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Multi-layered Regulation of Plant Cell Wall Thickening

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Plants need to develop thickened cell walls with appropriate localization through precise regulation during the process of growth and development in order to support their body weight and to build long distance transportation systems. Wall thickening is achieved through a multitude of regulatory networks in various tissues under changeable environments. In this mini-review, we summarize current understanding of the regulatory pathways and mechanisms involved in cell wall thickening. Regulation of cell wall thickening is not only mechanistically essential to understand the plant structure accretion but also has applicable significance to plant cell wall biomass utilization.

Keywords: Cellulose • Lignin Protein modification • Regulation • Secondary cell walls • Transcription

Introduction

Cell wall thickening occurs after cell expansion in a variety of cell types and is also modified in response to specific environmental cues. The timing and localization of cell wall thickening in distinct cell types is essential for the proper function and adaptation of plants to their environment. Thickened cell walls are developed in various tissues, such as in tracheary elements (TEs), vessel elements, xylary fibers in xylem, sieve elements, sclerenchyma cells in phloem and bark, endothecium cells in anther, guard cells in stomata on leaf surface and Casparian strip (CS) in root endodermis. The cell wall thickening process occurs in an accurate spatio-temporal manner through precise regulation. A multitude of regulatory networks are involved in the formation of thickened-walls to confer cells with their proper functions to ensure vigorous plant growth and development.

Thickened walls are formed usually as secondary cell walls (SCWs), deposited at the inner side of primary cell walls (PCWs). SCWs are organized in laminar and patterned structure, mainly composed of cellulose, hemicellulose and lignin (Zhong et al. 2019). In SCW, cellulose is structured with a higher degree of crystallinity and polymerization than in PCW, and the SCW hemicelluloses are composed of monosaccharides proportionally different to those in PCW (Scheller and Ulvskov 2010, Polko and Kieber 2019). However, the deposition of lignin is closely linked with the process of cell wall thickening, making cell walls rigid and waterproof. As most of the plant photosynthetic products are converted into cellulose, hemicellulose and lignin and stored in thickened walls, plant cell walls are considered the main biomass resource for renewable biofuel and biomaterial production.

Multidecadal efforts to characterize the regulation of cell wall thickening have revealed various regulatory networks, molecular pathways and signaling in this process. Here, we consider how the formation of the thickened walls is regulated with respect to the regulatory pathways of wall thickening at multiple levels.

Atlas of the transcriptional regulatory networks for wall thickening

A variety of cells undergo wall thickening in plants. The most studied wall-thickened cell is the vessel element in xylem, which transports water and minerals from underground to the aerial parts of plants. Vessel elements in eudicots are differentiated from cambium/procambium cells and undergo a developmental process of cell expansion, SCW thickening and programmed cell death. Hollow vessel elements are connected together to form a circulatory system in plants (Evert 2006). In addition to vessel elements, fiber cells are another type of founding cells with heavily thickened walls in the xylem tissue of angiosperms (Evert 2006). In flowers, the anther endothecium develops peculiar cell walls called fibrous bands to provide mechanical force to help anther dehiscence and pollen release (Van der Linde and Walbot 2019). The endodermis in roots has a ring-like cell wall thickening band called the CS, which helps plants control the absorption of water and minerals from the soil (Geldner 2013). Guard cells in stomata have thickened walls at specific ledges, providing a structure needed for stomata function (Zhao and Sack 1999). In C4 plants, the bundle sheath cells around the vein of a leaf have thickened walls for the compartmentalization of the photosynthesis process in separate locations (Griffiths et al. 2013). Thus, cell walls are thickened in

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various circumstances for different biological functions, which need to be specifically programmed and regulated during plant growth and development.

Although wall thickening occurs in a variety of tissues, our current understanding of the initiation of wall thickening is primarily based on evidence from studies of vessel elements and interfascicular fiber cells. In recent years, the analysis of the SCW deposition in xylem development has depicted transcriptional regulatory networks that guide the induction of wall thickening and SCW biosynthesis (summarized in **Fig. 1**). The transcriptional regulators at the top layer of the networks are considered 'master switches' for wall thickening and include VASCULAR-RELATED NAC DOMAIN (VND1-VND7), SECONDARY WALL-ASSOCIATED NAC DOMAIN (SND1/NST3 and SND2) and NAC SECONDARY WALL THICKENING PROMOTING FACTORs (NST1 and NST2) transcription factor genes. They belong to the NAC transcription factor gene family and, among them, VND6 and VND7 play specific roles for wall thickening in vessels, NST1 and SND1/NST3 in interfascicular fiber cells and NST1 and NST2 in anther endothecium (Kubo et al. 2005, Mitsuda et al. 2005, 2007, Zhong et al. 2006, Yamaguchi et al. 2011). The transcription factor genes are organized in a hierarchy of networks regulating cell wall thickening. The transcription factors of the top layer pass the regulatory cascade down to the second layer via direct control of expressions of the transcription factor genes such as MYB46 and MYB83 (McCarthy et al. 2009). It is generally thought that the third-layer regulators, controlled by the second-layer transcription factors, act as activators or repressors to control expression of wall polymer biosynthesis genes (Rao and Dixon 2018, Zhong et al. 2019). For example, MYB6, MYB42 and MYB43 promote cellulose biosynthesis, while MYB52 inhibits cellulose biosynthesis. KNAT7 acts as a repressor of both hemicellulose and lignin biosynthesis (Zhong et al. 2008, Liu et al. 2014). MYB4 represses lignin biosynthesis (Wang et al. 2020b, Xiao et al. 2021). In addition, the transcription factors at the top layer can also directly regulate the third-layer TFs and wall polymer biosynthesis genes. For example, VND7 regulates *MYB103* as well as *CesA*s for SCW synthesis by binding their promoters (Yamaguchi et al. 2011). F5H, which catalyzes a branch point in lignin biosynthetic metabolism toward the formation of syringyl monolignol, is regulated directly by SND1 (Zhao et al. 2010).

Wall thickening in xylem vessels and fibers follows a hierarchical transcriptional regulation. However, it is yet to be investigated how such transcriptional regulatory networks are activated in the first place. In recent years, evidence shows there are upstream regulators manipulating the transcriptional regulatory cascade for wall thickening in various circumstances. During the initiation of cell wall thickening in Arabidopsis roots, the expression of VND7 can be regulated by E2Fc through

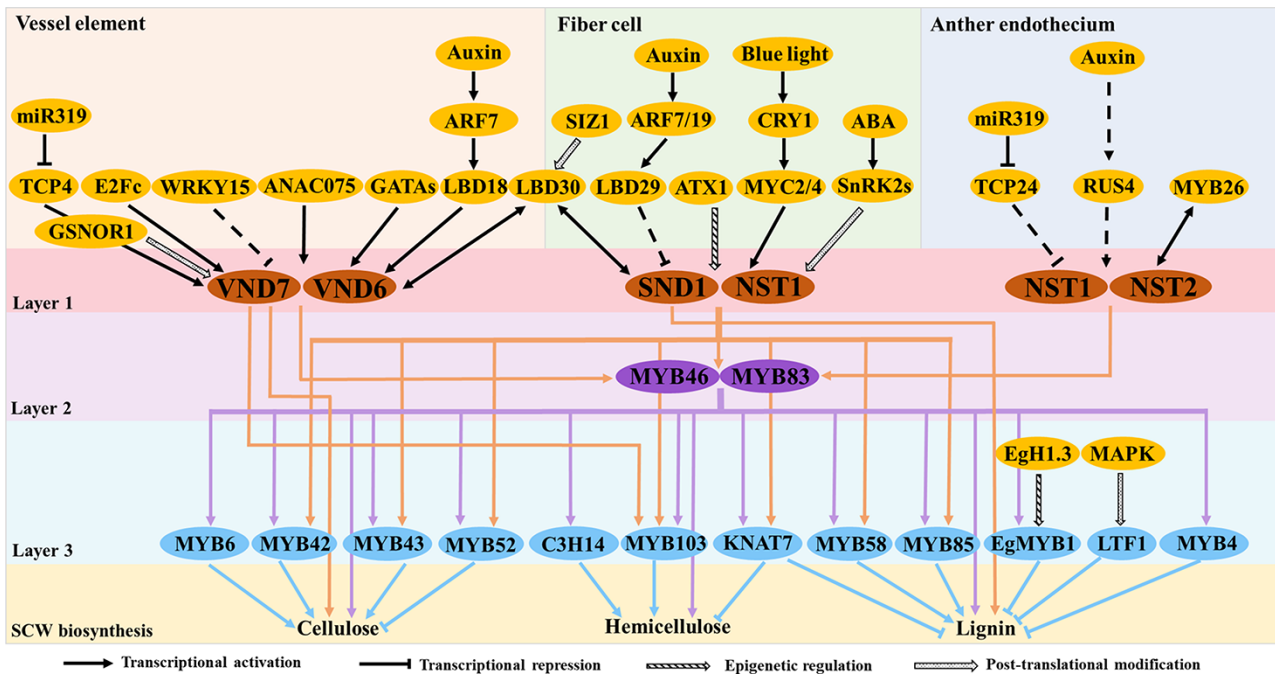

Fig. 1 Outline of the transcriptional regulatory networks involved in plant cell wall thickening. The transcriptional regulatory networks are hierarchically organized on multiple levels. Highlighted in brown are the transcription factors in Layer 1 that control wall thickening and are specifically expressed in vessel elements, interfascicular fiber cells and anther endothecium. The transcription factors in Layer 2, which are regulated by Layer 1 factors, either tune the Layer 3 factors or directly activate or repress the biosynthesis genes of SCW components (cellulose, hemicellulose and lignin) during wall thickening. The transcription factors in Layer 1 can also either tune the Layer 3 factors or the biosynthesis genes. A variety of signals and regulatory factors have been identified to modulate Layer 1 gene expression during plant development or in response to environmental stimuli.

binding to the VND7 promoter (Taylor-Teeples et al. 2015). Furthermore, the expression of VND7 is transcriptional regulated by several transcription factor genes, such as ANAC075, GATA5 and GATA12, during TE differentiation (Endo et al. 2015). Also in Arabidopsis roots, WRKY15 is expressed in the procambial cells and shows the inhibition of VND7 expression in the vascular protoxylem (Ge et al. 2020). In xylary fiber cells, LATERAL ORGAN BOUNDARIES DOMAIN29 (LBD29) acts as an upstream repressor of SCW biosynthesis through auxin signaling (Lee et al. 2019). In anther endothecium, MYB26 is expressed in the endothecium cell layer to control cell wall thickening via the direct induction of NST1 and NST2 expression (Yang et al. 2017). Epigenetic changes can also affect wall thickening. ARABIDOPSIS HOMOLOG of TRITHORAX1 (ATX1), a H3K4-histone methyltransferase, is involved in the activation of SND1 and NST1 transcription (Wang et al. 2020c).

Formation of wood in trees results from intensive cell wall thickening of vessel elements and fiber cells in the secondary xylem. The transcriptional regulatory networks in wood formation in trees are similar to those in xylem differentiation of herbaceous Arabidopsis (Chen et al. 2019), while particular regulatory mechanisms have also evolved in perennial trees. Alternative splicing of the first-layer regulator *SND1/WND1B* occurs in trees and plays a role in maintaining homeostatic wall thickening in wood fiber cells, which does not occur in Arabidopsis (Li et al. 2012, Zhao et al. 2014). Wall thickening is the foremost activity during perennial tree growth, providing mechanical support for the constant increment in body weight. Specific regulatory mechanisms for wall thickening are implicated in trees and most of them are yet to be elucidated.

The expression of many plant micro-RNAs affects cell wall thickening and mechanical strength (Lu et al. 2005). The overexpression of miR319 results in a reduction of SCW thickness. MiR319 targets TCP4 (TEOSINTE BRANCHED1/CYCLOIDEA/PCF4) and the TCP4 transcription factor directly activates VND7 expression (Sun et al. 2017). However, TCP24, which is also a target of miR319, inhibits wall thickening in anther endothecium, probably via the suppression of NST1 and NST2 expression (Wang et al. 2015a). Certainly, more studies are needed to fully understand how miR319 acts differentially in wall thickening by targeting different genes in different cells.

Studies have depicted a hierarchy transcriptional regulatory cascade dictating the process of wall thickening. What are the signals that initiate this transcriptional regulatory cascade? In Arabidopsis, a leucine-rich repeat receptor-like kinase, encoded by AtVRLK1 (Vascular-Related Receptor-Like Kinase1), is expressed specifically in cells undergoing SCW thickening and plays a role as a signaling component in coordinating cell elongation and cell wall thickening during growth and development (Huang et al. 2018). Clearly, the signaling underlying initiation of the transcriptional regulatory cascade for wall thickening is yet to be revealed but would aid in understanding how wall thickening is specifically initiated in a variety of cells and tissues.

Wall thickening is regulated through protein modifications

Recent findings indicate that wall thickening is tuned through posttranslational modifications, such as phosphorylation, SUMOylation and ubiquitination. Sucrose Non-Fermenting Related Kinase 2 (SnRK2) kinases are central components in the core ABA (abscisic acid)-signaling pathway (Cutler et al. 2010). Arabidopsis SnRK2s triple mutants show much thinner SCW in inflorescence stems. SnRK2 phosphorylates NST1 to affect the NST1 transcriptional activity for wall thickening, a mechanism of the ABA enhancement of wall thickening (Liu et al. 2021). VND7 can be modified by S-NITROSOGLUTATHIONE REDUCTASE 1 (GSNOR1) and S-nitrosylated VND7 leads to alteration of its transcriptional activity (Kawabe et al. 2018). Lateral Organ Boundaries Domain (LBD) genes, LBD30 and LBD18, play a role in regulating VND7 expression in TE differentiation (Soyano et al. 2008). A recent study showed that LBD30 SUMOylating acts as a regulatory mechanism to tune SND1 and NST1 transcriptional activity, subsequently modulating wall thickening (Liu et al. 2019).

Posttranslational modifications also occur on the proteins responsible for the biosynthesis of wall polymers. Cellulose synthase (CesA) is a primary enzyme for the biosynthesis of cellulose. Multiple cellulose synthases are assembled into a cellulose synthase complex (CSC) in the Golgi, going through the cellular trafficking pathway and inserting into the plasma membrane (Bashline et al. 2014). Posttranslational modifications on CesAs may affect their stability or localizability. Phosphorylation of CesA7 results in its degradation via a 26S proteasome-dependent pathway (Taylor 2007). In addition, when CesA7 is S-acylated on cysteines at its variable region 2 or the carboxy terminus, its CSC is unable to localize to the plasma membrane (Kumar et al. 2016). KORRIGAN (KOR), a membrane-bound endo-(1,4)-β-glucanase, is of high importance in SCW cellulose formation in both Arabidopsis and *Populus* (Szyjanowicz et al. 2004, Yu et al. 2014). The highly conserved N-glycosylation on KOR1 is associated with KOR1 localization and cellulose deposition (Rips et al. 2014). *Populus* PtMAN6 encodes an endo-1,4-β-mannanase to convert the mannan-type polysaccharides to oligosaccharides in cell walls. PtMAN6 activity is dependent on its N-glycosylation and plays a role in coordinating cell wall remodeling and thickening through the generation of oligosaccharide signal (Zhao et al. 2013a, 2013b).

The enzymes involved in monolignol biosynthesis are largely regulated at the post translational level (Sulis and Wang 2020). For example, a group of Kelch repeat F-box proteins mediate phenylalanine ammonia-lyase (PAL) ubiquitination and made PAL degradation via 26S proteasome pathway, further leading to a decrease in monolignol biosynthesis (Zhang et al. 2013). In addition, PAL degradation may also be related to the metabolism reprogram driven by the cellular carbon availability (Wang et al. 2020a). For monolignol biosynthesis, 5-hydroxyconiferaldehyde O-methyltransferase in another enzyme to catalyze 5-methylation of 5-hydroxyconiferaldehyde

(Li et al. 2000), and its enzymatic activity is tuned by phosphorylation (Wang et al. 2015b).

From the above, it is clear that wall thickening is subject to highly dynamic biosynthetic processes. Protein modifications are crucial for controlling protein stability, activity, localization and conformation, able to swiftly modulate the cellular and metabolic activities. Certainly, much more is yet to be understood about the mechanisms underlying dynamic wall thickening and how it is modulated through various protein modifications.

Environmental stimuli affect cell wall thickening

Plant cell walls can be thickened in response to environmental stimuli, abiotic and biotic stress as defensive measures (Philippe et al. 2015). Under drought or dehydration conditions, lignin content and structure are changed to strengthen SCWs and avoid cell wall damage (Miedes et al. 2014). CRYPTOCHROME1 (CRY1) is a blue light receptor in Arabidopsis, and cry1 mutant displays much thinner SCWs in the fiber cells of the inflorescence stem. Blue light is able to enhance wall thickening via the CRY1-mediated modulation of NST1 transcriptional activity (Zhang et al. 2018). By contrast, in anther endothecium, a plastid-localized ROOE UVB SENSITIVE4 leads to the activation of *NST1* and *NST2* expressions indirectly, likely through the alteration of auxin distribution within the anther tissue, to direct anther endothecium wall thickening (Zhao et al. 2019). Under salt and osmotic stress, the expression of *BpNAC012*, an *AtSND1* ortholog in white birch (*Betula platyphylla*), was substantially induced, and *BpNAC012* overexpression caused 30% more lignin content in stem (Hu et al. 2019). In biosynthesis of monolignols, 4-coumarate-CoA ligase (4CL) catalyzes a rate-limiting step, and *4CL* expression is tightly controlled by lignin biosynthesis-associated transcription factor 1 (LTF1) in *Populus* (Gui et al. 2019). In responding to environmental stimuli, such as wounding and drought stress, LTF1 can be phosphorylated by MPK6 and then degraded via a proteasome pathway (Gui et al. 2019). Such an environmental sensory switch turns on/off the monolignol biosynthesis and has applicable significance for lignin genetic modification (Gui et al. 2020).

The *lew2* mutants, alleles of *AtCesA8/IRX1*, which encodes CesA8 responsible for cellulose biosynthesis of SCWs, have shown more tolerance to drought stress, suggesting that SCW cellulose synthesis is related to plant response to drought and osmotic stresses (Chen et al. 2005). A specific mechanism that safeguards the cellulose synthesis under salt stress is proposed. Companion of Cellulose synthase 1 (CC) protein in Arabidopsis-mediated CSCs migration along cortical microtubule during cellulose production by interaction with microtubules (Endler et al. 2015). When exposed to salt stress, CC1 plays a role in the re-establishment of microtubule arrays and CSCs rerecruitment on the plasma membrane, thereby restoring cellulose synthesis via its cytosolic N-terminus to affect microtubule bundling or dynamics (Endler et al. 2015, Kesten et al. 2019). In responding to environmental stimuli, plants have evolved a complex of mechanisms to form thickened cell walls as defense barriers.

SCW deposition pattern is precisely controlled in different cell types

Wall components are deposited into SCWs, which is controlled in a precisely guided manner; however, our understanding of the deposition is largely incomplete. The SCW displays a characteristic architecture in various cells. For example, vessel elements in protoxylem display spiral or helical deposition pattern and in metaxylem show a reticulate or pitted deposition pattern (Evert 2006). In addition, hypodermis cells in the cortex undergo wall thickening particularly at the corners (Evert 2006), while the CS ring-like structure results from a wall buildup that only occurs on the side facing the root surface (Geldner 2013). Thus precise wall buildup provides proper cell function.

Cortical microtubules play a critical role in directing wall deposition in distinct patterns through the spatiotemporal assembly and disassembly of cortical microtubules. Several regulators have been identified that participate in the control of cortical microtubule dynamics (Oda and Fukuda 2012a). Microtubule-associated proteins (MAPs), such as MAP65 and MAP70, are involved in arrangements of cortical microtubules, contributing to shaping SCW pattern (Mao et al. 2006, Fache et al. 2010, Pesquet et al. 2010). ROP GTPases (plant-specific Rho/Rac-like small GTPases) signaling is involved in the formation of the pits in SCW (Oda and Fukuda 2012b). The coordination of Microtubule-Depletion Domain 1 (Oda et al. 2010), kinesin13A (Oda and Fukuda 2013) and IQD13 (Sugiyama et al. 2017) may also be required to properly form the SCW pits. Other proteins, such as Cortical microtubule Disordering 1 (Sasaki et al. 2017), Walllin and Boundary of ROP domain 1 (Sugiyama et al. 2019), participate in the control of pit size and boundaries.

Laccases (LAC) and peroxidases (PRX) catalyze the polymerization of monolignols deposited in the polysaccharide matrix (Berthet et al., 2011). AtLAC4, AtLAC17 and AtPRX72 are localized in the SCWs wall of both vessels and fiber, while AtLAC4, AtPRX64 and AtPRX71 are localized at the corner of fiber cells (Hoffmann et al. 2020). Does the location of laccases and peroxidases determine the polymerization of lignin in particular patterns? The CS is composed of 70% lignin, reflecting a unique pattern of lignin deposition. During the formation of the CS, it is proposed that the CS domain proteins (CASPs) provide a scaffolding platform to restrict PRX64 activity to particular positions of the walls to guide lignin deposition (Lee et al. 2013). A recent study shows that mutations of Endodermis-specific Receptor-like Kinase 1 caused CASP1 mis-localization and ectopic lignin deposition outside of the CS, suggesting the involvement of a signaling factor in regulating CS formation (Durr et al. 2021). It would be highly interesting to know how the signaling cascade controls the localization of CASPs, which, in turn, recruit LACs and PRXs to the specific locations in guiding lignin deposition.

Concluding Remarks

Cell wall thickening occurs in a variety of specialized cells to implement specific physiological functions in plants, for which complex regulatory mechanisms are involved. Wall thickening occurrences can be regulated at the transcriptional, posttranscriptional and posttranslational levels in response to developmental programs, as well as environmental stimuli. As summarized above, tremendous progress has been achieved in revealing the regulatory networks and pathways that guide wall thickening under different circumstances. This knowledge is valuable to help understand how wall-thickened structures in plants are established through precise regulatory networks. Nevertheless, there are still many yet to be dissected and elucidated. For example, how is the wall thickening program initiated in the first place? Is there any true 'master' regulator to unlock the wall thickening program? Are there any intercellular communications or signals involved? Because the composition of biopolymer components varies substantially in different wall-thickened cells, an understanding of the regulation underlying the biosynthesis of various biopolymers during cell wall thickening would be of great importance for modifying the wall materials utilized by human society. Another interesting question is how the deposition patterns in SCWs are controlled. The answer to this question will help decipher how the wall biopolymers can be concentrated into different structures, which may be related to the specific cell function in plant.

In plants, the majority of the photosynthetic products are stored in cell walls through wall thickening, primarily by the process of plant trunk increment, providing abundant biomass for the production of cellulosic fibers, chemicals, biofuels and other biomaterials. A full mechanistic unveiling of wall thickening processes will contribute to building the knowledge foundation needed for efficient utilization of renewable carbon-neutral resources stored within thickened plant walls.

Data Availability

No new data were generated or analyzed in support of this research.

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Disclosures

The authors have no conflicts of interest to declare.

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