Transition of primary to secondary cell wall synthesis

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Abstract

The construction of a secondary cell wall is an important and necessary developmental decision that supports cell function and plant stature. Unlike the primary cell walls, which are initiated during cell division and develop along with the expansion of the cells, secondary cell walls are constructed after the cells have stopped growing. Hence, the transition from primary to secondary wall synthesis marks an important and distinct metabolic investment by the plant. This transition requires a coordinated change of a plethora of cellular processes, including hormonal, transcriptional and post-transcriptional activities, metabolic flux re-distributions and enzymatic activities. In this review, we briefly summarize the hormonal and transcriptional control of the primary to secondary wall transition, and highlight important gaps in our understanding of the metabolic framework that support the transition. Several tools that may aid in future research efforts to better understand the changes in cell wall synthesis during the trans-differentiation are also discussed.

1 Introduction

A major developmental difference between animals and plants is that plant cell morphogenesis and pattern formation are accomplished largely through asymmetric cell divisions and cell enlargement [1, 2]. These events are underpinned by extensive synthesis and re-modelling of the plant cell wall, a glycan-enriched extracellular structure. In the course of cell division, a nascent cell wall is deposited usually perpendicular to the dividing axis of a mother cell following mitosis [1, 3]. The completion of this cross wall marks the end of cytokinesis and thus the formation of two daughter cells. Importantly, the position of the cross wall may be regarded as a first "decision point" of cell fate as it results in two daughter cells with different shape [1]. After cytokinesis, the two daughter cells typically expand to obtain their final form and function. Cell expansion requires rapid synthesis, localized deposition, and extensive re-modelling of cell wall material to allow anisotropic cell growth. One impressive example of cell form and function is the dramatic differentiation of the xylem tissue [4]. Xylem vessels consist of interconnected cells that can reach over a meter in length, which is approximately 10,000 times larger than the size of newborn cells [5]. However, the elongation of these cells, and their interconnectedness, are not sufficient to ensure solute transport through the vessels. To sustain this capacity, the maturing vessel cells produce a secondary and much thicker cell wall. Due to the developmental order, these two types of cell walls were historically termed as primary and secondary cell walls (PCWs and SCWs), respectively.

SCWs are specialized cell wall structures that support plant stature and cell functions [6, 7]. While SCWs are deposited around xylem vessels, they may also be found at anther endothecium, and around tracheids and fibres in woody tissues. The SCW structure represents a key advance for plants to adapt to terrestrial environments, and was a necessity for land plants to evolve. The PCWs of most plants consist of three major glycan structures; cellulose, hemicelluloses and pectins. The relative amounts of these three components can vary depending on species, on cell and tissue types, and on the developmental and environmental contexts [8]. For example, a typical dicot primary wall consists of cellulose, xyloglucans, a mixture of homo- and rhamnogalacturonans, and heavily glycosylated protein [8]. While the SCWs also contain large amounts of cellulose, they have a different set of hemicelluloses and reduced amounts of pectins [7]. Hence, the SCWs typically contain xylans and mannans, but also a significant amount of the polyphenolic structure lignin [7]. In contrast to PCWs, which are relatively elastic due to effective cell wall re-modelling, SCWs are characterised by their high rigidity. In both cell wall types, cellulose provides the main mechanical strength and steers plant cell morphology. For example, in the SCW, cellulose prevents vessels from collapsing due to the negative pressure inside of the cells [7]. Nevertheless, both xylan and lignin provide SCW strength as they aid in cross-linking cellulose microfibrils, and to an interconnected SCW framework. In addition, lignin confers water-repellent characteristics to the SCWs due to its inherent hydrophobicity and is thus crucial for the water transporting efficiency of the vessels [9]. Understanding how plants produce SCWs is therefore of significant biological interest.

Apart from the biological significance, SCWs also constitute the bulk of biomass a plant produces. This biomass is of major economic importance as it is used for a variety of industries, such as textile, lumber, feed and fuel [8, 10, 11]. While we understand a great deal of the enzymes involved in SCW synthesis, and how the corresponding genes are transcriptionally regulated, we know surprisingly little concerning the transition of the primary to SCW synthesis. That is, what aspects of the PCW synthesis are shut down when SCW synthesis is turned on, and what are the drivers for these processes? How is the metabolic framework changing during this transition? And, how can we begin to address these questions with the tools we currently have at hand? Here, we highlight aspects that are related to some of these questions, and also attempt to underline tools that may be used for future endeavours in this direction. With a few exceptions, this review mainly focuses on tracheary elements and xylem/interfascicular fibres due to length restrictions.

2 Hormonal control of the initial onset of secondary wall production

Almost every aspect of plant growth and development is under the control of various hormones [12-15]. It is therefore not surprising that also the transition from the primary to the SCW is controlled by their coordinated action [16, 17]. Given the pleiotropic effect of single hormones, and the added combinatorial effects of multiple ones, it is difficult to dissect aspects of SCW formation the hormones regulate. However, it is clear that auxin, cytokinin and brassinosteroids contribute to the onset of SCW in vessels of *Arabidopsis thaliana* [18-20]. Various combinations of these hormones have also been used to induce SCW synthesis in cell suspension cultures, e.g. in *Zinnia elegans* and *Arabidopsis* [21-24], supporting an active role of them in SCW production.

Several vessel-specific transcription factors (TFs) that drive SCW synthesis are under the control of auxin, cytokinin and brassinosteroids. These include the central NAC TFs, VASCULAR-RELATED NAC-DOMAIN (VND)6 and VND7 that are referred to as master TFs as expression of them in cells that do not normally undergo SCW synthesis triggers SCW production [7, 25]. Notably, robust activation of these TFs requires the presence of auxin, cytokinins and brassinosteroids, while each hormone alone has no major impact [25] (Fig. 1). Other VND TFs, namely *VND1-5* and the *NAC SECONDARY WALL THICKENING PROMOTING FACTOR (NST)3/ SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN (SND)1*, may be induced by abscisic acid (ABA) [26]. While the ABA induction of SCW inducing TFs is interesting it is currently not clear whether this activation is part of an *in planta* activation process [26] (Fig. 1). Based on recent reports it appears that there may be at least an additional layer between the hormone tier and the master TFs. For example, the expression of *VND6* and *VND7* is regulated via a feedback-loop by *ASYMMETRIC LEAVES-LIKE* (*ASL)19* that in turn are controlled by the auxin induced *AUXIN RESPONSE FACTOR7* (*ARF7*) [27] (Fig. 1).

Both primary and secondary wall cellulose synthesis is regulated by cortical microtubules that guide the cellulose synthase complex (CSC) [10, 28-31]. This guidance is essential to maintain cell wall strength, cell morphology and function [32-35]. The microtubule array undergoes a dramatic re-organization during the transition between the two wall types. In elongating cells, the microtubule array is evenly dispersed across the cell cortex and is typically transversely oriented with regards to the cell's growth axis [36]. However, during SCW synthesis the microtubules form distinct and evenly distributed bands and/or lattices that mark synthesis of cellulose and other cell wall components [30, 37-39] (Fig 1). Hormones, such as AUX, BR and GA, impact on the microtubule organization [40-42], which could influence the patterning of SCWs and might affect the transition from primary to secondary wall synthesis (Fig. 1). For example, auxin can either induce reorientation or depolymerize the microtubule cytoskeleton, possibly due to different experimental conditions and materials [43-47]. However, the mechanistic details behind the impact of hormones on microtubule organization and dynamics remain largely obscure. Perhaps the recently reported AUXIN BINDING PROTEIN (ABP)1-mediated effects on microtubules might shed light on the relationship between auxin and microtubule organization [48]. However, the importance of ABP1 in auxin signalling has been questioned [49, 50], so care has to be taken when evaluating this protein in context of microtubule organization [51]. Moreover, insights gained from salt stress-related changes in the SCW synthesis regulatory network, and COMPANION OF CELLULOSE SYNTHASE (CC) proteins that affect microtubule dynamics may provide some links between ABA and microtubule organization [52, 53].

3 The secondary wall transcription factor network

During the past two decades, the transcriptional framework that underpins SCW formation has been extensively investigated [54-57]. There are many excellent reviews that give detailed information about the action and regulation of the TFs involved in SCW synthesis, including [54-58]. We are therefore only briefly summarizing these aspects here (Fig. 1). The first identified "master" TFs were the VND1-7 [25, 59-61], and ectopic expression of VND6 and VND7 resulted in trans-differentiation of cells that normally do not undergo xylogenesis, including mesophyll cells. These studies concluded that VND6 and VND7 regulated meta- and protoxylem formation, respectively, in both *Arabidopsis* and poplar [25]. Analogously, over-expression of the five other *VND* genes led to ectopic SCW deposition and activated the expression of secondary wall biosynthetic genes for cellulose, xylan and lignin [61]. The fact that these *VND* genes are preferentially expressed in developing vascular tissues suggested that other SCW-containing cell types need different TFs to turn on SCW biosynthesis. Indeed, in *Arabidopsis*, the two TFs NST1 and NST2 function redundantly to initiate SCW biosynthesis in anther endothecium [62], and SND1/NST1 controls SCW deposition in fibre cells [63]. The complex relationship amongst the "master" TFs is also reflected by the unexpected result that over-expression of *SND1* or *NST1* induced SCWs with a xylem-vessel-like pattern (helical and reticulated) despite the fact that fibre SCWs are uniformly thickened [62-64]. Furthermore, the proposed roles of the "master switches" do not necessarily mean that they are the only responsible regulators that determine the SCW cell fate. This is illustrated by the fact that dominant repression of the TFs only resulted in a reduced SCW thickening, but the cell identity was not affected [25]. For example, xylem vessels still underwent elongation and programmed cell death in VND6 or VND7 dominant repression *Arabidopsis* plants [25]. However, it remains unclear if there are other regulators that impact on cell differentiation without turning on SCW biosynthesis. Also, whether the initiation of SCW formation is the signal for cell growth termination (i.e., the end of cell enlargement and PCW formation) or vice versa is still an open question.

The master TFs regulate an intricate network of downstream TFs and genes encoding biosynthetic and hydrolytic enzymes (Fig. 1). In this network, MYB TFs account for a majority of the targets. These TFs are mainly activated, directly or indirectly, by the master TFs [54] and positively regulate the expression of various cell wall related genes. Two of the most prominent MYBs in this network, MYB46 and MYB83, work downstream of the master TFs, but they are also capable of triggering the expression of the majority of the SCW biosynthetic genes. Thus, these MYBs are considered as the converging point of the SCW transcriptional network. However, this model has been challenged as there exist complex feedback (e.g., downstream TFs MYB4, MYB7, and MYB32 can repress SND1 independently of MYB46 and MYB83) and feed-forward loops in the SCW TF network [53, 65], and it is, therefore, possible that the inferred conversion point is overestimated (Fig. 1). These multiple layers of regulation might precisely determine structural features of the SCW, and perhaps also be beneficial for plants to endure abiotic stress [53]. It is interesting to note that some of the SCW related TFs may maintain or promote the expression of the "master" TFs via a positive feedback loop. For example, over-expression of the TF *ASL19* led to activation of *VND7* while it also is a direct target of VND7 [27]. Hence, *ASL19* expression is therefore also induced by VND7. In addition to activation, transcriptional repression in the SCW regulatory framework has also been recently reported. VNI2 and WRKY12 are transcriptional repressors of *VND7* and *NST2*, respectively [66, 67]. In addition, the recently identified E2Fc, an upstream regulator of *VND6 and VND7*, can act either as an activator or a repressor in a dose-dependent manner [53]. These types of relationships therefore add additional dimensions of control to the regulatory network, and support the notion that plants need a precise transcriptional regulatory system to control SCW formation.

4 Metabolic transitions

The formation of SCW is a very rapid and energy consuming process, so it is clear that a dramatic metabolic switch needs to occur to effectively supply precursors for the SCW components. In addition, the onset of SCW synthesis also implies that the primary wall biosynthesis needs to be terminated, which should promote re-direction of metabolic fluxes. However, our knowledge of the metabolic alterations during these events is very limited. This is surprising, given that the cell wall, and in particular the SCW, is the major carbon sink in a plant. Nevertheless, the bulk of research has focused on the immediate precursors of the cell wall polymers [68-70]. With the exception of lignin, most cell wall components are made from nucleotide sugars. Given the composition of the different polymers, it is clear that the nucleotide sugar pools must change when different cell wall polymers are made. For example, a xylan is made largely from UDP-xylose [71], whereas pectin is largely based on UDP-GalA and other nucleotide sugars depending on the type of pectin [72]. These differences raise important questions about how the carbon flux is redirected during the primary to SCW transition, and what factors control this change. Both UDP-GalA and UDP-Xyl can be generated from UDP-GlcA [73, 74]. Thus, the flux emanating from UDP-GlcA needs to be re-routed when SCW synthesis is initiated (Fig. 1). Intriguingly, although most nucleotide interconversion reactions are reversible, the reaction from UDP-GlcA to UDP-Xyl is not. The latter is a highly exergonic process, which prevents the reaction from being reversible, and indicates a commitment of the system to irreversibly support SCW synthesis [70]. To further understand how the nucleotide sugar supply changes during the transition it will be important to explore regulatory aspects of the enzymes involved in the nucleotide sugar interconversion pathway.

Metabolic flux analyses to understand cell wall synthesis have only been undertaken in a handful of studies largely focussed at understanding starch biosynthesis in potato [75-78]. However, in the vast majority of studies neither environmental nor genetic perturbation of enzymes involved in the sucrose to starch transition had a considerable effect on cell wall biosynthesis. Nevertheless, inhibition of the expression of the nucleotide sugar biosynthetic enzyme uridine monophosphate synthase resulted in a compensatory increase in the expression of the uridine salvage pathway, an overall increase in uridinylate content and a corresponding increase in the rate of cell wall biosynthesis [76]. Additional studies, done in tomato fruit development, revealed that the carbon flux towards cell wall production decreased dramatically during later stages of fruit development [79]. It will certainly also be of interest to similarly explore carbon flux changes in the transition between PCW and SCW synthesis.

In *Arabidopsis*, xylem cell differentiation is concluded by lignification of the tissue [80, 81]. The mono-lignol producing framework has been extensively reviewed elsewhere [71, 82, 83], and we will only briefly summarize the pathway, and the impacts of its perturbation, here. The shikimate, the phenylpropanoid and associated pathways support lignin synthesis through a range of oxidation, methylation and hydroxylation steps [82]. Attempts to modify these pathways have provided considerable insights into the metabolic adjustments during lignin formation. Interestingly, lignin-modified plants have revealed unexpected effects on metabolic processes [84-86]. For example, silencing of hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), involved in lignin precursor formation , in *Arabidopsis* caused severe growth defects and led to an accumulation of flavonoids instead of lignin [84]. These effects appear to be the result of re-routing of metabolic fluxes when enzymes in the phenylpropanoid and mono-lignol metabolism are perturbed. Therefore, it is not surprising that some changes can affect other secondary metabolites, for which precursors are intimately linked to phenylpropanoids [84, 85, 87-94]. Perhaps more interestingly, a trade-off between lignin and other cell wall polymers has been observed [95, 96], suggesting a feedback loop between the phenylpropanoid pathway and the precursor pool, or synthesis machineries, of other cell wall components. This implies either a resource distribution strategy, or a cell wall mechanical need, by the plant during SCW deposition. Nevertheless, large-scale metabolic flux studies would be necessary for a better understanding of these possible feedback processes.

While many metabolic profiling analyses have facilitated a general picture of plant growth-related metabolic signatures [97-101], very few studies have attempted to address the metabolic changes in *Arabidopsis* that the primary to secondary wall transition necessitate. That said, it may be challenging to directly link metabolic changes to SCW accumulation as the tissues undergoing differentiation typically are surrounded by other cell types with different fates. This would mask metabolic changes directly linked to SCW synthesis. Nevertheless, alterations in general metabolic processes do affect SCW synthesis. For example, changes in energy metabolism alter SCW accumulation and composition. Transgenic tomato plants with altered enzyme activities associated with the tricarboxylic acid (TCA) cycle exhibited a substantial reduction in cellulose and lignin, and in xylem vessel development, in roots [102]. In this study, the authors also evaluated the metabolic flux by feeding $^{[U\text{-}14C]}$Glc into the system. Under energy limited conditions, a depressed cell wall synthesis rate coupled with constant glycolysis, starch and Sucrose biosynthesis indicated that the TCA impaired plants maintained their primary metabolic pathways at the expense of other processes. Taken together, these data indicate that reduced TCA cycle activity inhibits SCW biosynthesis, linking this process to the ability of the plant to produce energy (Fig 1). While these studies provide a first insight into how changes in metabolism impact on bulk biomass production in *Arabidopsis* it is unclear how the metabolic fluxes proceed in a system where only primary to SCW transition is being monitored.

Apart from using *Arabidopsis* to address PCW to SCW transitions, valuable insights have been gained from studies on cotton fibre development. Here, the major metabolic changes seem to mainly support cellulose synthesis [103]. Decreases in glucose and fructose concentrations have been observed, which imply changes in sugar metabolism [104]. Further biochemical studies revealed that sucrose phosphate synthase may have a critical role in recycling fructose to support cellulose synthesis [105]. In addition, repression of the pectin biosynthesis pathway was also reported, supporting a low pectic content in maturing cotton fibres [103]. Furthermore, reactive oxygen species are important factors for the transition to SCW synthesis. For example, it has been shown that the cotton fibre maturation depends on a spike of H_2O_2, which might be a signal for initiating the dimerization of cellulose synthases [106].

5 Tools to evaluate the primary to secondary wall transition

In planta, certain characteristics prevent us from evaluating the primary to SCW transition easily as: the developmental programs are not synchronous in cells with the same developmental fate; SCWs are synthesized very rapidly and the transition is therefore difficult to clearly outline in a plant and, SCW-containing cells are usually located deep inside plant tissues with surrounding cells obscuring visualization and monitoring of the transitions. Nevertheless, several systems that overcome one or several of these difficulties have been developed and used.

The developmental program of wood forming tissues of trees makes them suitable to address the progression of SCW production. The cells in tree stems are arranged in a developmental order, in which different cell layers in a cross-section represent different stages of cell differentiation [107]. Therefore, using precise sampling approaches may provide snapshots of SCW progression [108], and this system has been used to perform large-scale omics analysis, such as transcriptomics and chemical profiling [109, 110]. The main shortcoming of this system is that the number of cells at each cross-section is limited, so large-scale studies and high-resolution analyses are time consuming tasks.

Trans-differentiating cell cultures offer an alternative to the tree cross-section approach. Over 30 years ago, *Zinnia* was used for trans-differentiation studies, i.e. induction of tracheary elements, using auxin and cytokinin as inducers [21]. Several pioneering analyses using this system dramatically improved our knowledge regarding the SCW formation, including the identification of the “master” TFs VND1-7 mentioned above [22, 25, 39, 111, 112]. These studies used transcriptomics, i.e., microarrays, to establish a time-resolved expression compendium of genes induced after trans-differentiation [22]. Here, many genes that are associated with SCW formation and regulation showed distinct induction patterns and have subsequently been shown to partake in the process. Recently, this *in vitro* system was further optimized by using *Arabidopsis* cell lines that may be trans-differentiated by adding a hormone cocktail [23]. Compared to the *Zinnia* system, this optimized system is more stable and efficient, with up to 40 % percent of cells being synchronously trans-differentiated [23]. Studies facilitated by these cell lines have uncovered proteins that regulate a range of different aspects of SCW pattering, including many microtubule-related proteins [23, 24]. However, a limitation of the system is that *in vitro* stimulation cannot accurately represent the *in vivo* situation, particularly considering the communication between different cell types that are of great importance for lignification of tissues.

A third system has been taking advantage of the “master” TFs, mainly the VNDs, to induce trans-differentiation of cells in a plant [113]. In these systems, VND6 or VND7 were fused to the activation domain of the herpes virus VP16 protein and the glucocorticoid receptor (GR) domain [113]. Transgenic plants harbouring the constructs can then be treated with dexamethasone (DEX), a glucocorticoid derivative, to re-direct the over-expressed VND6 or VND7 to the nucleus [113]. This *in vivo* system is not only ideal for large-scale omics studies, but also for direct visualization of the transition [30]. In addition, these lines offer great potential to introgress SCW deficient mutants into them and to therefore study how the SCW synthesis process is affected when individual components are impaired. Hence, together these systems offer ways to measure metabolic re-routing during the transition of primary and SCW synthesis, and potentiate system wide analyses where metabolic and transcript changes are put in context to live cell imaging data.

Lastly, cotton fibres, which are highly elongated and thickened single cells of the seed epidermis, are excellent as a system to understand the PCW to SCW transition [114]. As the SCW thickening of cotton fibre is almost only sustained by cellulose biosynthesis it may offer a great way to better understanding the transition between primary and secondary wall cellulose synthesis. Indeed, several reports have concluded that the SCW thickening starts with a dramatic increase of cellulose synthesis during which cellulose fibrils are deposited in steep helixes relative to the fibre axis [103, 106, 115, 116]. Furthermore, due to the relative ease of isolating cotton fibres and culturing cotton ovules/fibres *in vitro*, this system has also been employed in several systems biology studies [103, 106]. It is noteworthy that while cotton fibres are of significant economic importance, data generated from this system might not be sufficient to provide an understanding of SCW synthesis from other species as the SCW contents differ substantially. Nevertheless, these differences might become of relevance for tailoring various characteristics of cell wall properties across species barriers.

6 Future perspectives

Due to its potential application for biomaterial and bioenergy, SCW biology has been attracting a great deal of attention and significant progress in understanding how the synthesis is initiated and controlled has been made in the past decade. From these analyses, a complex interleaved TF-hormone regulation system has emerged. Yet, many major gaps remain: First, although it is evident that hormones play fundamental roles in triggering SCW biosynthesis, mechanistic links (e.g., direct links between hormone signalling cascades and “master switches”) are largely unknown. In addition, it is also unclear whether hormone-related signaling impacts on the termination of PCW biosynthesis. Second, a detailed map of the metabolic framework that underpins SCW synthesis is lacking. A better understanding of the metabolic changes during the onset of SCW production will most likely bring new biomaterial applications, and potential approaches to increase biomass production. For example, changing enzyme activities related to cell wall synthesis have generated wood with different characteristics, suitable for diverse biomaterials [117]. Third, while transcriptional regulation has been extensively explored, translational and post-translational control of SCW formation remains tenuous. This may be especially relevant during the PCW to SCW transition as metabolic fluxes are likely to be substantially different. Lastly, while most of these relationships have been gleaned from *Arabidopsis*, the level of which this can be transferred to energy crop species remain unclear but is a necessary step towards achieving the goal of renewable energy and new biomaterials.

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Fig. 1 Hormonal and transcriptional control, and metabolic adjustment, associated with the transition from PCW to SCW synthesis. A complex transcriptional network is employed by plants to regulate the SCW biosynthetic program. In *Arabidopsis*, several tissue-specific master TFs, namely, VND1-7, NST1/2 and SND1, have the capacity to trigger trans-differentiation of cells. Upstream regulators, and multiple levels of downstream TFs, comprise an intricate feed-forward /feedback loop system that control the expression of SCW biosynthetic genes. Several hormones control the expression of the master TFs via unknown signalling pathways or other TF tiers (e.g., ASL19/20). During the transition, hormones might also impact on the microtubules re-organization. To rapidly synthesize SCWs, the flux of carbon needs to be redirected through the nucleotide sugar pool as different cell wall polymers need different precursors. In addition, altered primary metabolism (e.g., TCA cycle) is required to provide energy for the PCW to SCW transition. Please note that the relative order and alignment between the different processes is largely unknown. ABA: abscisic acid; AUX: auxin; CK: cytokinin; BR: brassinosteroid; GA: gibberellic acid.