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The role of HD-ZIP III transcription factors and *miR165/166* in vascular development and secondary cell wall formation

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The Arabidopsis vascular system is composed of xylem and phloem, which form a well-defined collateral pattern in vascular bundles. Xylary element and fibers develop secondary cell walls (SCWs) that provide mechanical strength to support plant growth and to transport water and minerals to all above ground organs. SCWs also constitute the majority of terrestrial biomass for biofuel production. The biosynthesis of secondary cell walls are known to be under transcriptional regulation. Transcription factors, such as NAC (NAM, ATAF1/2 and CUC2) and MYB domain proteins, serve as master regulators in SCW development. Recent studies indicated that Class III homeodomain leucine zipper transcription factors (HD-ZIP III TFs) and *microRNA 165/166* (*miR165/166*) may play important roles in SCW formation. Here we discuss the diverse functions of *miR165/166* and HD-ZIPIII in vascular development and their interaction with the regulatory pathways of SCW biosynthesis.

Plant vascular tissues are composed of xylem and phloem; xylem transports water and minerals from roots to above ground organs, while phloem transports photosynthates from sources to sinks. Tracheary elements, xylary fibers and interfascicular fibers develop secondary cell walls that consist of cellulose, hemicellulose and lignin.[1,2] These wall materials deposit between primary cell wall and plasma membrane soon after cells completing expansion. The accumulation of SCW gives rigidity to fiber and vessel cells facilitating their functions of mechanical support and water transport.[3] In addition to the developmental importance, secondary cell walls (or better known as lignocellulosic biomass) have been used as feedstocks in biofuel industry. Therefore, understanding the regulatory mechanisms of vascular development and secondary cell wall biosynthesis has significant importance in fundamental biology as well as in biotechnological applications.

Vascular tissues are produced through proliferation and differentiation of the vascular cambium. In tree species, vascular cambium is responsible for the formation of secondary xylem or wood. Whereas, the slow growth and long life cycle impedes genetics and molecular biology studies of vascular development and wood formation in trees.[4] In contrast, Arabidopsis is a fast growing model plants with notable secondary growth at the bottom part of the inflorescence stem, making Arabidopsis an excellent system to study xylem development.[5] In Arabidopsis, xylem and phloem are organized in a collateral pattern, with xylem located inside and phloem outside of the vascular bundle.[6,7] During secondary growth, vascular cambium and interfascicular cambium connect and form a cylindrical structure, producing secondary xylem and phloem.[7] A group of HD-ZIP III TFs, whose expression is post-transcriptionally regulated by *miR165/166*, play key roles in the establishment of the vascular pattern.[6,8-10] Mutants of HD-ZIP III TFs also exhibited obvious defects in secondary cell wall formation.[11-13] In this review, we discuss the recent progress on the functions of miR165/166 and HD-ZIP III TFs in determining vascular pattern and secondary cell wall formation.

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The Functions of HD-ZIP III TFs in Vascular Differentiation and Patterning

The HD-ZIP III TFs have 5 members, i.e. REVOLUTA/INTERFASCICULAR FIBERLESS1 (REV/IFL1), PHABULOSA (PHB), PHAVOLUTA (PHV), CORONA (CAN/AtHB15) and AtHB8. These proteins are characterized by an HD-ZIP domain for DNA binding and protein dimerization[14] and a highly conserved lipid or steroid binding START (Steroidogenic acute regulatory protein-related lipid transfer) domain[15] (**Fig. 1**). The HD-ZIP III TFs regulate a number of developmental processes, such as embryo patterning, meristem initiation and homeostasis, lateral organ polarity and vascular development in Arabidopsis.[11-13,16-19] In this review, we mainly focus on the functions of HD-ZIP III TFs in vascular differentiation and secondary cell wall development.

The functions of HD-ZIP III TFs in vascular differentiation and patterning have been revealed through analyses of loss- and gain-of-function mutants. Double mutant of *phb phv* showed no obvious phenotypes in vascular patterning or plant growth, but aberrant amphicribral vasculature structure with xylem surrounded by phloem were observed in the triple mutant of *phb phv rev*.[20] In contrast, gain-of-function point mutations of any of these 3 genes, *PHB*, *PHV* or *REV*, resulted in amphivasal vasculatures with

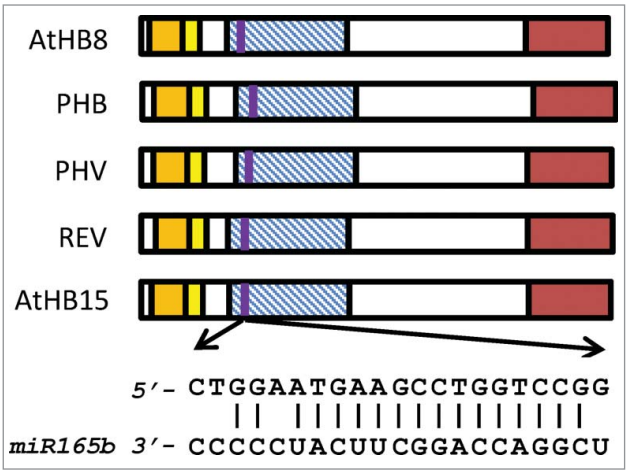

Figure 1. Domain structures of the 5 HD-ZIP III transcription factors (TFs) and the complementary sequence of miR165b. The four known functional domains (from N-terminus to C-terminus) of the HD-ZIP III TFs are: the homeodomain that is responsible for DNA binding (orange); the basic Leucine Zipper domain (b-ZIP), which is responsible for DNA binding and dimerization (yellow); the START domain, a putative lipid or steroid binding domain (blue diagonal lined). The miR165B binding site is located in the coding sequence of START domain (purple bar); the C-terminal MEKHLA domain (red). The paring of complementary sequence of AtHB15 and miR165b is shown at the bottom of the diagram.

phloem surrounded by xylem.[17,21,22] These results indicate that PHB, PHV and REV function redundantly in determining the collateral vascular bundle organization. The function of AtHB15 in vascular development was determined through a comprehensive mutant analysis of the HD-ZIP III TFs.[13] Triple mutant *phb phv cna/athb15* developed amphivasal vasculature internally away from the stem periphery.[13] The distinct vasculature phenotypes (amphicribral vs amphivasal) between *phb phv rev* and *phb phv cna/athb15* indicates that REV and CNA/AtHB15 may have antagonistic functions in vascular patterning. This is supported by the fact that the vascular defects of *rev/ifl1* were partially suppressed in the *rev cna athb8* triple mutant.[13,23] A dominant-negative mutation of CNA/AtHB15 further supports its function in vascular patterning as manifested by the development of ectopic amphivasal bundles in the *clv3–1cna-1* double mutant.[24] The fifth member of HD-ZIP III TF, AtHB8 interacts with auxin signal and functions as a positive regulator of procambium and cambium development in vascular tissues.[25-29] The functions of HD-ZIP III TFs in vascular differentiation and patterning may be conserved in different plant species as shown by characterization of their orthologs in *Zinnia elegans* and rice.[11,12,30]

MiR165/166 Mediated Regulation in Vascular Development and Patterning

MicroRNAs are small (around 21 nucleotides) non-coding RNAs that post-transcriptionally regulate target genes by paring with the complementary sequences located in the target transcripts. The binding of microRNAs causes translational repression or degradation of target mRNAs.[31,32] The expression of the 5 HD-ZIP III TFs is predicted to be regulated by *microRNA 165/166* (*miR165/166*) .[33] In Arabidopsis, there are 9 members in *miR165/166 g*ene family, with 2 *miR165* (*miR165a* and *miR165b*) and 7 *miR166* (*miR166a* to *miR166g*). The mature microRNAs from these 2 species differ at only one single nucleotide.[33]

The complementary sequence of miR165/166 locates in the putative lipid/sterol-binding START domain of the HD-ZIP III family members (**Fig. 1**). Missense mutation in the START domain resulted in resistance to mRNA cleavage, and in turn over-accumulation of corresponding transcripts in the gain-of-function mutants *phb-1d*, *phv-1d* and *avb*.[17,20,22,33] The common phenotype of these dominant mutants is the development of radialized amphivasal vascular bundles, despite their phenotypic difference in meristem patterning and lateral organ development. In contrast, a point mutation in the START domain in AtHB15/INCURVATA4/CORONA resulted in a higher expression of the *AtHB15/ICU4* transcripts and incurvature in leaves, but vascular patterning (bilateral organization) stays normal,[18,19] indicating that the function of AtHB15/ICU4/CNA is different from PHB, PHV and REV in vascular development. Taken together, interruption of the *miR165/166* binding to the complementary sequence in HD-ZIP III TFs may affect vascular development.

Direct evidence of *miR165/166* function in vascular development comes from the identification and analysis of the corresponding activation tagged mutants. Activation tagging of *miR166a* and *miR166g* resulted in significant reduction of the transcript levels of *PHB*, *PHV* and *AtHB15*, but less or no obvious effect on the expression level of *REV* or *AtHB8*.[34,35] We recently reported that activation-tagging of *miR165b* that showed similar effect on the expression of HD-ZIP III genes, with *PHB*, *PHV* and *AtHB15* as that mainly affected targets.[36] The common phenotype of ectopic internalized amphivasal bundle(s) in all 3 activation-tagged lines in *miR166a*, *miR166g* and *miR165b* may reflect the fact that the same subset of HD-ZIP III genes were significantly repressed in these lines. In contrast, transgenic overexpression of *miR165a* under the control of 35S promoter caused repression on all of the 5 HD-ZIP III genes.[10] The difference between overexpression (driven by 35S promoter) and activation-tagging of *miR165/166* in regulating HD-ZIP III genes may be explained by the difference in their promoters. While 35S promoter causes a constitutive overexpression, activation tagging leads primarily to an enhancement of the endogenous expression pattern.[37] In fact, different microRNAs showed distinct and dynamic expression patterns as characterized by P*romoter:GUS* analyses.[38] It is also possible that the activation tagging preserved the sequence integrity around the *miR165/166* loci, which may contain regulatory elements, such as small peptides.[39]

Transcriptional Pathways in Secondary Cell Wall Biosynthesis

NAC domain and MYB domain transcription factors (NACs and MYBs) function as master switches in secondary wall

biosynthesis (**Fig. 2**). NACs are plant-specific proteins with a conserved NAC domain in the N-terminal region and an activation domain in the C-terminal region.[40,41] NAC SECONDARY WALL THICKENING FACOTRS1 (NST1) and NST2 activate secondary cell wall thickening in anther endothecium,[42] while NST1 together with SECONDARY WALL-ASSOCIATED NAC-DOMAIN PROTEIN 1 (SND1) control secondary wall biosynthesis in fiber cells.[2,43] VASCULAR-RELATED NAC DOMAIN6 (VND6) and VND7 play important roles in metaxylem and protoxylem formation, respectively.[44] The other VND members (VND1–5) positively regulate secondary cell wall deposition in fibers.[45] Direct downstream of the NACs are the MYB master regulators, *MYB46* and *MYB83*[46,47]. A number other transcription factors, including many other NACs, MYBs and KNATs, function downstream of master NACs/MYBs and form complex regulatory networks in secondary wall biosynthesis (**Fig. 2**).[48-53]

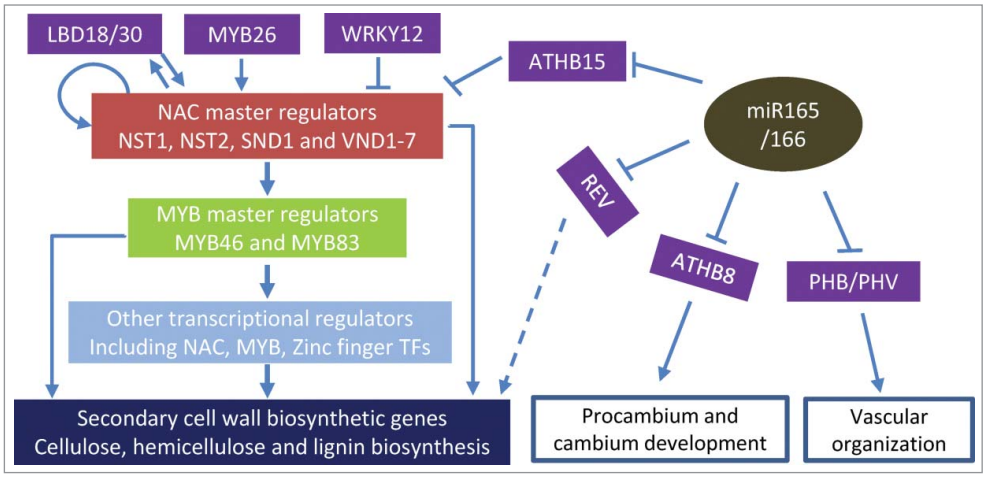

Figure 2. Transcriptional regulation of secondary cell wall biosynthesis and the role of *miR165/166-ATHB15*.

Both positive and negative regulators of the master switches have been identified in recent years. MYB26 positively regulate the expression of NST1 and NST2, and in turn activate secondary cell all thickening in anther endothecium.[54,55] In dicotyledonous plants, WRKY12 functions as a negative regulator of secondary cell wall thickening by directly repressing the expression of *NST2* in pith cells.[1,56] Feedback regulation is also common in regulating secondary cell wall development. SND1 actually binds to its own promoter and functions as a positive regulator for its own expression.[57] Another example is *MYB32*, a direct target of SND1, represses *SND1* expression and secondary cell wall biosynthesis.[57,58] Expression of LOB domain proteins *LBD18/ASL20* and *LBD30/ASL19* is dependent on VND6 and VND7, while ectopic expression of *VND7* was detected in *LBD18* overexpressing plants indicating a positive feedback regulatory mechanism.[59-61]

HD-ZIP III TFs and *miR165/miR166* Interact with Secondary Wall Regulatory Pathways

The first line of evidence showing that HD-ZIP III TFs involve in secondary cell wall biosynthesis comes from the characterization of *rev/ifl1* mutant. The null mutation of *REV* resulted in disruption of interfascicular fiber differentiation in stems.[23,62-64] These studies indicate that REV functions as a positive regulator of the differentiation of interfascicular fibers, or secondary cell wall synthesis in general.[50] AtHB8 also functions as a positive regulator of secondary wall development. Although the knock-out mutants of *athb-8* showed no phenotypes in secondary cell wall development, overexpression of AtHB8 promoted xylem differentiation.[16] In contrast to REV and AtHB8, AtHB15 functions as a negative regulator of secondary wall development. In Populous, synthetic miRNA knock-down of the AtHB15 ortholog, POPCORONA, resulted in an abnormal lignification in pith cells, while overexpression of a miRNA-resistant POPCORONA delayed lignification of xylem and phloem fibers during secondary growth.[65] In Arabidopsis, examination of 2 *athb15* knockout mutants confirmed *AtHB15* function as a negative regulator of secondary wall development.[36] Secondarily thickened cell walls and over-accumulation of all 3 major secondary wall components were observed in the pith cells of the *athb15* mutants (**Fig. 2**).[36]

Activation tagging of *miR166a* resulted in meristem enlargement (the mutant was hence named as *men1*) and greatly expanded protoxylem and metaxylem.[34] It is proposed that *miR166*-mediated *ATHB15* mRNA cleavage is a principal mechanism for the observed vascular development phenotype.[34] In both vascular and interfascicular regions, vascular cambium are formed in the periphery of the existing secondary wall bearing vessel and fiber cells. Indeed, the expanded protoxylem and metaxylem in *men1* was believed to be the results of promoting the activity of fascicular cambium and interfascicular cambium. Recently, we reported a *miR165b* activation-tagging line, *stp-2d*, which showed a dominant secondary cell wall thickening phenotype. The stems of *stp-2d* are much thinner than the wild type indicating that the cambium activity is unlikely enhanced in the *stp-2d* mutant. Actually, the activation tagging of *miR165b* in the *stp-2d* mutant resulted in secondary cell wall biosynthesis in pith cells, which are located in the center of the stem. Transgenic overexpression of a microRNA resistant *AtHB15* (*mAtHB15*) further indicate that *miR165b* functions through *AtHB15* in regulating secondary wall development in pith.[36]

The *miR165/166* mediated *ATHB15* cleavage impact the known regulatory pathways in secondary wall development (**Fig. 2**). Two NAC master switches, *SND1* and *NST2*, are upregulated in *athb15* mutants confirmed that AtHB15 functions as

a negative regulator of the secondary wall related regulatory pathway.[36] The pith phenotypes of *stp-2d* and *athb15* mutants are quite similar to that of previously described *wrky12* mutants.[56] Gene expression analysis indicated that *WRKY12* and *AtHB15* may not directly regulate each other.[36] Although the exact relations between WRKY12 and *miR165b-AtHB15* involved regulatory pathways are still unknown, these studies shed new light in our understanding of the regulation of secondary cell wall formation.

Conclusions

Plants have to invest a large amount of resources and energy to produce secondary cell walls, which once made cannot be recycled and reused by the plants. Therefore, plants have established elegant regulatory pathways to ensure only certain cell types develop secondary cell walls at certain developmental stages.[66] Accumulation of secondary walls in ground tissues, such as pith, increases biomass density and may boost biofuel production.[56,67] The miR165/166 and HD-ZIP III TFs control many aspects of plant growth and development. In this review, we discussed the functions of miR165/166 and HD-ZIP III TFs in regulating vascular development and secondary cell wall biosynthesis. Future studies should focus on elucidation of the functional specificity of the close members in miR165/166 family, as well as their interaction with existing regulatory pathways. The studies on miR165/166 and HD-ZIP III TFs will enhance our understanding of vascular development and facilitate genetic engineering of biomass feedstocks for biofuels production.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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