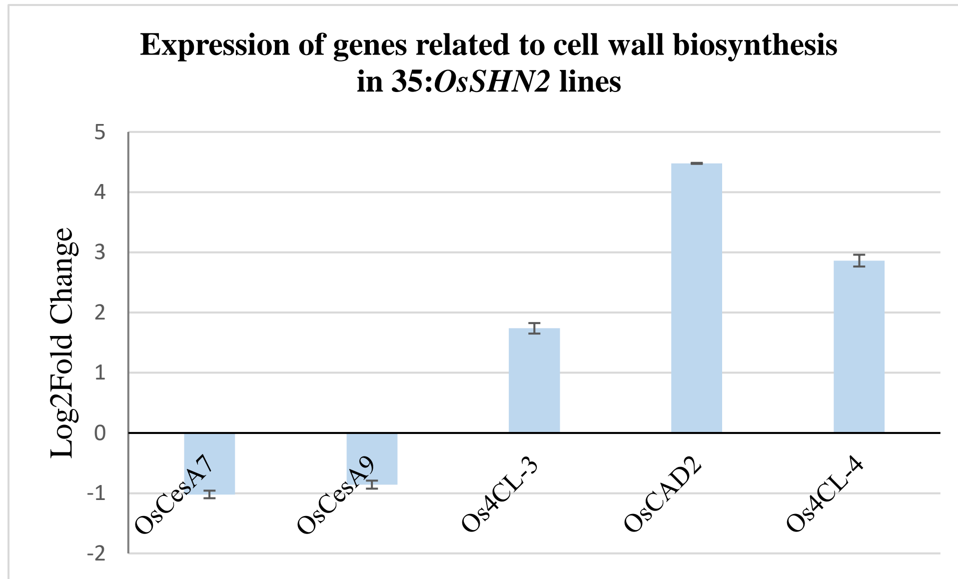



**Figure 2: Relative expression levels of cell wall related genes from 35s:*OsSHN2* lines.** Data are expressed as mean relative transcript levels in *OsSHN2* lines compared with the wild type (log2 ratio). Error bars represent SE (n = 3; three wild-type and three SHN lines).

**Alignment and Quality metrics of RNA-Seq data**

To study global transcriptomic changes in 35S:*OsSHN2* rice transgenic lines, RNA-Seq analysis of 35S:*OsSHN2* lines and WT plants (n=2 for each) was performed. Alignment of the Fastq reads to release 7 of Oryza Sativa reference genome from Michigan State University was performed using Tophat. Alignment statistics are enlisted in Table 1. About 25 million reads were obtained for each sample. The overall mapping rate was ~89% for WT plants and ~88% for 35S:*OsSHN2* lines. The concordance between the replicates was calculated by determining the Pearson correlation co-efficient by using normalized counts obtained from the reads using HTSeq-counts and It was observed that the correlation co-efficient between WT plants was 0.9995 and between 35S:*OsSHN2* replicates was 0.9993 (Figure 3) proving high reproducibility between the two biological replicates for each condition.

**Table 1: Alignment Statistics for RNA-Seq data.** The table represents the total reads obtained for each sample sequenced. The table also enlists the percentage of mapped, multimapped reads and the overall mapping rate as obtained by Tophat alignments.

| | **Total Reads** | **Mapped Reads** | **Multi-mapped Reads** | **Overall mapping Rate** |
|---|---|---|---|---|
| WT1 | 24451523 | 22831108 | 10.30% | 89.10% |
| WT2 | 25775032 | 22938090 | 9.50% | 89.00% |
| *OsSHN1* | 23956299 | 21048127 | 9.5% | 87.9% |
| *OsSHN2* | 25642869 | 22831108 | 21.0% | 89.0% |

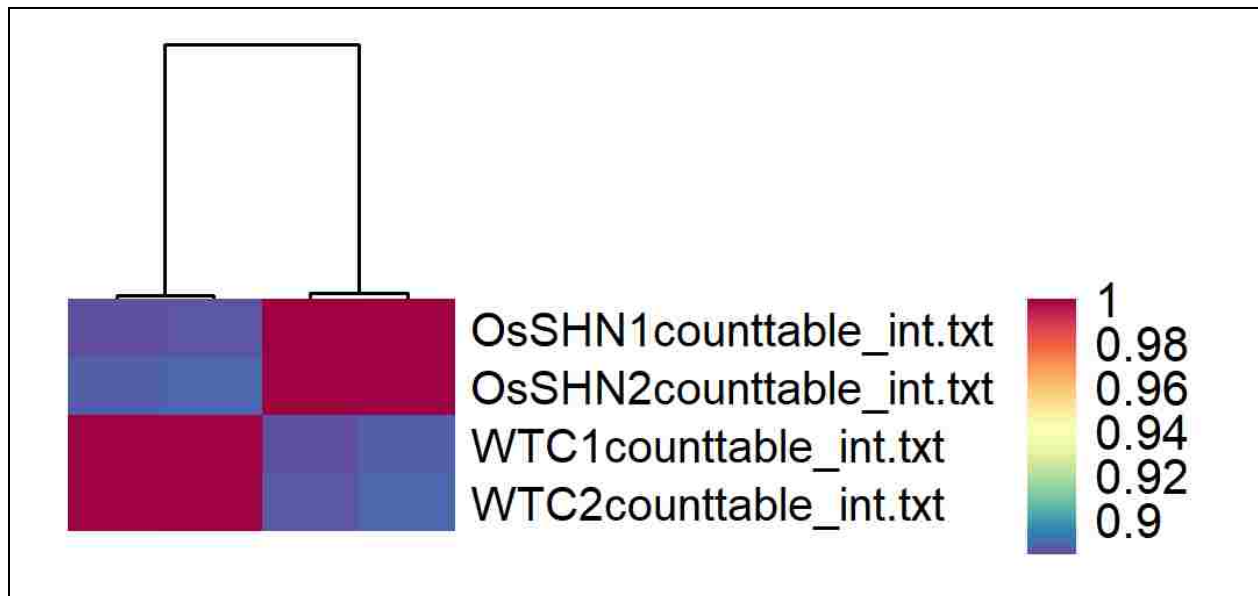



**Figure 3: Heatmap of correlation coefficients between the replicates for each condition.** Mapped reads obtained using TopHat were converted to counts by HTSeq. Pearson's correlation coefficient was computed between counts obtained for each condition post normalization. High correlation co-efficient between normalized counts can be seen for the replicates belonging to same condition

**Identification of Differentially Expressed Genes (DEGs)**

For calling DEGs, DESeq2 was used. DESeq2 performs differential gene expression analysis using a negative binomial generalized linearized model to calculate dispersion estimates and log2FoldChange based on prior distribution of the data (Love, et al., 2014). In total, 11,790 genes were found to be expressed using a cut-off FDR < 0.05. Of these, 662 genes were upregulated with FC >2 and 359 genes were downregulated with FC < -2. Of the 662 genes, 106 genes were expressed only in 35S: *OsSHN2* lines as evidenced by zero counts for the same genes in WT plants. Also, 38 genes out of 359 genes were completely shut down in 35S: *OsSHN2* rice lines.

Genes exclusively expressed in 35S:*OsSHN2* lines include LOC_Os07g48560 *(OxWox11)*, a homeobox domain containing TF involved in plant developmental response and responsive to hormonal signaling and abiotic stress (Cheng, et al., 2014), LOC_Os02g46970 *(Os4CL2)*, a 4-coumarate ligase gene responsible for monolignol biosynthesis in rice (Sun, et al., 2013). LOC_Os12g37690, an uncharacterized MYB was also upregulated by 3-fold in 35S:*OsSHN2* lines. 2.3-fold upregulation of LOC_Os02g15350 (*OsDof3),* a pyrimidine box-binding protein was seen in 35S:*OsSHN2* lines. OsDof3 interacts with GAMYB and regulates the expression of GA responsive genes in rice (Washio, 2003). Among the genes whose expression was completely shut down by *OsSHN2* misregulation were LOC_Os06g06300 *(OsRFT1)*, a florigen gene essential for flowering pathway in rice (Komiya, et al., 2009) and LOC_Os01g60770, an uncharacterized expansin gene. Members of expansin gene family have been attributed to regulate various functions in plant cell wall remodeling, fruit ripening and germination (Marowa, et al., 2016). Moreover, LOC_Os03g40720, UDP-glucose 6-dehydrogenase 2 *(UGD2)* gene was shut down by *OsSHN2* misregulation by 3.29-fold. *UGD2* is involved in the biosynthesis of UDP-glucuronic acid (UDP-GlcA), which acts a substrate for synthesis for cell wall polymers (Klinghammer, et al., 2007). LOC_Os02g40530 *(OsMPS*), a MYB TF was also repressed in 35S:*OsSHN2* lines. *OsMPS* is involved in the regulation of plant cell wall regulation by regulating expansins and endoglucanase genes. In addition, *OsMPS* is responsive to multiple hormones such as Auxins, gibberellins (GA) and brassinolides (Schmidt, et al., 2013). LOC_Os06g06320 *(hd3a)* encoding HEADING DATE 3A was also suppressed in 35S:OsSHN2 lines. Hd3a and RFT1 are crucial genes controlling flowering in rice (Komiya, et al., 2008). These observations suggest that changes in *OsSHN2* expression misregulates the expression of many genes involved in a variety of processes related to plant developmental processes such as cell wall biosynthesis, hormonal

signaling and flowering pathway, indicating that *OsSHN2* might serve as a central hub for many pathways.

**Functional classification of DEGs**

To gain further insight into the role of *OsSHN2*, functional enrichment of DEGs was performed. All genes with FDR<0.05 were probed against rice regulatory network (RRN) available at https://plantstress-pereira.uark.edu/RRN/ with a Q-value threshold of 0.05. RRN facilitates functional annotation of high-throughput datasets by providing coregulated clusters along with associated biological processes, KEGG pathways and the regulator for each cluster. Using *OsSHN2* expression dataset, 46 clusters were enriched by RRN (Table 2). Of these, 18 clusters had annotations for known KEGG pathways and 29 clusters were annotated with complex biological processes. Upregulated clusters with known KEGG pathways were cl_10 with a 12-fold upregulation belonging to plant-pathogen interaction and LOC_Os02g45450 as the central regulator for the cluster with 100% overlap. Cl_4 belonging to phenylpropanoid biosynthesis was also enriched with a FC of 7.4 and LOC_Os09g26170 as the TF regulator with 97% overlap. Clusters which were downregulated in the dataset were cluster 2 associated with photosynthesis and regulator LOC_Os03g24590 with 100% overlap, carbohydrate metabolic processes (cl_126), N-Glycan biosynthesis (cl_23), pentose and glucoronate interconversion (cl_11) with downregulation by -3.822, -2.475, -2.376-folds respectively. Interestingly, cl_7 pertaining to phenylpropanoid biosynthesis albeit with a different regulator LOC_Os02g46780 was also downregulated indicating the role of *OsSHN2* in regulation of phenylpropanoid biosynthesis by two independent mechanisms.

**Table 3: Functional enrichment of DEGs:** Cluster enrichment using RRN. The table lists the clusters, the log2FoldChange of the cluster, the biological process, KEGG pathway and the regulator for the pathway. The overlap column represents the overlap of the regulator with the genes in the cluster.

| Cluster | Size | FC | Bio_Proc | KEGG | Regulator | Over lap |
|---|---|---|---|---|---|---|
| **cl_10** | 199 | 12.008 | regulation_of_ transcription_DNA-dependent | PLANT-PATHOGEN_ INTERACTION | LOC_Os02g45450 | 1 |
| **cl_4** | 238 | 7.483 | response_to_ o xidative_stress | PHENYLPROPANOID_ BIOSYNTHESIS | LOC_Os09g26170 | 0.97 |
| **cl_98** | 28 | 7.243 | response_to_ oxidative_stress | NA | LOC_Os07g42510 | 0.72 |
| **cl_109** | 37 | 6.395 | NA | NA | LOC_Os06g51070 | 1 |
| **cl_25** | 100 | 6.385 | drug_ transmembrane _transport | NA | LOC_Os02g44360 | 0.73 |
| **cl_195** | 19 | 5.159 | NA | NA | LOC_Os11g29870 | 0.77 |
| **cl_1** | 87 | 4.011 | Death | NA | LOC_Os12g03660 | 1 |
| **cl_157** | 21 | 3.241 | NA | NA | LOC_Os07g40000 | 0.93 |
| **cl_237** | 11 | 3.006 | NA | NA | LOC_Os06g41770 | 0.9 |
| **cl_235** | 16 | 2.965 | NA | NA | LOC_Os10g30880 | 1 |
| **cl_167** | 25 | 2.933 | vesicle-mediated_transport | NA | LOC_Os06g41100 | 0.93 |
| **cl_90** | 29 | 2.892 | NA | NA | LOC_Os06g43860 | 0.67 |
| **cl_95** | 32 | 2.807 | NA | NA | LOC_Os03g12370 | 0.68 |
| **cl_87** | 22 | 2.613 | Electron_transport | NA | LOC_Os01g01840 | 0.98 |
| **cl_309** | 10 | 2.507 | NA | NA | LOC_Os01g62130 | 1 |
| **cl_8** | 21 | 2.504 | ion_ homeostasis | NA | LOC_Os02g51090 | 0.87 |
| **cl_40** | 29 | 2.45 | NA | NA | LOC_Os01g10504 | 1 |
| **cl_75** | 35 | 2.315 | vesicle-mediated_ transport | NA | LOC_Os03g20310 | 0.82 |
| **cl_67** | 43 | 2.306 | protein_catabolic_ process | PROTEASOME | LOC_Os01g61810 | 1 |
| **cl_114** | 27 | 2.292 | carboxylic_acid_metabolic_ process | FATTY_ACID_ METABOLISM | LOC_Os03g58250 | 0.81 |
| **cl_113** | 22 | 2.29 | protein_modification by_small_protein_conjugation | NA | LOC_Os03g43840 | 0.93 |
| **cl_44** | 46 | 2.149 | establishment_of_ localization_in_cell | PHAGOSOME | LOC_Os03g09860 | 0.99 |
| **cl_281** | 13 | 2.13 | NA | NA | LOC_Os10g22430 | 0.71 |
| **cl_18** | 95 | 2.076 | establishment_of_ protein_localization | MRNA_SURVEILLANCE _ PATHWAY | LOC_Os06g45840 | 0.73 |
| **cl_100** | 29 | 1.979 | NA | NA | LOC_Os12g43950 | 0.98 |
| **cl_34** | 64 | -1.961 | cellular_catabolic_ process | PROTEIN_ PROCESSING_IN_ENDO P LASMIC_RETICULUM | LOC_Os01g15460 | 0.95 |
| | | | | | | |
| **cl_262** | 16 | -2.077 | NA | NA | LOC_Os06g21330 | 1 |
| **cl_200** | 14 | -2.288 | NA | NA | LOC_Os02g33610 | 0.88 |

**Table 3 Continued**

| Cluster | Size | FC | Bio_Proc | KEGG | Regulator | Over lap |
|---|---|---|---|---|---|---|
| | | | | | | |
| **cl_11** | 74 | -2.376 | lipid_localization | PENTOSE_AND_ GLUCURONATE_ | LOC_Os11g32100 | 0.95 |
| **cl_205** | 14 | -2.376 | NA | NA | LOC_Os12g42430 | 1 |
| **cl_7** | 151 | -2.383 | Carbohydrate_metabolic _process | PHENYLPROPANOID_ BIOSYNTHESIS | LOC_Os02g46780 | 1 |
| **cl_224** | 10 | -2.399 | NA | NA | LOC_Os12g07730 | 1 |
| **cl_23** | 51 | -2.475 | RNA_ processing | N-GLYCAN_ BIOSYNTHESIS | LOC_Os04g34720 | 0.99 |
| **cl_158** | 11 | -2.732 | NA | NA | LOC_Os02g07650 | 0.97 |
| **cl_128** | 25 | -3.188 | protein_folding | PROTEIN_PROCESSING | LOC_Os09g35790 | 0.96 |
| **cl_38** | 64 | -3.217 | NA | MRNA_SURVEILLANCE _ PATHWAY | LOC_Os01g66140 | 0.75 |
| **cl_196** | 20 | -3.308 | cellular_nitrogen_ compound_metabolic_process | NA | LOC_Os08g40430 | 0.92 |
| **cl_65** | 42 | -3.478 | catabolic_process | NA | LOC_Os10g19860 | 0.93 |
| **cl_5** | 418 | -3.569 | Translation | RIBOSOME | LOC_Os02g03730 | 0.98 |
| **cl_47** | 46 | -3.667 | apoptotic_process | NA | LOC_Os02g33750 | 1 |
| **cl_126** | 20 | -3.822 | carbohydrate_metabolic_proces s | NA | LOC_Os02g47640 | 0.82 |
| **cl_39** | 54 | -3.894 | ribonucleoside_monophosphate _biosynthetic_process | PURINE_METABOLISM | LOC_Os03g12120 | 0.99 |
| **cl_6** | 148 | -4.316 | microtubule-based_movement | DNA_ REPLICATION | LOC_Os12g25120 | 0.98 |
| **cl_9** | 298 | -4.385 | apoptotic_process | HOMOLOGOUS_ RECOMBINATION | LOC_Os08g32440 | 0.98 |
| **cl_13** | 196 | -6.052 | cellular_component_ organization | SPLICEOSOME | LOC_Os02g02290 | 0.82 |
| **cl_2** | 1148 | -6.222 | Photosynthesis | PHOTOSYNTHESIS | LOC_Os03g24590 | 1 |

**35S:*OsSHN2* lines exhibit altered cell wall biosynthesis**

Functional classification of DEGs showed enrichment of two clusters cl_4 and cl_7, both associated with the phenylpropanoid biosynthesis pathway. The two clusters, although belonging to the same KEGG pathway, exhibited some differences. cl_4 was upregulated by 7.4-fold with LOC_Os09g26170 as the master regulator for the pathway. LOC_Os09g26170, a MYB TF has a role in stress responsive signal pathways (Kaur, et al., 2015). It is also highly co-expressed with LOC_Os01g51260 and LOC_Os01g74410 MYB TFs (Smita, et al., 2015). Ortholog of LOC_Os01g51260 from Arabidopsis has been known to be involved in secondary cell wall biosynthesis (Yang, et al., 2007), and the ortholog LOC_Os01g74410 plays a key role in fructan biosynthesis in wheat (Xue, et al., 2011). cl_7, also enriched for phenylpropanoid biosynthesis, is regulated by LOC_Os02g46780 and exhibit repression by 2.3-fold. The ortholog of LOC_Os02g46780 (*OsMYB58*/63) from Arabidopsis is the master regulator for lignin biosynthesis pathway (Zhou, et al., 2009). Contrasting reports have been published regarding the function of *OsMYB58*/63 for its role in cell wall biosynthesis in rice. While glucocorticoid-fused *OsMYB58*/63 overexpression plants exhibited stronger phloroglucinol staining and no change in calcofluor staining, indicating possible role of *OsMYB58*/63 in lignin biosynthesis in rice (Hirano, et al., 2013), *OsMYB58*/63 has also been shown to bind to *OsCESA7*, one of the primary genes for secondary cell wall biosynthesis in rice (Noda, et al., 2015).

Another difference between the two clusters are the associated Gene Ontology (GO) Biological process (BP). Cl_7 showed enrichment of carbohydrate metabolic process with a score (-log q value) of 7.5. Other BPs belonging to the cluster include cellulose metabolic process, cellulose biosynthetic process, glucan metabolic process, and polysaccharide metabolic process, all with a score of at least 3. For cl_4, response to oxidative stress was the key BP with a score (-

log q value) of 14.115. Some other BP associated with the cluster were response to chemical stimulus, electron transport, response to stress and response to stimulus each with a score (-log qvalue) > 3. BPs like cell wall organization and carbohydrate metabolic process also belong to the cluster, though with a lower score (-log q value), indicating that different processes might be involved in the regulation of phenylpropanoid biosynthesis pathway in 35S:*OsSHN2* lines.

Cl_7 included critical phenylpropanoid biosynthesis genes like LOC_Os02g41650 (*OsPAL4*). *OsPAL4* is also implicated in the process of broad spectrum disease resistance and can be induced by drought in rice (Hu, et al., 2008; Tonnessen, et al., 2015). Other genes belonging to the cluster were LOC_Os08g34280, cinnamyl co-A reductase, a key enzyme involved in the biosynthesis of lignin (Lacombe, et al., 1997), LOC_Os02g08100 (*Os4CL3*) encoding 4-Coumarate:coenzyme A ligase, suppression of which leads to reduction in lignin and plant height (Gui, et al., 2011). Taken together, these results indicate that 35S:*OsSHN2* rice plants may have a higher lignin content. Although the expression of secondary cell wall cellulose synthase gene was not quantified by RNA-seq, the transcript counts for many glycosyl transferases, expansins and other cell wall related genes were perturbed (Supplementary Table 1), further demonstrating that 35S:*OsSHN2* lines have an altered cell wall content compared to WT plants.

In addition, N-Glycan biosynthesis(cl_23) shows downregulation in 35S:*OsSHN2* lines. Recent reports have implicated the role of N-glucan biosynthesis in cellulose biosynthesis in Arabidopsis (Burn, et al., 2002; Lukowitz, et al., 2001). In rice, it was shown that *gnt1* (N-acetylglucosaminyltransferase I) mutant plants exhibited reduced cellulose content (Fanata, et al., 2013). Moreover, cl_11 associated with pentose and glucose interconversions pathway was downregulated by 2.4-fold. The Pentose and Glucose interconversion pathways are a subset of carbon metabolism pathway. The cluster is associated with *OsSHN2* as one of the central regulators

with an overlap of 81% with all the 74 genes in the pathway, demonstrating that *OsSHN2* might lead to changes in cell wall biosynthesis by redistribution of carbon allocation through changes in key carbon metabolic pathways. (Supplementary table 2).

Cl_4, the cluster annotated with phenylpropanoid biosynthesis, shows upregulation of genes such as LOC_Os07g35560 a beta-glucosidase, LOC_Os11g37960 (*OsWIP4*) a wound-induced protein, LOC_Os01g45200 (Snl6) a cinnamoyl-CoA reductase (CCR)-like gene, LOC_Os02g38840 (*OsG6PDH1*), and LOC_Os01g53040 (*OsWRKY14*). *OsG6PDH1* is involved in rice defense response to *Xanthomonas oryzae* via reactive oxygen species (ROS) generation by supplying NADPH (Kano, et al., 2013). Snl6 also mediates rice immune response and mutant plants of the gene have been shown to have lesser lignin and higher sugar extractability (Bart, et al., 2010). *OsWRKY14* is involved in tryptophan and tryptophan-derived secondary metabolites (Kang, et al., 2011). Taken together, these observations suggest that upregulation of oxidative and defense-induced genes cause activation of oxidative stress induced phenylpropanoid biosynthesis in 35S:*OsSHN2* lines as the two processes are interconnected with each other.

**Expression of OsSHN2 is strongly correlated with OsCesA7, a cellulose biosynthetic gene in rice.**

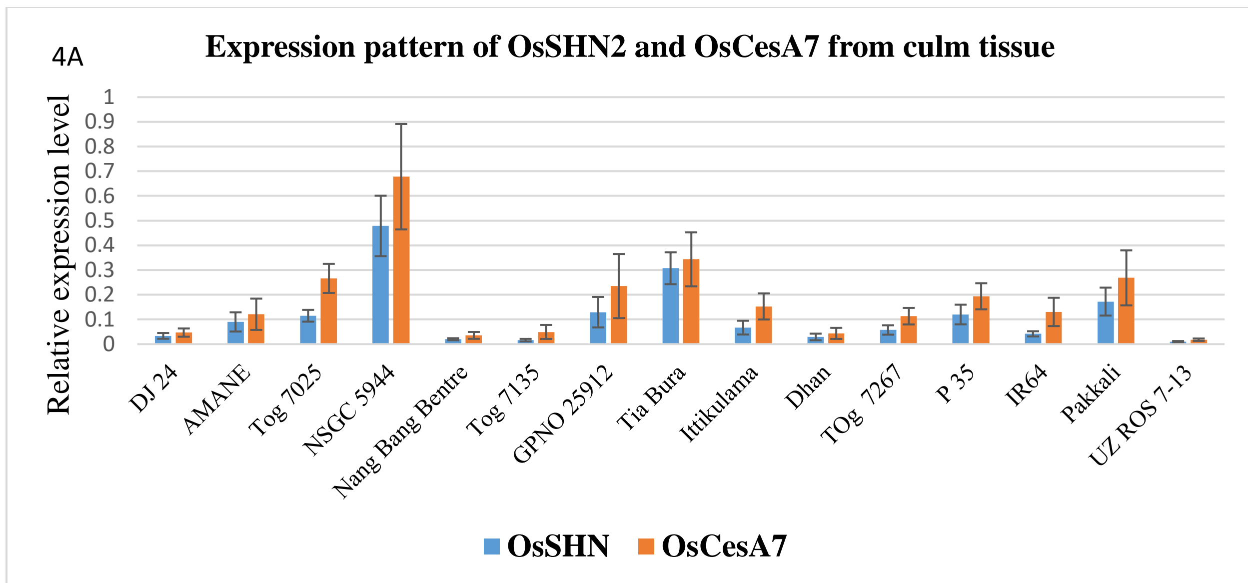
Given the fact that 35S:OsSHN2 lines exhibit altered cell wall biosynthesis, quantification of *OsSHN2* and *OsCesA7* was performed simultaneously from seventeen diverse rice genotypes using culm and leaf samples (Table 4). Correlation analysis was performed between the Ct values of the two genes obtained using δδCt method. The results show that the expression of *OsSHN2* from both culm and leaf samples varies in different rice genotypes suggesting an inherent variation in the expression pattern of *OsSHN2* gene occurs in nature (Figure 2, Figure 3). Furthermore, it

was found that correlation co-efficient ($R^2$) value between *OsSHN2* and *OsCesA7* between leaf samples was 0.7981 suggesting a linear relationship between the genes (Figure 5). In addition, $R^2$ value between *OsSHN2* and *OsCesA7* from culm samples was 0.9471 further demonstrating that a positive correlation exists between the expression of *OsSHN2* and *OsCesA7* as demonstrated by quantification from 17 diverse genotypes (Figure 4).

We also quantified the expression of lignin biosynthetic genes namely 4CL, CAD and PAL using the same samples. However, no significant correlation between *OsSHN2* and lignin biosynthetic genes was found (Supplementary Figure 1), further signifying that cellulose biosynthesis is specifically and directly correlated with *OsSHN2* expression among diverse naturally occurring genotypes.

**Table 4: List of genotypes used for quantification of OsSHN2 and OsCesA7.**

| **GSOR#** | **Taxonomy** | **Genotype** | **Origin country** | **Ancestry* (sub) species** |
|---|---|---|---|---|
| **311572** | *O. sativa* | DJ 24 | Bangladesh | *aus* |
| **311635** | *O. sativa* | AMANE | Sri Lanka | *indica* |
| **311603** | *O. glaberrima* | Tog 7025 | Sierra Leone | *glaberrima* |
| **311695** | *O. glumaepatula* | NSGC 5944 | United States | *glumaepatula* |
| **311561** | *O. sativa* | Nang Bang Bentre | Vietnam | *aus* |
| **311690** | *O. glaberrima* | Tog 7135 | Senegal | *glaberrima* |
| **311688** | *O. glaberrima* | GPNO 25912 | El Salvador | *glaberrima* |
| **311642** | *O. sativa* | Tia Bura | Indonesia | tropical *japonica* |
| **311545** | *O. sativa* | Ittikulama | SriLanka | *aus* |
| **311606** | *O. sativa* | Dhan | Nepal | *indica* |
| **311693** | *O. glaberrima* | TOg 7267 | Cameroon | *glaberrima* |
| **311644** | *O. sativa* | P 35 | India | *aus* |
| **311793** | *O. sativa* | IR64 | Philippines | *indica* |
| **311769** | *O. sativa* | Pakkali | Philippines | aromatic |
| **311592** | *O. sativa* | UZ ROS 7-13 | Uzbekistan | *aus* |
| **-** | *O. sativa* | N 22 | India | *indica* |
| **-** | *O. sativa* | Vandana | India | *aus* |



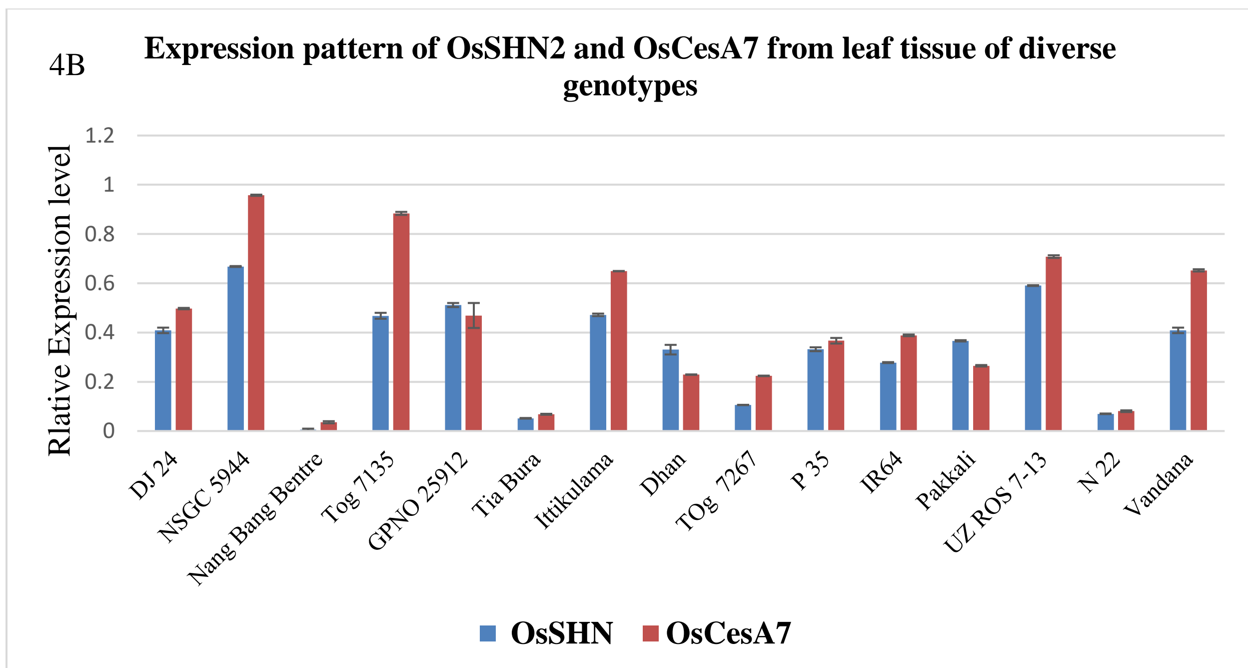




**Figure 4: Relative expression level of *OsSHN2* and *OsCesA7* assessed through qRT-PCR**. Quantification of both genes from culm (4A) and leaf (4B) tissue from diverse rice genotypes is shown. Data are expressed as relative transcript levels of *OsSHN2* and *OsCesA7* in the respective plants compared with the intrinsic actin. Error bars represent +/-SE (n = 3)

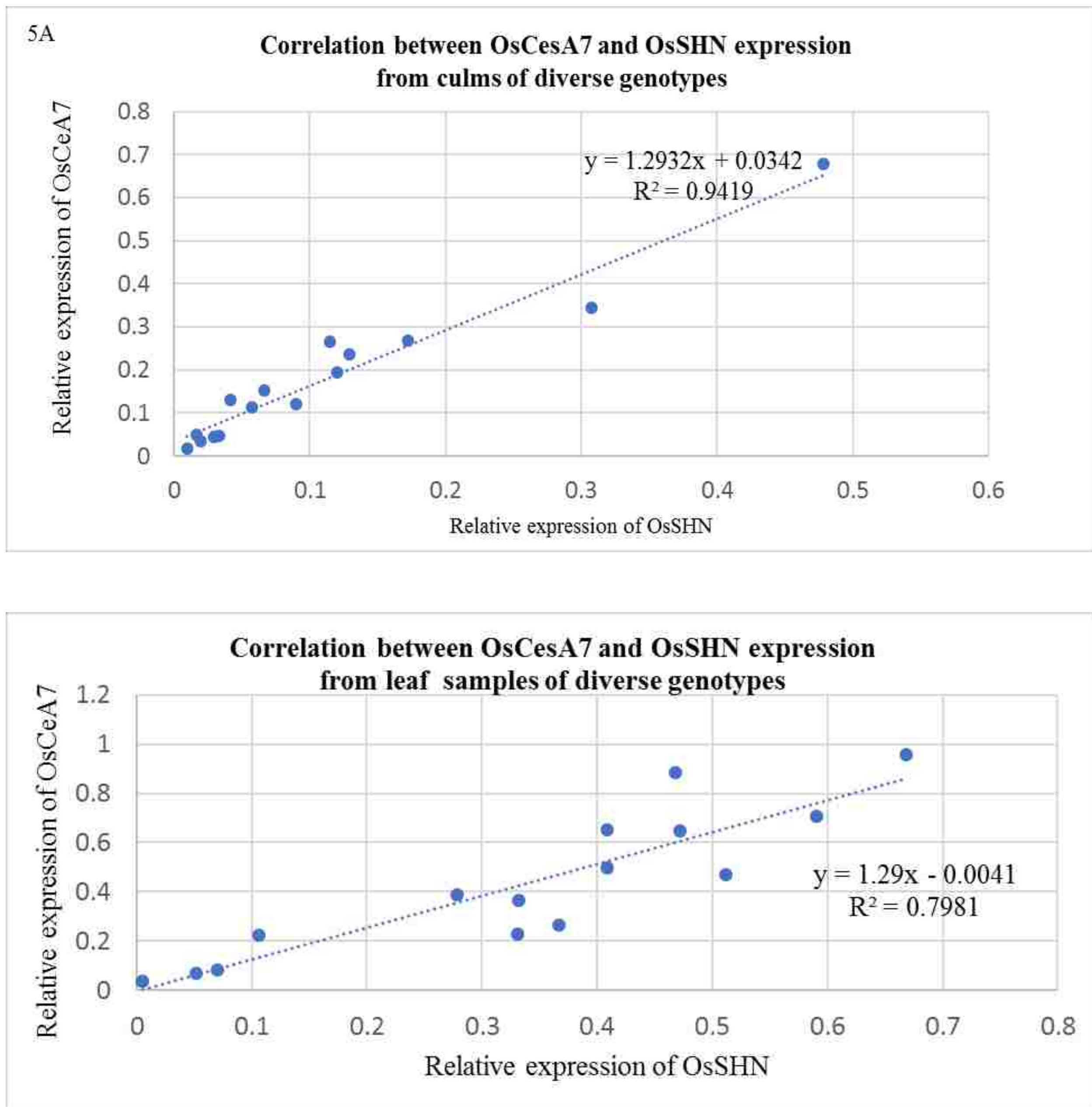



**Figure 5: Correlation analysis between *OsSHN2* and *OsCesA7*.** (5A) Expression levels of *OsSHN2* and *OsCesA7* from culm tissues are plotted against each other and $R^2$ value calculated between the two. The equation represents the linear relationship between *OsCesA7* and *OsSHN2* expression values. $R^2$ value of 0.9419 represents that 94% of the variance in *OsCesA7* values can be explained by relative *OsSHN2* expression levels across diverse genotypes. (5B) $R^2$ value of 0.7981 was estimated between expression levels of *OsSHN2* and *OsCesA7*, representing that 74% of the variance in *OsCesA7* value can be explained by relative *OsSHN2* expression levels across diverse genotypes from leaf samples.

***OsSHN2* can directly activate the expression of *OsMYB61* and *OsMYB15* to regulate cell wall biosynthesis.**

To find out genes directly activated by *OsSHN2*, a steroid-inducible gene expression system was used, a system widely used to identify targets of TFs in plants (Basu, et al., 2017; Baudry, et al., 2004; Zhou, et al., 2009). This system has been widely used to find out downstream genes whose expression is directly modulated by a TF. *OsSHN2*, belongs to the AP2/ERF class of TFs. These TFs bind to the GCC box motif present in the promoter sequence of downstream genes to regulate their expression. Based on this information and literature survey, three candidate genes were chosen LOC_Os01g18240 (*OsMYB61*), LOC_ Os02g46780 and LOC_ Os10g33810 (*OsMYB15*).

For this purpose, a 35S:*OsSHN2*: HER (Human estrogen receptor) fusion constructs were made and transfected into rice protoplasts. The fusion of *OsSHN2* with HER causes binding of the TF to a cytoplasmic complex, thus inactivating the TF. Addition of estradiol causes estradiol to bind to HER instead, thus allowing movement of the TF to nucleus and resume its activity. Cycloheximide was added to the system to inhibit new protein synthesis. Addition of estradiol following cycloheximide treatment causes translocation of trapped TF into the nucleus and nascent transcription of downstream genes. Using this inducible system, it was found that estradiol-based activation of OsSHN2 can lead to an increase in transcripts of *OsMYB61* and *OsMYB15*, however there was a slight reduction in the in the transcripts of *OsMYB58*/63. The induction of gene expression occurred even in the presence of cycloheximide for *OsMYB61* and *OsMYB15*, but not for *OsMYB58*/63 (Figure 6), indicating that *OsSHN2* can directly activate the expression of *OsMYB61* and *OsMYB15* but not *OsMYB58*/63.

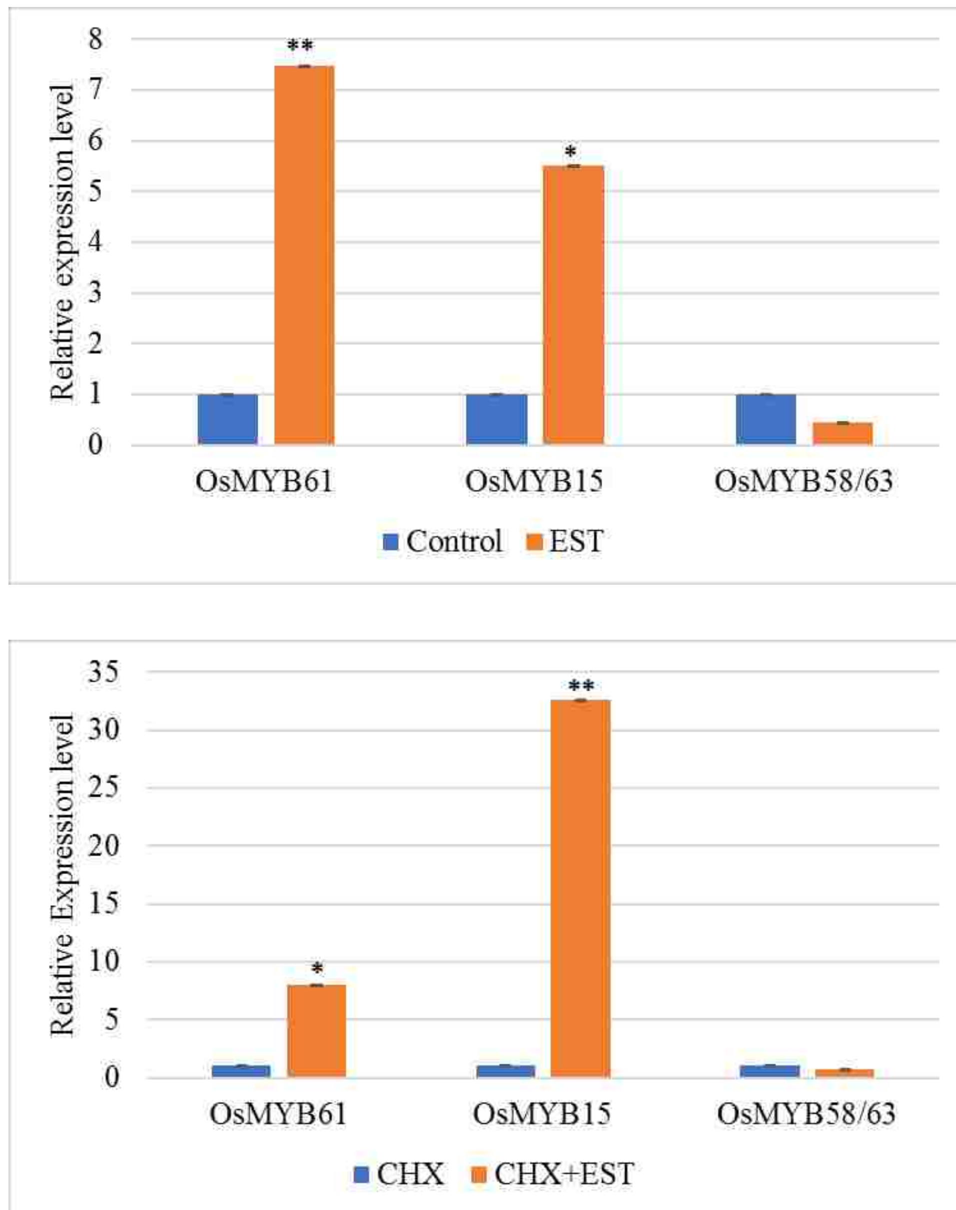



**Figure 6: Direct activation of downstream genes by OsSHN.** To study transcriptional regulation by *OsSHN2* gene, 35S:*OsSHN2*:HER was transfected into rice leaf protoplasts. Following transfection, the protoplasts were treated with estradiol (EST), cycloheximide (CHX) and cycloheximide + estradiol (CHX+EST). qRT-PCR was used to quantify the expression of individual transcripts. Quantitative differences in the expression of the tested genes between the control and treated samples that are statistically significant are designated (* represents p-value < 0.05, ** indicates p-value<0.01 and *** indicates p-value < 0.001). The expression level of each gene in the mock-treated (control) or cycloheximide-treated protoplasts (CHX) is set to 1. Error bars represent SEs of three biological replicates.

*OsMYB61* is involved in cell wall biosynthesis through a GA mediated SLR1-NAC dependent pathway (Huang, et al., 2015). *OsMYB15* is an uncharacterized MYB TF. A query of *OsMYB15* with RECoN, available at https://plantstress-pereira.uark.edu/RECoN/, enabled the identification of clusters of genes functionally associated with each other. A small network of eight genes (Table 5) was found associated with *OsMYB15* (Fig 7), including strong association of *OsMYB15* with *OsMYB4* (LOC_Os04g43680). Heterologous expression of *OsMYB4* in Arabidopsis has been shown to enhance tolerance to chilling and freezing (Vannini, et al., 2004). In addition, it was seen that overexpression of *OsMYB4* in rice led to increase in phenylpropanoid induced defense and oxidative response under cold conditions with changes in panicle development (Park, et al., 2010). Thus, *OsSHN2* appears to have a significant role in cell wall biosynthesis in rice by mediating the expression of MYB TFs.

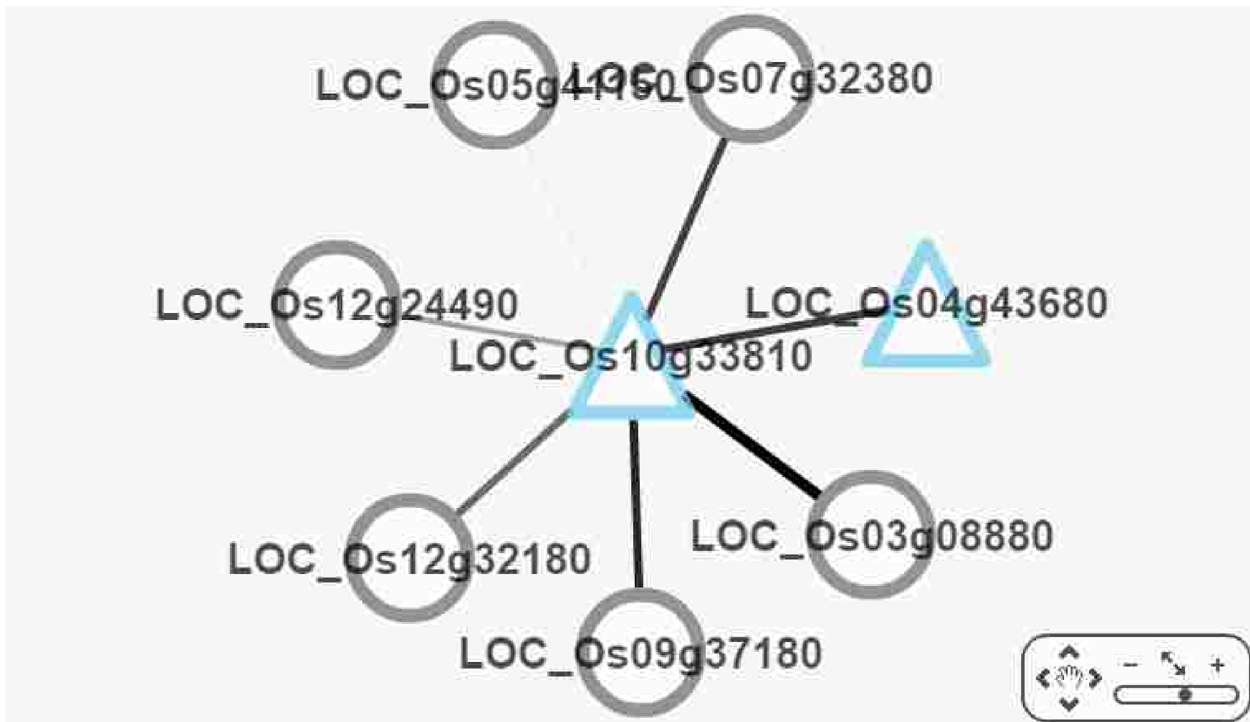



**Figure 7: Co-expression network for *OsMYB15*:** *OsMYB15* is co-expressed with eight genes with weight of 0.8 for the module. Triangles represent TFs while circles denote other genes. The thickness of the edges connecting different genes represent the degree of co-expression.

## Table 5: List of genes co-expressed with OsMYB15

| Gene | Annotation | Arabidopsis homolog | Molecular Function |
|---|---|---|---|
| **LOC_Os03g08880** | purine permease, putative, expressed | AT1G28220 | other |
| **LOC_Os04g43680** | MYB family transcription factor, putative, expressed | AT3G23250 | TF |
| **LOC_Os05g41150** | expressed protein | AT5G17910 | other |
| **LOC_Os07g32380** | protein phosphatase 2C, putative, expressed | AT3G15260 | other |
| **LOC_Os09g37180** | transferase family protein, putative, expressed | AT5G48930 | other |
| **LOC_Os10g33810** | myb-related protein Myb4, putative, expressed | AT3G23250 | TF |
| **LOC_Os12g24490** | zinc finger, C3HC4 type domain containing protein, expressed | AT1G72200 | Gene/TF |
| **LOC_Os12g32180** | cornichon protein, putative, expressed | AT3G12180 | other |

**Section 4.5: Discussion**

AP2/ERF (APETALA2/ethylene responsive factor) class of transcription factor have been known to be involved in a variety of processes in rice growth, development, and response to environment stimuli (Sharoni, et al., 2011). Members of the SHINE clade of AP2/ERF TF are responsible for regulating cuticle and wax biosynthesis in Arabidopsis (Aharoni, et al., 2004). The clade consists of three TFs, *AtSHN1* (At1g15360), *AtSHN2* (At5g11190) and *AtSHN3* (At5g25390). Overexpression of *AtSHN1*/*WIN1* in Arabidopsis led to glossy-leaf phenotype and increased drought tolerance. Recently, it was also shown that overexpression of *AtSHN2* in rice leads to changes in cell wall biosynthesis (Ambavaram, et al., 2011). Although the exact role of *AtSHN3* is not known, it has been speculated that the SHN proteins function in cell-separation by inducing the formation of separating layers.

In rice, *OsSHN1*/*WIN1* (*OsWR1*) is involved in drought tolerance modulated through wax biosynthesis. However, the functions of *OsSHN2*, *OsSHN3* and *OsSHN4* are unknown. Here, we characterize the function of *OsSHN2* in rice and show that co-suppression of *OsSHN2* in rice causes changes in cell wall biosynthesis with overall changes in plant growth and development.

Transcriptional changes associated with *OsSHN2* co-suppression showed enrichment of 46 clusters, each associated with a TF possibly regulating the expression of downstream genes associated with each cluster. *OsSHN2* co-suppression led to downregulation of core processes such as photosynthesis in rice leading to a significant reduction of carbon fixation and carbohydrate metabolic processes.

Interestingly clusters belonging to phenylpropanoid biosynthesis and plant-pathogen interaction were upregulated. Phenylpropanoid biosynthesis in plants is an intrinsic intricate

pathway leading to synthesis of many secondary metabolites including flavonoids, sinapate esters and lignin. Lignin being the second most abundant polymer on earth after cellulose, is a major product of the phenylpropanoid biosynthesis (Raes, et al., 2003). Phenylalanine ammonia-lyase (PAL) catalyzes the first step towards phenylpropanoid biosynthesis in plants. Downregulation of PAL leads to systemic changes in plant structure and morphology such as altered leaf shape alteration, reduced plant growth and pollen viability in addition to changes in flower morphology and reduced lignification (Song, et al., 2011). Cinnamic acid 4-hydroxylase (C4H) catalyzes the second step in the pathway and functions in the conversion of cinnamic acid to p-coumaric acid. Mutants of C4H exhibit similar phenotypes as PAL mutants i.e., stunted growth, male sterility and reduction in lignin biosynthesis Downregulation of other key genes in the pathway such as Os4CL3 causes a reduction of lignin biosynthesis in rice with no effects on plant morphology and agronomic traits (Gui, et al., 2011). Given the relation of 4CL3 and PAL with phenylpropanoid biosynthesis, it is believed that Upregulation of key biosynthetic genes *Os4CL*-3 and *OsPAL2* caused due to misexpression of *OsSHN2* results in an increase in phenylpropanoid biosynthesis specifically modulated by LOC_Os09g26170. We also speculate that this increase in phenylpropanoid biosynthesis further leads to activation of defense related genes due to innate role of phenylpropanoids in plant immunity, causing enrichment of plant pathogen defense pathways. About 29 known and 18 unknown clusters were enriched in response to *OsSHN2* overexpression, indicating that changes in *OsSHN2* expression can lead to broad function changes in plant growth and developmental response.

Based on the studies of *AtSHN2*, *OsSHN2* was proposed to have a role in cell wall biosynthesis pathway in rice (Ambavaram, et al., 2011). A recent study from switchgrass revealed that overexpression of PvERF001, the closest homolog of Arabidopsis SHN clade of TFs, results

in increased sugar release and higher xylose content (Wuddineh, et al., 2015) with no changes in cutin and wax biosynthesis. In the present study, global transcriptomic studies revealed that *OsSHN2* has a native association with carbon partitioning particularly related to pentose-glucuronate interconversions. Based on the above information we propose that *OsSHN2* plays a critical role in the regulation of sugar responsive transcriptome regulation in rice.

Our study also reveals the direct association of *OsSHN2* with cell wall biosynthesis by its capacity to activate the expression of *OsMYB61*, which in turn activates CesA biosynthesis. *OsMYB61* is a GA responsive MYB and interacts with NAC to activate the transcription of downstream cellulose biosynthetic genes (Huang, et al., 2015). Members of the SHN family in Arabidopsis been shown to be GA responsive (Shi, et al., 2011). We propose that *OsSHN2* might also be responsive to GA in rice. Besides direct activation of *OsMYB15* suggest that *OsSHN2* might activate a suite of TFs capable of orchestrating cell wall biosynthesis in rice.

**Summary**

In summary, we have characterized the role of OsSHN2 in cell wall biosynthesis in rice. Information from this study can also be used to study the role of uncharacterized genes associated with known Kegg pathways in rice. In addition, we identify a new TF, OsMYB15 and suggest it might have a potential role in cellulose biosynthesis in rice. With this current research, this inventory of genes and the information provided in this study could facilitate the regulation of carbon partitioning in rice.

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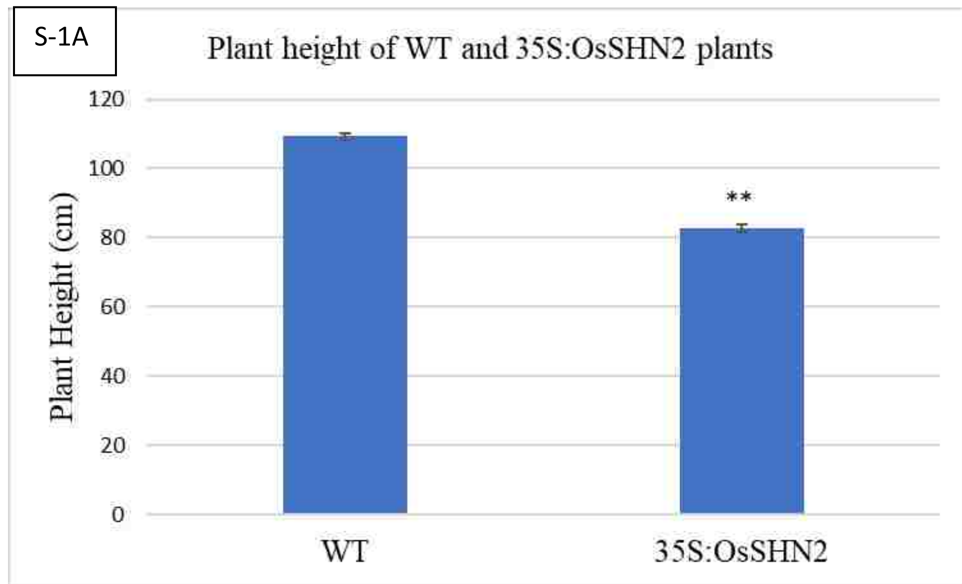
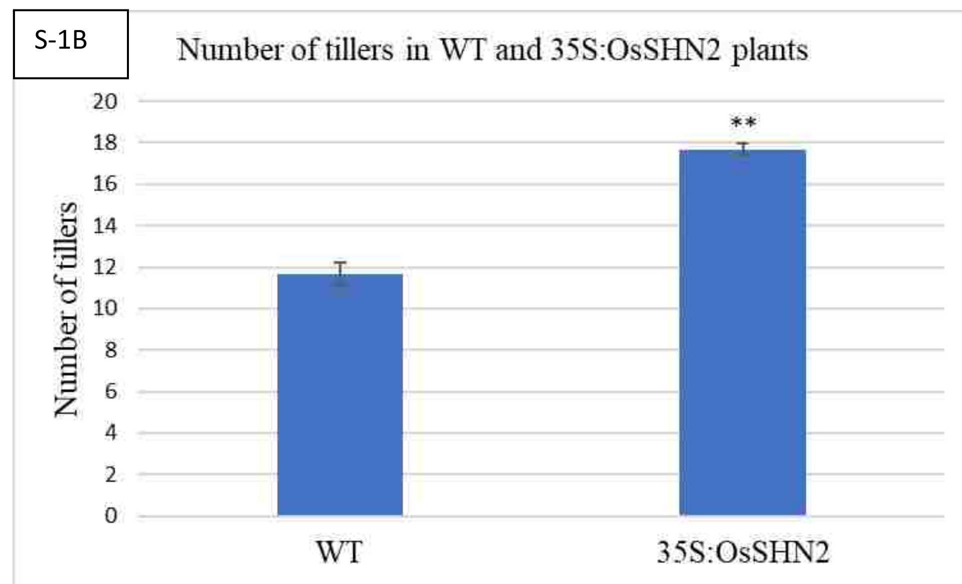
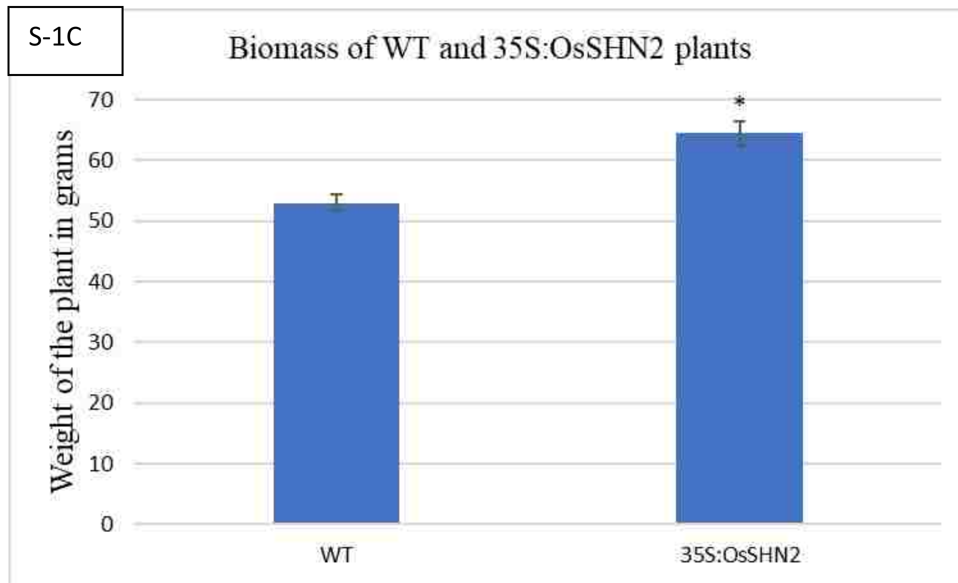
## Section 4.6: Supplementary Figures






Supplementary Figure 1: Physiological characteristics of WT and 35S:OsSHN2 plants: Comparison of plant height (1A), number of tillers (1B) and biomass (1C) between WT and 35S:OsSHN2 transgenic rice. Data are shown as the means ± s.d. (Student's t tests, *P< 0.05, **P < 0.01, n = 10).

## CHAPTER 5: Conclusions and Future Directions

### A new model of cell wall regulation using *AtSHN2* and *OsSHN2* as central tools

Plant cell walls remain a major source of biomass, thus biofuel on Earth. The biosynthetic machinery orchestrates a highly complex process constituting an interplay of a variety of TFs, biosynthetic wall formation by the SHN clade of TF, particularly AtSHN2 and OsSHN2 in rice using a systems-study demonstrated that AtSHN2 can regulate the expression of many key downstream target genes thus suggesting AtSHN2 activates through a hierarchical network

ChIP-TAP enabled sequencing enabled finding of genome wide putative targets of *AtSHN2*. The study identified *AtSHN2* can regulate the expression of many key downstream target genes regulating multiple pathways by binding to a variety of motifs present in the promoter regions of these genes (Chapter 2, Figure 4). This in turn leads to regulation of many sub-regulons under *AtSHN2* overexpression thus suggesting *AtSHN2* activates by the virtue of hierarchical network. In addition, presence of WRKY and MYB binding motifs in the downstream target sequences of *AtSHN2* also suggests that *AtSHN2* might work in conjunction with these TFs to regulate processes in rice, suggesting a complex regulatory network is under the control of *AtSHN2* expression.

Multiple evidences show *AtSHN2* regulates the expression of several MYB TFs specifically by binding to the GCC box motif. In addition, the function of MYB TF LOC_Os02g49986 was also characterized. LOC_Os02g49986 binds to the promoter of CesA-4, 7, and -9, the secondary cell wall specific cellulose biosynthetic genes. Direct activation of OsCesA9 by LOC_Os02g49986 and possible co-expression with *OsWRKY36* and *OsWRKY34* show that LOC_Os02g49986 plays a significant role in cell wall biosynthesis in rice. It was interesting to see that LOC_Os02g49986

could slightly increase the expression of primary cell wall biosynthetic genes (data not shown) and overexpression/ RNAi transformation of LOC_Os02g49986 were not successful in three independent attempts. Taken together it is speculated that LOC_Os02g49986 might be involved in cellulose biosynthesis pathway by regulation of a sub-regulatory network of genes and can be an interesting candidate to further unravel mechanism of cell wall formation in rice.

RNA-seq mediated expression profiling of genes helped unravel the global transcriptomic changes associated with *AtSHN2* overexpression in rice. DEG analysis along with functional annotations has provided a model for cell wall synthesis regulated by AtSHN2 expression and helped dissection of a complex pathway into respective clusters for each process. From the results presented in Chapter 3, it is seen that *AtSHN2*-TAP overexpression results in change in the expression of 11627 genes many of which belong to cell wall biosynthesis and interconnected processes. Functional enrichment of DEGs show downregulation of phenylpropanoid pathway with a concomitant increase in pentose-glucuronate pathway. Phenylpropanoid pathway in rice and its connection with lignin biosynthesis has been well documented (Boerjan, et al., 2003). Downregulation of lignin biosynthetic genes and their association of with the phenylpropanoid pathway in the cluster fortify the results of decreased lignin in *AtSHN2*-TAP rice lines. Pentose-glucuronate pathway is a subset of carbon metabolism pathway in rice and is used in the generation of plant cell wall precursors. The results also show that genes belonging to photosynthesis process were upregulated in *AtSHN2*-TAP rice lines and related processes such as glucolysis and gluconeogenesis along with starch metabolism were perturbed. This cluster included sugar signaling genes such as *OsMYBS1* (LOC_Os01g34060). *OsMYBS1* in conjunction with *OsGAMYB* has been shown to negate the effect of high sugar induced repression of genes containing TATCCA element in their promoter region (Lu, et al., 2002). It has been known that sugar signals can induce

or repress the transduction of various genes (Koch, 1996). Recently the importance of sugar transport and carbon fixation with respect to cell wall biosynthesis has gained a lot of attention (Ivakov, et al., 2017; Verbančič, et al., 2017). Decreased assimilation of glucose in the growing cell wall polymer chain was seen during night in Arabidopsis WT plants using isotope labelling technique. This decrease was more so in the starchless phosphoglucomutase (pgm) mutant where it was seen that cell wall polymer formation occurs during the first 4 h in the night subsequently following complete shutdown of polymer synthesis suggesting that a close association between the available sugars and the incorporation of monomers exists for cell wall biosynthesis (Ishihara et al., 2015) . Many factors such as hexokinases, sucrose transporters and enzymes such as glycosyltransferases and sucrose synthase are involved in cell wall formation (Fujii, et al., 2010; Klinghammer, et al., 2007; Wang, et al., 2017; Zhang, et al., 2014). RNA-Seq analysis of *AtSHN2*-TAP lines show that many of these genes were differentially expressed under *AtSHN2* overexpression. Taken together all the above facts with the observation that *OsSHN2*, a true ortholog of *AtSHN2* is predicted to be a regulator for carbon metabolism in rice, it is hypothesized that *AtSHN2*-TAP causes a change in the carbon flux directing carbon generated from photosynthesis towards polysaccharide generation in rice plants.

To further unravel the mechanism of cell wall formation, the role of *OsSHN2*, the ortholog of *AtSHN2* in rice was studied. 35S:*OsSHN2* lines were generated and studied for this purpose. The results from Chapter 4 show that *OsSHN2* was suppressed in 35S:*OsSHN2* lines as compared to WT plants through some unknown mechanism. We speculate that the expression of *OsSHN2* like many TFs is controlled by an autoregulatory mechanism that might participate in a feedback loop to regulate its own expression (Agarwal, et al., 2016; Strayer, et al., 2000). This is further supported by the repression of suppression of OsSHN2 under osmotic stress since induction of SHN clade of

TF is seen under osmotic stress in many plant species (Lashbrooke, et al., 2015; Wang, et al., 2012). Moreover, OsSHN2 misexpression causes costly tradeoffs for plant growth and stature as seen by the phenotypic observations of 35S:*OsSHN2* lines particularly with respect to cell wall formation. This is peculiar of CAMV 35S promoter and has been widely studied in plants further implying that OsSHN2 functions in a quantitative matter and it might be imperative to express the TF in optimal amount to study its function. (Ito, et al., 2006; Kasuga, et al., 1999).

Functional annotation of DEGs by RRN helped establish that misregulation of *OsSHN2* can lead to changes in multiple transcriptional sub-networks, bolstering the fact that *OsSHN2* acts as a transcriptional hub for controlling numerous plant growth and developmental processes. Observations such as thinner cell walls, increase in expression of genes related to lignin biosynthesis and activation of reactive oxygen species mediated phenylpropanoid pathway clusters with diminished N-glucan, pentose-glucuronate and photosynthesis clusters and the expression patterns of these datasets which are opposite of *AtSHN2*-TAP rice lines suggest that *OsSHN2* like *AtSHN2* plays a central role in cell wall formation in rice plants. This result is further supported by high correlation of *OsSHN2* expression with *OsCesA7* expression pattern from diverse rice genotypes. (Vannini, et al., 2004)

In conclusion, the results from this study imply that SHN2 TFs work in a hierarchical fashion and can activate a suite of TFs capable of activating multiple sub-regulons. The negative effects of *OsSHN2* overexpression in 35S:*OsSHN2* lines suggest that *OsSHN2* must be expressed in optimal amounts to achieve a true picture of regulation of cell wall formation by *OsSHN2*. Based on this observation, we suggest that downstream targets of *OsSHN2* might be better tools for studying the mechanisms towards cell wall formation in rice.

**Systemic view of the results:**

To unravel the layers involved in cell wall formation in rice mediated by cis-regulatory and transcriptomic regulation of SHN controlled regulon, integration of the three datasets from the study i.e., *AtSHN2*-TAP ChIP-Seq, *AtSHN2* RNA-Seq and *OsSHN2*-RNA seq was done. Out of the 1766 putative targets of AtSHN2 found by *AtSHN2*-TAP ChIP-Seq results, 284 genes were differentially expressed in both *AtSHN2*-TAP and 35S:*OsSHN2* lines. Functional classification of these genes categorized them into 26 TFs, 13 Kinases and 245 biosynthetic genes (Supplementary excel file 3). The expression profile of all the above genes was studied and it was observed that many genes had opposite expression patterns in both the lines (Figure 1). To mention a few, LOC_Os06g01890, an uncharacterized MADS box TF was upregulated in 35S:*OsSHN*2 lines but downregulated by 4-fold in *AtSHN2*-TAP lines. *OsWRKY76* (LOC_Os09g25060), a TF involved in cold stress and phenylpropanoid biosynthesis in rice was upregulated by 4-fold in 35S:*OsSHN2* lines and showed downregulation by 1.8-fold in *AtSHN2*-TAP rice transgenics. *OsWAK34* (LOC_Os04g21790), was upregulated in 35S:*OsSHN2* lines by 1.5-fold but showed repression in *AtSHN2*-TAP lines by 4.2-fold. LOC_Os02g40340, an uncharacterized protein was downregulation in *AtSHN2*-TAP lines by 6-fold but its expression was not significantly perturbed in 35s:*OsSHN2* lines. OsPAL2, (LOC_Os02g41650) was upregulated in 35S:*OsSHN2* lines by 3.5-fold but downregulated in *AtSHN2*-TAP rice lines by 1-fold. LOC_Os01g25010, a flavanol synthase gene, and LOC_Os02g16630 a tryptophan biosynthetic gene were upregulated in 35S:*OsSHN2* lines but downregulated in *AtSHN2*-TAP rice lines.

We were also able to fish out some genes which were expressed only in *AtSHN2*-TAP lines or 35S:*OsSHN2* lines (Supplementary excel file 3). 45 genes had $-1.5<FC<1.5$. Genes such as LOC_Os04g52810, NAC domain TF, LOC_Os04g56990, MYB TF, LOC_Os04g24840, glucosyl

transferase, LOC_Os08g20440 (*OsMADS69*) were highly upregulated in 35S:*OsSHN2* lines whereas, LOC_Os01g58910, an auxin induced protein, LOC_*Os03g40720*, UDP-glucose dehydrogenase, LOC_Os09g30130, *OsCSLE6* were highly downregulated in 35S:*OsSHN2* lines.

For *AtSHN2*-TAP lines, 56 genes were exclusively expressed (Supplementary excel file 3). LOC_Os01g63160, an uncharacterized MYB was a direct target of *AtSHN2* and highly expressed in *AtSHN2*-TAP lines but not in 35S:*OsSHN2* lines. LOC_Os08g39630, homolog of Arabidopsis flowering bHLH1 was downregulated only in *AtSHN2*-TAP ovexpression lines. Genes such as LOC_Os06g36560, an inositol oxygenase, LOC_Os04g43410, beta glucoside homolog, LOC_Os09g25850, *OsWAX2* were directly modulated by AtSHN2 and had higher expression in *AtSHN2*-TAP lines whereas genes such as LOC_Os01g71810, a glycosyl hydrolase and LOC_Os03g20780, an ethylene insensitive gene were downregulated only in *AtSHN2*-TAP lines.

Based on their expression patterns, we speculate that these downstream targets of *AtSHN2* can be used to further enhance our understanding towards cell wall biosynthesis in rice.

**Directions for the future:**

The role of *AtSHN2* in cuticle biosynthesis has been well documented. The ChIP-TAP enabled study of *AtSHN2* showed direct binding capability of *OsWAX2* promoter by *AtSHN2* and further increase in its expression. It was also seen that wax biosynthesis genes were upregulated when 35S:*OsSHN2* lines were subjected to osmotic stress. Analysis of performance of *AtSHN2*-TAP lines and 35S:*OsSHN2* lines under water deficit conditions should clarify the role of *OsSHN2* in wax biosynthesis in rice. Further, since overexpression of *OsSHN2* in rice plants had costly trade-offs for plant growth, moderate expression studies of *OsSHN2* in rice should be performed supplemented by parallel analysis with *OsSHN2* knock out lines.

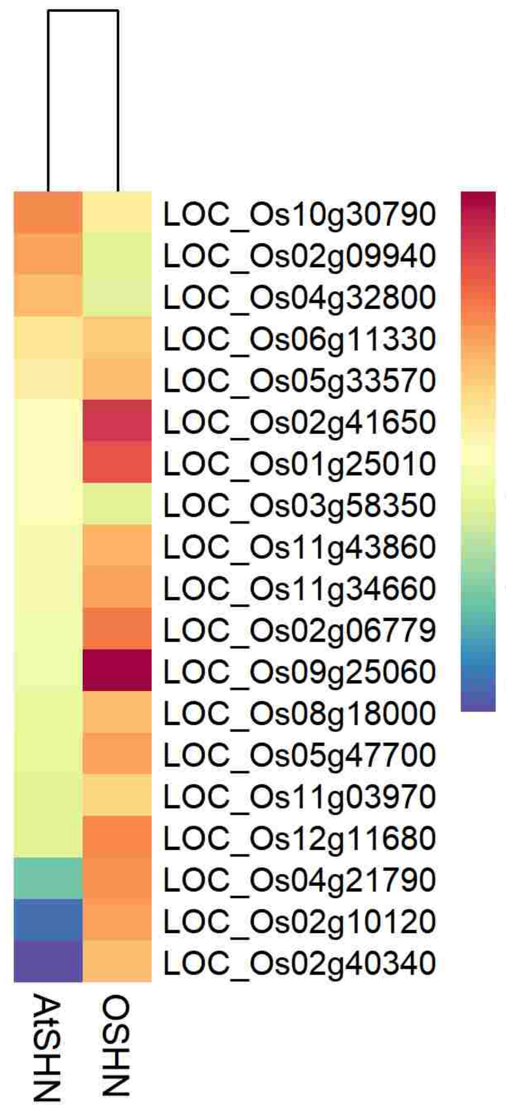



**Figure 1**: **Heatmap depicting the fold change in the expression profile of genes from *AtSHN2*-TAP and *OsSHN2* lines.** Common genes between *AtSHN2*-TAP ChIP-Seq, *AtSHN2* RNA-Seq and *OsSHN2* RNA-Seq were selected and log2FoldChange of some genes is plotted as obtained by DESeq2 analysis.

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