Class II KNOX Transcription Factors

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Knotted-like homeobox (KNOX) genes encode homeodomain-containing transcription factors (TFs) that modulate various important developmental processes in plants. While *Class I KNOX TF* genes are mainly expressed in the shoot apical meristems of both monocot and eudicot plants and are involved in meristem maintenance and/or formation, *Class II KNOXTF* genes exhibit diverse expression patterns and their precise functions have mostly remained unknown. The expression patterns of *Class II KNOX TF* genes in Arabidopsis, namely *KNAT3, KNAT4, KNAT5,* and *KNAT7*, suggest that TFs encoded by at least some of these genes, such as *KNAT7* and *KNAT3*, may play a significant role in secondary cell wall formation.

bioethanol KNOX II transcription factors saccharification secondary cell walls xylan xylem and fiber development

1. *KNOX* Genes and Encoded KNOX Proteins in Plants

The *KNOX* genes are members of one of the ancestral gene families involved in the transition of plants from an aquatic to a terrestrial habitat during evolution [1]. *KNOX* genes encode homeodomain (HD)-containing TFs involved in various developmental processes. Typical HD proteins contain 60 amino acids, while the HD of *KNOX* proteins contains a highly conserved 63-amino acid stretch consisting of three ∝-helices that form a helix-turn-helix-type DNA binding motif [2] (**Figure 1**). Due to the presence of three extra amino acids between the first and second helices, all KNOX TF proteins are included in the TALE (three amino acid loop extension) superclass, the members of which are evolutionarily conserved from single-cell algae to higher plants [3]. The sequence immediately upstream of the HD, the ELK domain, has been suggested to function as a nuclear localization signal and be involved in protein–protein interactions [3]. In addition to the HD and ELK domains, a stretch of 100 amino acids located at the N terminus of almost all KNOX proteins, the MEINOX domain, also functions in protein–protein interactions [3]. This MEINOX domain in plants consists of two smaller domains, KNOX1 and KNOX2, separated by a poorly conserved linker sequence (**Figure 1**).

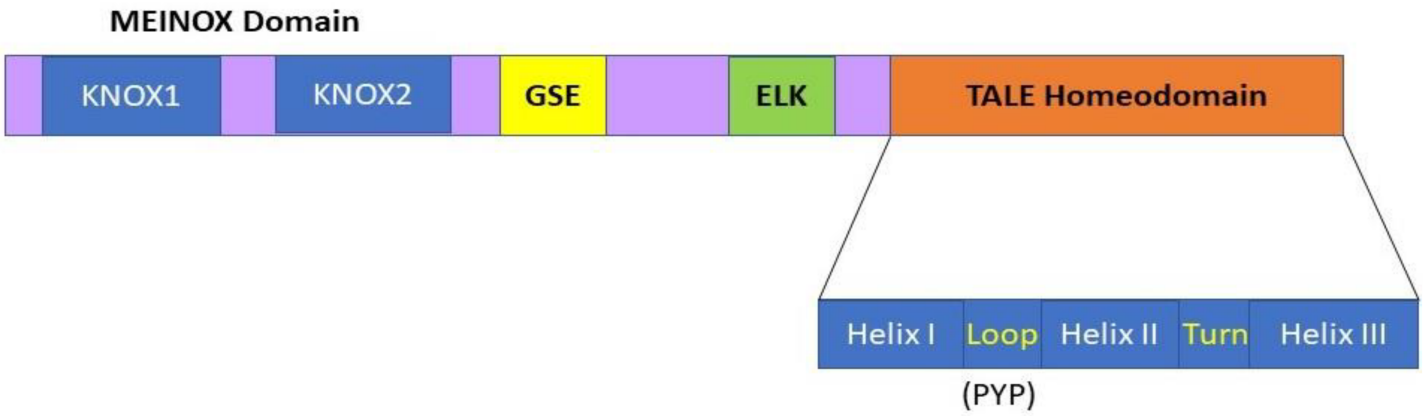

Figure 1. KNOX protein domain organization comprising MEINOX, ELK, and the TALE homeodomain (HD). The TALE homeodomain consists of three α-helices which comprise a helix-turn-helix type DNA binding motif, and contains three extra residues (PYP) in the loop between the first and second helices as compared to typical HDs. The MEINOX domain is present at the N terminus of the KNOX proteins, and it functions during protein–protein interactions. This MEINOX domain in plants is made of two smaller domains, KNOX1 and KNOX2, separated by a linker sequence. The ELK domain has been suggested to function as a nuclear localization signal and be involved in protein–protein interactions. The relatively small and less well-conserved amino acid motif located between the MEINOX and ELK domains is called the GSE domain; its function is not well understood.

Plant *KNOX* genes are divided into three subclasses based on their sequence similarity within the HD encoding regions, intron positions, expression patterns, and phylogenetic analysis [4][5][6][7]. *Class I KNOX* genes are similar to the *knotted1* gene of maize [8] and are mainly expressed in the shoot apical meristems (SAMs) of both monocot and eudicot plants. The *Class I KNOX* genes *STM*, *KNAT1*/*BP*, *KNAT2*, and *KNAT6* in *Arabidopsis* play an important role in the transcriptional regulation of meristem development, leaf shape control, and hormone homeostasis [9]. Loss-of-function mutations in these genes affect meristem maintenance and/or formation [10]. The only member of *Class III KNOX* gene, *KNATM*, is involved in the regulation of leaf polarity, leaf shape, and compound leaf development [11]. Four *Class II KNOX* genes (*KNAT3, KNAT4, KNAT5,* and *KNAT7*) in *Arabidopsis* form a separate monophyletic group and have several orthologues in higher plant genomes with few known functions [6][7][12]. Interestingly, *Class II KNOX* genes have been suggested to regulate the haploid-to-diploid morphological transition in land plants [1]. The first plant homeobox gene was discovered over 25 years ago; however, researchers only recently began to decipher the roles of *Class II KNOX* genes in higher plant growth and development. This entry focuses on the functions of *Class II KNOX* genes and their encoded proteins in higher plants.

2. The Expression Patterns of *Class II KNOX* Genes in Plants Provide Some Clues about Their Functionality in SCW Formation

The only *Class II KNOX* gene that has recently been well characterized and extensively studied is *KNAT7* [13][14][15][16][17][18][19]. The role of KNAT7 TF as a regulator in SCW biosynthesis was first reported in *Arabidopsis* through the

observation of the *irx* (irregular xylem) phenotype that occurred in a loss-of-function *knat7* mutant, *irx11* [18]. At the same time, the tight co-expression of the *KNAT7* TF gene along with SCW-specific *CesA* genes was reported using microarrays of *Arabidopsis* inflorescence stems undergoing SCW formation [20][21]. Promoter-GUS expression studies of *AtKNAT7* in *Arabidopsis* showed that it is highly expressed in developing xylem, phloem fibers, and cambium cells of inflorescence stems [14]. Wang et al. [16] recently examined whether several *Class II KNOX* genes from *Arabidopsis*, *KNAT3, KNAT4, KNAT5,* and *KNAT7*, were expressed during SCW deposition. All these *Class II KNOX* gene promoters regulated GUS expression in the vascular bundles in younger stems and intrafascicular fibers and vessel cells in older stems. These observations suggest that these *Class II KNOX* genes have similar expression patterns during the deposition of the SCWs. Qin et al. [17] also showed that while *KNAT7* expression was much higher in stem tissues, *KNAT3* expression remained similar in all tissues examined. Promoter-GUS fusions confirmed that *KNAT3* and *KNAT7* genes are co-expressed in developing xylem and interfascicular fibers in the *Arabidopsis* stem.

In poplar (*Populus balsamifera*), the expression of *PtKNAT7* gradually increases from the primary cell wall expansion stage to the mature xylem tissue formation stage, and from the youngest to the older internodes of stem [14]. Cotton *GhKNL1* was reported to be preferentially expressed in developing cotton fibers during SCW biosynthesis [22]. Switchgrass *KNAT7* also appears to be a functional ortholog of *Arabidopsis KNAT7*, based on its expression patterns [23]. In researchers' laboratory, researchers studied the expression patterns of two *Class II KNOX* genes, *KNAT3* and *KNAT7*, in tobacco (*Nicotiana benthamiana)* [15]. Higher expression of *NbKNAT7* was seen in older stems of tobacco showing secondary growth followed by young stems and old leaves, while *NbKNAT3* displayed higher expression in older leaves followed by roots and young leaves. These two *Class II KNOX* genes were also found to be highly expressed during tension wood formation in aspen. The expression of *NbKNAT3* and *NbKNAT7* in young and old stems indicates that they play a role in wood formation. Thus, *Class II KNOX* genes are associated with SCW formation during xylem and fiber development.

3. Genetic Mutations in *Class II KNOX* Genes Further Clarify Their Role in SCW Formation

Until 2005, *KNAT7* was not often discussed in mutation studies of the *Class II KNOX* genes; however, a number of *Class II KNOX* mutations have recently been studied in detail (**Table 1**). A T-DNA insertion in the intron of the *KNAT7* gene resulted in a loss-of-function mutant, *irx11*, that showed only a moderately weak growth phenotype. The *irx11* mutant also exhibited the typical *irx* phenotype in xylem vessels that were collapsed due to weak SCW formation. The *irx11* mutant did not have significantly altered cellulose or xylan content compared to controls. No lignin content of these mutants was reported at that time. While discovering a set of novel TFs involved in SCW biosynthesis, Zhong et al. [13] associated *KNAT7* expression with SCW formation, and the dominant repression of *KNAT7* (DR-*KNAT7* mutants) affected SCW formation in both xylem and fiber cells (Table 1). Curiously, they did not observe the typical *irx* phenomenon in these DR-*KNAT7* mutants, a tell-tale sign of weak SCW formation; however, the cell wall thicknesses of both xylem vessels and fibers were reduced compared to controls (28% down in

interfascicular fibers (IF), 26% down in vessels (V), and 80% down in xylary fibers (XF)). Several monosaccharides from the cell walls of DR-*KNAT7* mutants were reduced by 20–30%, except for arabinose, which was increased by 18%. The overexpression of *KNAT7* did not increase the SCW thickness of fibers and vessels. These results indicated that KNAT7 could be a positive regulator of SCW formation in *Arabidopsis*. However, Li et al. [14] reported a contrasting observation that loss-of-function mutants in the *AtKNAT7* gene resulted in differential thicknesses of interfascicular and xylary fibers compared to vessels (58% up in IF, 35% down in V, and 31% up in XF; **Table 1**). The vessels walls were thinner, resulting in collapsed xylem vessels that showed the *irx* phenotype (similar to [18]); however, the interfascicular fibers were significantly thicker than in the wild type control, suggesting that KNAT7 is a transcriptional repressor of fiber SCW formation (but a transcriptional activator of vessel SCW formation). *KNAT7* overexpression lines exhibited thinner fiber walls (57% down in IF) with normal vessel and xylary fiber cell walls. Interestingly, even though many SCW-specific cellulose and xylan synthesis genes were upregulated in these mutants, no quantitative changes in cellulose or xylan were reported. All ten lignin synthesis genes tested were upregulated along with an 11% increase in lignin content of cell walls from the stem. Li et al. [18] speculated that KNAT7 interacts with different partner proteins in different cell types to form functionally distinct complexes. Recently, the regulatory roles of other members of the *Class II KNOX* gene family, *KNAT3, KNAT4,* and *KNAT5*, in SCW formation were explored in *Arabidopsis* inflorescence stems [16][17] (**Table 1**). Loss-of-function mutants of *knat3, knat4,* and *knat5* did not produce any *irx* phenotype, as observed in the case of loss-of-function mutants of *knat7* [16]. This could be due to the functional redundancy of *KNOX II* genes. However, *knat3/knat7* double mutants displayed an enhanced *irx* phenotype compared to single *knat7* mutants. These double mutants had thinner interfascicular fiber cell walls compared to the single mutants and wild-type plants (40% down in IF) indicating a potentially positive regulatory role of KNAT3 in combination with KNAT7 in xylem SCW development. Even though many SCW genes were highly expressed in the *knat3/knat7* double mutants, the cellulose and xylan contents of their cell walls were reduced by 19% and 43%, respectively, and the changes in lignin content were not significant. The Syringyl to Guaicyl (S/G) lignin ratio was down by 83%; however, it was not possible to correlate all these cell wall content changes with the changes in gene expression patterns. In addition, the severe *irx* phenotype in these double mutants indicated the overlapping roles and partial functional redundancy of KNAT3 and KNAT7 in xylem vessel development during SCW formation. Furthermore, *KNAT3* overexpression in *Arabidopsis* resulted in thickened interfascicular fibers in the SCW of inflorescence stems [16]. This entry described KNAT3 as a potential transcriptional activator, working together with KNAT7 to promote SCW biosynthesis in xylem vessels. A synergistic interaction of KNAT3 and KNAT7 to regulate monolignol biosynthesis in *Arabidopsis* was also reported in another study [17]. Most importantly, they attempted to link S-lignin formation with *KNAT3* and *KNAT7* expression; however, they could not show the direct transcriptional regulation of a key gene, ferulate 5-hydroxylase (*F5H*), involved in S-lignin formation by KNAT3 or KNAT7. Similar to the earlier observation by Wang et al. [16], the overexpression of KNAT3 also caused thickening in the interfascicular fiber walls, indicating the positive regulation of interfascicular fiber wall development by KNAT3. These studies by Wang et al. and Qin et al. [16][17] reconciled the paradoxical observations about *KNAT7* mutants in *Arabidopsis* and indicated that KNAT3 and KNAT7 might be working synergistically in fibers, but antagonistically in vessels, during the regulation of SCW biosynthesis (**Table 1**).

Table 1. Gene Mutations in *Class II KNOX* genes and their effect on SCW formation.

Target Gene	Mutation	Type of Mutation	Anatomy of Mutants	References
AtKNAT7	*irx11*	T-DNA insertion	Irregular xylem with collapsed vessels.	[18]
AtKNAT7	-	Dominant repression	Reduced cell wall thickness of both xylem vessels and fibers; reduced composition of several monosaccharides from the cell walls.	[13]
AtKNAT7	*irx11*	Loss-of-function mutation	Thinner vessels walls resulted in a collapse of xylem vessels that showed the *irx* phenotype and thicker interfascicular fibers compared to controls; increase in lignin content.	[14]
AtKNAT3, AtKNAT4, AtKNAT5	Single mutants	T-DNA insertion	No *irx* phenotype.	[16]
KNAT3/KNAT7	Double mutant	T-DNA insertion	Enhanced irregular xylem (*irx*) phenotype characterized by weak inflorescence stem; reduced interfascicular fiber wall thickness and modified cell wall composition.	[16]
KNAT3/KNAT7	Double mutant	Chimeric repression	Thinner interfascicular fiber cell walls compared to single mutants and wild type (WT); reduced cellulose and xylan and reduced S/G lignin ratio.	[17]
OsKNAT7	*CRISPR/CAS9*	T-DNA insertion	Thicker fiber cell walls; larger grain size due to cell expansion in spikelet bracts.	[24]
GhKNL1	-	Dominant repression	Abnormal shorter fiber length.	[22]

4. Targeted Genetic Manipulations in *Class II KNOX* Genes Confirm Their Role in SCW Formation

Apart from the detailed study of *Class II KNOX* gene mutants, targeted genetic manipulations of *Class II KNOX* genes, especially, *KNAT7* genes have offered some additional clues regarding the functions of these genes (**Table 2**). While the overexpression of *KNAT7* in *Arabidopsis* did not produce any specific SCW phenotype [13], subsequently, Li et al. [14] reported that such experiments produced thin interfascicular fibers without any changes in wall thickness of vessels suggesting that KNAT7 TF is indeed a regulator of SCW formation.

Table 2. Genetic manipulation of *Class II KNOX* genes in different plant species.

Gene Used	Target Plant	Gene Modification Method	Impact on Transgenic Plants	References
AtKNAT7	Arabidopsis	Overexpression	Thin interfascicular fiber walls, but no change in vessel wall thickness.	[14]
Cotton GhKNL1	Arabidopsis	Overexpression	Thinner interfascicular fibers and slightly thinner vessel walls, but no change in xylary fibers.	[22]
Cotton GhKNAT7	Arabidopsis	Overexpression	Reduced deposition of lignocellulose in interfascicular fibers, but no change in the SCWs of xylem fibers and vessels.	[7]
NbKNAT7	Tobacco	Downregulation by VIGS and *RNAi*	Increased xylem proliferation with thin-walled fiber cells, increased polysaccharide extractability, and higher saccharification rate.	[15]
AtKNAT7	Arabidopsis	Dominant repression	Reduced expression of SCW genes that resulted in thinner fiber cell walls with altered cell wall composition.	[13]
PtKNAT7	Poplar	Overexpression	Enhanced expression of SCW genes, CesA8, IRX9, PAL, and CCR.	[25]
PtKNAT7	Poplar	Downregulation by antisense	Reduced expression of SCW genes, reduced lignin content, altered lignin composition (S/G ratio), and increased saccharification.	[25]

[22] and poplar *PtKNAT7* [14] rescued the defective *irx* phenotype of the *knat7* mutants, suggesting the *NL1* gene functional conservation of *KNAT7* genes among Arabidopsis, cotton, and poplar. The overexpression of cotton *GhKNL1* in *Arabidopsis* resulted in thinner interfascicular fibers and slightly thinner vessels walls without any change in the xylary fibers compared to control plants [22]. The overexpression of cotton *GhKNAT7* significantly reduced the deposition of lignocellulose in the interfascicular fibers of *Arabidopsis* [7]. However, the SCWs of the xylem fibers and vessels in the transgenic plants did not show any difference from the control plants. The dominant repression of the same cotton *KNAT7* orthologue in *Arabidopsis* produced thinner interfascicular fibers, but thicker vessels and xylary fiber walls, suggesting that KNAT7 can act as a negative or positive regulator of SCW formation in different cell types.

In researchers' laboratory, researchers generated *RNAi* lines of tobacco (*N. benthamiana*) that exhibited reduced expression of *KNAT7* [15]. *NbKNAT7* downregulated through a transient virus-induced gene silencing (VIGS) system resulted in increased xylem proliferation with thin-walled fiber cells. The glycome analyses of the cell walls showed increased polysaccharide extractability in 1 M KOH extracts of the VIGS-*NbKNAT7* lines, suggestive of SCW loosening. In addition, there were increased saccharification rates (40% higher than control) in stems of VIGS-*NbKNAT7* lines, which indicated the alteration of cell wall composition in VIGS lines downregulated for the *NbKNAT7* gene. Similar to the VIGS results, the stems of stable *RNAi* lines also showed increased xylem area in their stems as compared to control stems [15]. The cell walls of xylem fibers were thinner (over 50%) in the *RNAi* lines as compared to vector control lines. The stems of *KNAT7* repression lines in tobacco showed reduced

expression of SCW genes that resulted in thinner fiber cell walls with altered cell wall composition [15]. All these results suggested that KNAT7 TF might act as a positive regulator of SCW formation in tobacco.

In a recent study performed in researchers' laboratory by Ahlawat et al. [25], transgenic poplar plants overexpressing *PtKNAT7* and *AtKNAT7* genes showed enhanced expression of the SCW genes *CesA8, IRX9, PAL,* and *CCR,* and reduced expression of the same genes in the poplar *PtKNAT7* antisense plants. These results further suggested a positive regulatory role of KNAT7 in SCW formation in poplars. In addition, the genetic suppression of *KNAT7* in transgenic poplar stems reduced lignin content by about 6% and altered the lignin composition (S/G ratio) of poplar wood with increased saccharification ability (44–53% higher saccharification efficiency over control plants). Yoo et al. [26] also reported a negative correlation between lignin content and the saccharification efficiency of woody tissues and a positive correlation between the S/G ratio and the saccharification efficiency of SCW biomass. Therefore, a change in the S/G ratio and reduction in lignin content might be important for improving the saccharification efficiency of SCW biomass. All the studies reported so far in Arabidopsis and other higher plants suggest that KNAT7 acts differentially as a negative and positive regulator of SCW biosynthesis in different cell types of the same plant or in different plant species.

5. Transcriptional Network of the *Class II KNOX* Genes Involved in SCW Formation

A complex network of transcription factors regulates SCW biosynthesis in plants [27][28][29][30][31][32]. Among these, some Class II KNOX TFs also regulate SCW biogenesis. The major constituents of the SCW are cellulose, lignin, and hemicelluloses [33]. Cellulose is a polymer of glucose synthesized at the plasma membrane by the cellulose synthase (CesA) complex [34], while lignin is composed of guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) units that are synthesized through the phenylpropanoid pathway [35]. Xylan is the major hemicellulose component in the SCW and consists of a linear backbone of β-(1–4)-linked D-xylosyl (Xyl) residues and α-linked (OMe(methyl)) glucuronic acid (GlcA) side branches [36]. Many specific genes involved in cellulose, hemicellulose, and lignin biosynthesis pathways have previously been identified in plants (e.g., [36][37][38]) and it was anticipated that Class II KNOX TF proteins might directly regulate the expression of some of these genes. The first direct evidence of KNAT7-mediated regulation of xylan biosynthesis in the SCW was reported only recently by He et al. [19], who demonstrated that KNAT7 physically binds to the promoters of the xylan biosynthetic genes, *IRREGULAR XYLEM 9* (*IRX9*)*, IRX10, IRX14L*, and *FRAGILE FIBER 8 (FRA8*; **Figure 2**). Wang et al. [39] also reported the involvement of KNAT7 in xylan synthesis during mucilage production. While various cellulose and lignin biosynthesis genes have been shown to be differentially expressed in various *knat7* mutants and during the ectopic expression of the *KNAT7* gene in transgenic plants, the direct regulation of any of these SCW genes by KNAT7 TF has not yet been reported. In addition, no information is currently available on transcriptional regulation by the TFs encoded by the other three *Class II KNOX* genes, namely *KNAT3*, *KNAT4*, and *KNAT5,* or their orthologs in any other plant species.

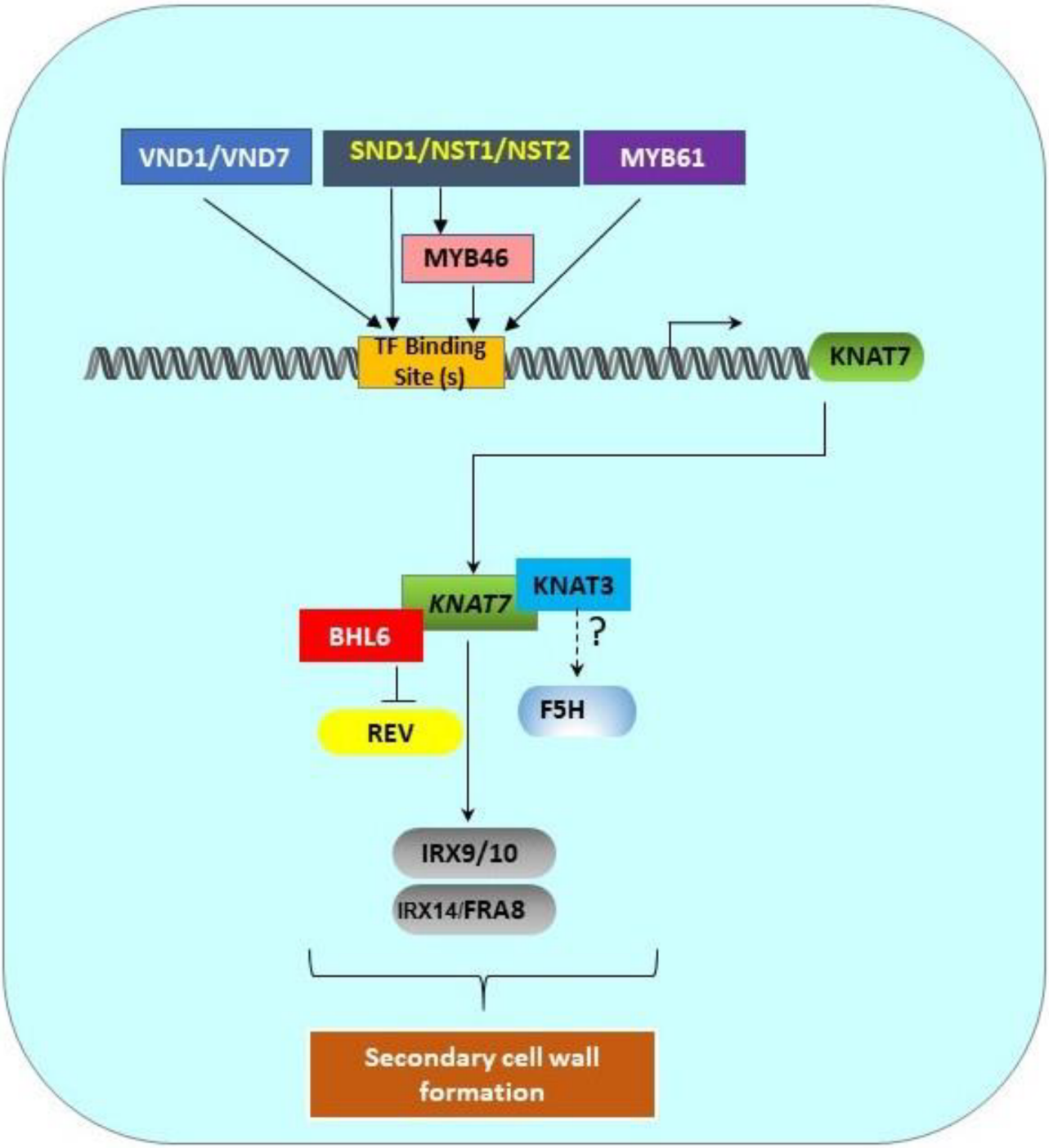

Figure 2. Transcriptional regulation pathway of *KNAT7* gene. SCW-associated upstream transcription factors (MYB61, SND1/NST1/NST2, VND1/VND7) and MYB46 directly bind the binding sites in the *KNAT7* gene promoter to regulate the expression of the *KNAT7* gene. KNAT7 positively regulates the expression of various xylan synthesis genes (*IRX9/10* and *IRX14L/FRA8*). Interactions between KNAT7 and KNAT3 TFs might regulate F5H expression, and the interactions between KNAT7 and BLH6 negatively regulate the expression of the homeodomain-ZIP (HD-ZIP) TF gene Revoluta. All these interactions ultimately regulate SCW formation in higher plants. All genes are shown as rounded rectangles and proteins are indicated by rectangles.

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