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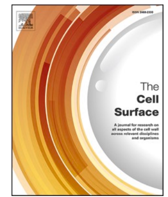

Insights into pectin *O*-acetylation in the plant cell wall: structure, synthesis, and modification

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ABSTRACT

O-Acetyl esterification is an important structural and functional feature of pectins present in the cell walls of all land plants. The amount and positions of pectin acetyl substituents varies across plant tissues and stages of development. Plant growth and response to biotic and abiotic stress are known to be significantly influenced by pectin *O*-acetylation. Gel formation is a key characteristic of pectins, and many studies have shown that gel formation is dependent upon the degree of acetylation. Previous studies have indicated that members of the TRICHOME BIREFRINGENCE-LIKE (TBL) family may play a role in the *O*-acetylation of pectin, however, biochemical evidence for acceptor specific pectin acetyltransferase activity remains to be confirmed and the exact mechanism(s) for catalysis must be determined. Pectin acetylesterases (PAEs) affect pectin acetylation as they hydrolyze acetylester bonds and have a role in the amount and distribution of *O*-acetylation. Several mutant studies suggest the critical role of pectin *O*-acetylation; however, additional research is required to fully understand this. This review aims to discuss the importance, role, and putative mechanism of pectin *O*-acetylation.

Introduction

Pectins are a family of plant cell wall glycans and glycoconjugates with essential roles in plant development and response to both biotic and abiotic stress. They are particularly abundant in the middle lamella and primary walls of dicotyledons where they account for about 30 % of cell wall dry mass (Caffall and Mohnen, 2009; O'Neill et al., 1990). Pectins are defined by the presence of 4-linked galacturonic acid (GalA) in the polymer backbone and include at least four glycan subclasses: homogalacturonan (HG), rhamnogalacturonan (RG-I), RG-II, and xylogalacturonan (XGA) (Mohnen et al., 2008). The backbones of HG, RG-II, and XGA consist of α-1,4-linked GalA (Fig. 1). In contrast, the RG-I backbone consists of the repeating GalA and rhamnose (Rha) disaccharide sequence [4)-α-D-GalA-(1,2)-α-L-Rha-(1,] (Fig. 1) (Voragen et al., 2009). This Surface Feature will focus on pectin *O*-acetylation, including structural aspects of acetylated pectin, pectin acetylesterases (PAEs), and the putative mechanism of and players involved in pectin *O*-acetylation.

Pectin *O*-acetylation across different plant species

O-Acetyl esterification is an important structural and functional feature of pectins (Ishii, 1997). GalA residues in the backbones of HG, XGA, and RG-I are *O*-acetylated at *O*-2 and/or *O*-3 whereas the aceric acid and fucosyl residues of sidechain B of RG-II are also modified by *O*-acetyl substituents (Fig. 1) (Ishii, 1997; Komalavilas and Mort, 1989; Perrone et al., 2002). In nuclear magnetic resonance (NMR) spectroscopic studies of okra RG-I, *O*-acetylation was reported on rhamnose residues at the *O*-3 position (Sengkhamparn et al., 2009), but more data is needed to determine if this is a ubiquitous substituent in pectins. The degree of acetylation (DA) of the GalA in the backbone of pectic polymers has been reported to range from 40 to 85 %, depending on tissue type and stage of the development (Ishii, 1997; Liners et al., 1994). For example, chicory root and sugar beet pulp pectins were found to be

Abbreviations: GalA, galacturonic acid; HG, homogalacturonan; RG-I, rhamnogalacturonan-I; XGA, xylogalacturonan; NMR, nuclear magnetic resonance; PAEs, pectin acetylesterases; DE, degree of esterification; DM, degree of methyl-esterification; DA, degree of acetyl-esterification; TBL, TRICHOME BIREFRINGENCE-LIKE; AXY9, ALTERED XYLOGLUCAN 9; RWA, REDUCED WALL *O*-ACETYLATION.

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highly acetylated with DAs of 43 and 35, respectively (Ramasamy et al., 2013). Liners et al. 1994 reported pectic fragments in friable and compact sugar beet calli are 85 % and 22 % acetyl-esterified, respectively (Liners et al., 1994). Enzymatic digestion of RG-I from sycamore cell-suspension cultures yielded oligosaccharide products having an acetyl group at either the *O*-2 or *O*-3 positions of the GalA (Lerouge et al., 1993). Taken together, pectin *O*-acetylation is a pervasive feature of plant pectins that varies remarkably across plants and across various tissues of a plant.

One key functional property of pectin is its ability to form gels through the interaction of polymer chains by hydrogen bonding, divalent cation cross-bridging (Ca^{2+}), and hydrophobic interactions (Bemiller et al., 1986). Gel formation is highly dependent on the type and distribution of substituents, pH, temperature, charge distribution on the backbone, molecular weight, and degree of esterification (DE), including both the degree of methyl-esterification (DM) and DA, (Gawkowska et al., 2018). Cacao pod husk pectins (CPHP) were shown to have moderate gelling ability owing to the GalA content (~60 %) and DE (DM = ~50 % and DA = ~3–10 %) (Yapo and Koffi, 2013). Other studies have indicated that a DA of ~4 % is critical for the gelling capacity of pectins (Iglesias and Lozano, 2004). High DA in pectins has been associated with reduced gelling capabilities due to steric hindrance of chain association (Willats et al., 2001; Williamson et al., 1990); however, high DA did not hinder gel formation in CPHPs (Vriesmann and Petkowicz, 2013). Steric hindrance due to acetylation also hinders polysaccharide interactions and eventually influences the entire dynamic of the plant cell wall (Busse-Wicher et al., 2014). Pectin acetylation might also contribute to viscosity by promoting hydrophobic interactions (Sengkhamparn et al., 2009).

Acetyl-esterification impacts the growth and development of plants. Gou et al (2012) reported the association of reduced acetylation in *Nicotiana tabacum* with wilting and retarded growth of flower organs as well as impaired development of pollen grains and pollen tubes resulting in inhibition of fertilization and seed development (Gou et al., 2012). Stranne et al (2018) suggested *O*-acetylated RG-I plays a role in abiotic stress responses in *Arabidopsis,* as *trichome birefringence like 10 (tbl10)* mutants, with decreased RG-I *O*-acetylation, exhibited elevated levels of drought tolerance indicating this modification may influence water uptake and transport (Stranne et al., 2018). Reduced levels of pectin *O*-acetylation also caused collapsed trichomes (Nafisi et al., 2015). A lower amount of pectin *O*-acetylation in *tbl44* mutants was associated with resistance to the powdery mildew (*Golovinomyces cichoracearum*) via triggering of an unknown defense pathway against the fungal pathogen (Chiniquy et al., 2019).

The role of pectin *O*-acetylation in the food and biofuel industries

Pectin's involvement is not confined to biological and physiological functions because of its complex and diversified structural makeup. It also plays a significant role in various industries, including both food and biofuel processing. Pectic polysaccharides serve as emulsifiers, stabilizers, and gelling and thickening ingredients in a variety of foods (Thiraviam and Mahejibin, 2019). Usually, pectin-rich products are extracted from citrus peel, apple pomace, and sugar beet at the industrial level. The physicochemical properties of pectins are influenced by the DA and DM. In a study on sugar beet pectin (SBP) and citrus pectin (CP), de-acetylated SBP pectin exhibited good emulsifying ability, while chemically acetylated CP showed better emulsifying properties than the deacetylated ones. This study suggested that acetyl groups might help in maintaining emulsion stability, which probably occurs due to the reduction in calcium bridging in flocculation (Leroux et al., 2003). This study suggested that the molecular weight, protein, and acetyl contents of pectin significantly influence its emulsifying properties (Leroux et al., 2003).

Pectin with a low molecular weight of about 60–70 kg mol^{-1} and a high degree of methoxylation showed the best emulsifying properties (Akhtar et al., 2002). It's been hypothesized that the enhanced hydrophobicity due to the presence of *O*-acetyl and *O*-methyl groups reduces the gelling abilities of pectins (Dea and Madden, 1986). Low gelation

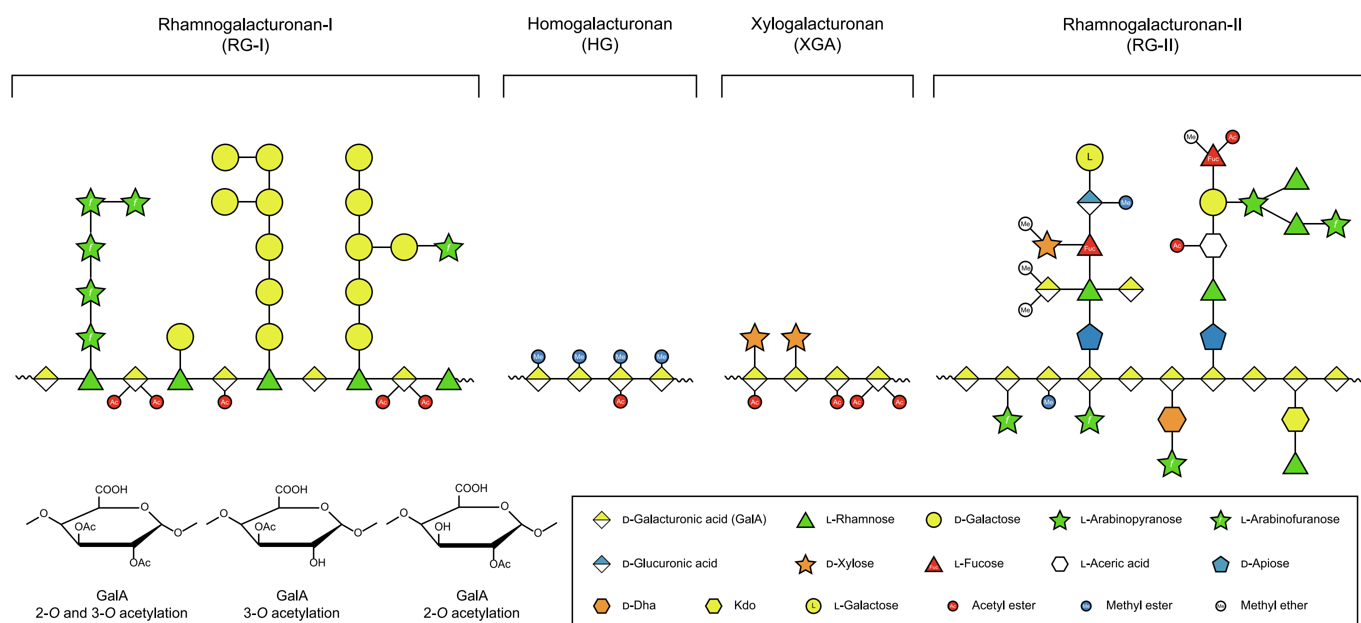

Fig. 1. A representative model structure of pectin. The schematic shown here highlights the four main structural domains; homogalacturonan (HG) and rhamnogalacturonan-I (RG-I) (Caffall and Mohnen, 2009), rhamnogalacturonan-II (RG-II) (Barnes et al., 2021), and xylogalacturonan (XGA) (Jensen et al., 2008). The order and degree of side chain substitution is not known. Several studies suggest that pectic domains are covalently linked (Coenen et al., 2007; Ishii and Matsunaga, 2001; Nakamura et al., 2002), but individual domains are shown for simplicity. The specific structure of the RG-I side chains is not known and is representative. *O*-acetyl and *O*-methyl esterification is shown by red and blue circles, respectively. The cyclic structure of a pyranose (six-membered) ring of galacturonic acid (GalA) with *O*-acetylation at 2-*O*/3-*O*, 3-*O*, and 2-*O* positions is representative and varies within the polymer. Monosaccharide symbols used in the representative schematic structure are taken from the Symbol and Text nomenclature from the Consortium for Functional Glycomics.

was reported in highly acetylated sugar beet pectin, and gelling ability was restored after pectin deacetylation by acid hydrolysis (Dea and Madden, 1986; Pippen et al., 1950). Taken together, these data suggest that *O*-acetyl substituents may prevent alignment and aggregation of the chains, possibly due to steric hindrance.

Pectins are typically found in primary cell walls; however, studies show that pectins may have an impact on secondary wall development (Goulao et al., 2011; Matsunaga et al., 2004). Conforming to cell wall architecture, pectic polymers that are integrated into the cellulose-hemicellulose network prevent the cellulose and hemicellulose from being exposed to degradative enzymes, minimizing the degradation of cell wall components for biomass processing (Marcus et al., 2008). It is reported that transgenic plants overexpressing pectin methyl esterase (PME) inhibitor accumulated less biomass, implying that de-esterified pectin can be a better substrate to produce biofuel and other commercial goods (Lionetti et al., 2010). Additionally, it is believed that pectin's acetyl substituents promote biomass recalcitrance by hindering the action of pectin-degrading enzymes (Gille and Pauly, 2012). Although deacetylated xylan is the main contributor to the acetate released during plant biomass deconstruction, it partly comes from pectin, therefore acetylated pectins may be a substantial barrier in the conversion of biomass to biofuels (Gille and Pauly, 2012).

Current state of knowledge on the pectin *O*-acetylation pathway

The *O*-acetylation of plant cell wall polymers is primarily mediated by three plant-specific protein families: TRICHOME BIREFRINGENCE-LIKE (TBL), ALTERED XYLOGLUCAN 9 (AXY9), and REDUCED WALL *O*-ACETYLATION (RWA) (Bischoff et al., 2010; Manabe et al., 2013; Schultink et al., 2015). Cell wall polymers isolated from *axy9* mutants showed reduced *O*-acetylation of hemicelluloses such as xylan and xyloglucan; however, pectin *O*-acetylation was unaffected (Schultink et al., 2015). The overall cell wall material of double, triple, and quadruple *rwa* mutant plants had significantly reduced acetyl content of the plant cell wall overall (Manabe et al., 2013). RWA2 and RWA3/4 proteins were reported to be crucial for xyloglucan (XyG) and xylan *O*-acetylation, respectively; however, no specificity for pectins was reported (Manabe et al., 2013). Current models suggest that RWAs are involved in transporting *O*-acetyl donors into the Golgi apparatus, but the identity of the *in vivo* donor and mechanism of action remains enigmatic.

Arabidopsis has 46 TBL proteins organized into five clusters (Bischoff et al., 2010). It has been shown that members of the TBL family catalyze plant polysaccharide *O*-acetylation. Within Clusters-IV and -V of this family, many hemicellulose *O*-acetyltransferases have been reported (Bischoff et al., 2010; Gille et al., 2011; Lunin et al., 2020; Urbanowicz et al., 2014; Zhong et al., 2018). On the other hand, no pectin-specific *O*-acetyltransferases have yet been thoroughly validated. Based on examination of *T*-DNA knockout mutants, several members of TBL Clusters-I and -II have been proposed as containing putative pectin acetyltransferases. RG-I *O*-acetylation levels in cell walls from *tbl10* (Cluster-I) null mutants are low, suggesting that TBL10 may be an RG-I *O*-acetyltransferase (Stranne et al., 2018). A report showing that heterologously expressed TBL44 (Cluster-II) transfers acetyl groups from [^{14}C]-acetyl-CoA to oligogalacturonides remaining at the origin of a paper chromatogram is the strongest evidence yet that TBL44 may be an HG acetyltransferase. However, in this case the radiolabeled products were not tested for endopolygalacturonase sensitivity and alternative acceptors were not evaluated (Chiniquy et al., 2019). Nonetheless, the reduced cell wall acetylation phenotype in *tbl44* knockout mutants provides further support that TBL44 may function as a pectin *O*-acetyltransferase (POAT) (Chiniquy et al., 2019). The cell walls of *tbr* plants (Cluster-I) have been reported to have reduced pectin *O*-esterification (Sinclair et al., 2017). Curiously, the *tbr* mutant also has enhanced pectin methylesterase (PME) activity and decreased levels of pectin methylesterification in cell wall material isolated from hypocotyls. Additionally, higher concentrations of GalA and Rha were found in the trichomes of *tbr*, suggesting that TBR may have a role in the biosynthesis of pectin (Bischoff et al., 2010). More biochemical data, combined with a structurally defined acceptor substrate library, are needed to further functionally define the members of these clades.

Mechanism of pectin *O*-acetylation

Plant growth and responses to biotic and abiotic stress are known to be significantly influenced by acetylation. However, little is understood about the mechanism underlying cell wall polymer *O*-acetylation (Lunin et al., 2020; Pauly and Ramírez, 2018). As mentioned, the TBL and RWA family members may participate in pectin *O*-acetylation. While RWA proteins are multimembrane spanning proteins with ten projected transmembrane domains (TMD) (Manabe et al., 2013), TBL proteins only have one TMD (Bischoff et al., 2010; Lunin et al., 2020; Urbanowicz et al., 2014), further suggesting a potential role of RWA proteins in the translocation of acetyl groups to the Golgi lumen (Manabe et al., 2013).

Based on several studies, TBL proteins are suggested to be polysaccharide *O*-acetyltransferases (Lunin et al., 2020; Pauly and Ramírez, 2018; Zhong et al., 2018). All TBL proteins characterized to date harbor a single transmembrane domain embedded in the Golgi membrane with the C-terminal catalytic domain in the Golgi lumen. The active site contains the conserved TBL (GD**S**) and DUF231 domains (**D**XX**H**) that together form a catalytic triad (Ser-His-Asp) (Bischoff et al., 2010; Lunin et al., 2020). Xylan *O*-acetyltransferase 1 XOAT1 (TBL29) is the most well-characterized *O*-acetyltransferases and is reported to function via a double-displacement bi-bi reaction mechanism (Fig. 2) (Lunin et al., 2020). This enzyme catalyzes the *O*-acetylation of xylan via two stages. First, the unprotonated Asp (**D**xxH) allows His (Dxx**H**) to act as a general base, abstracting a proton from Ser (GD**S**) and driving the nucleophilic attack of Ser on the acetyl moiety of the donor. This results in the formation of an acyl-enzyme intermediate with the acetate covalently attached to the Ser of the catalytic triad (GD**S**). Next, the abstraction of a proton from His by Ser allows the transfer of the proton onto the donor's deprotonated oxygen group facilitating the release of the deacylated donor. In the second step of the reaction, the acetylated Ser intermediate then transfers the acetyl group onto an acceptor substrate that is specified by the enzyme (Fig. 2) (Lunin et al., 2020; Wang et al., 2021). Due to the presence of these conserved motifs in all 46 TBL proteins, it is suggested that members of the TBL family also catalyze the *O*-acetylation of all pectins using the same bi-bi reaction mechanism (Lunin et al., 2020), however, this must be biochemically validated.

Pectin acetylesterases

Pectin acetylesterases (PAEs) hydrolyze acetylester bonds at *O*-2 and/or *O*-3 of GalA residues. Plant PAEs belong to carbohydrate esterase family 13 (CE13) in the Carbohydrate-Active enZYmes Database (CAZY; https://www.cazy.org), while bacterial and fungal PAEs belong to family CE12. Plant PAEs share low sequence similarity with bacterial and fungal PAEs, and the conserved motifs that are part of the catalytic triad in plant PAEs are not found in bacterial or fungal PAEs (Philippe et al., 2017), indicating an early diversification of *PAE genes* during evolution. Most plant species have multiple *PAE* genes. For example, the *Arabidopsis* genome encodes *PAEs* that are expressed in different tissues, developmental stages, and under various environmental stresses (Philippe et al., 2017), suggesting divergent functions of the corresponding enzymes.

Several studies indicated that plant PAEs are involved in tissue elongation, fruit ripening, and stress response. *Arabidopsis pae* knock-out mutants exhibit reduced inflorescence growth (de Souza et al., 2014). Overexpression of the *Populus PAE1* gene in tobacco resulted in shorter styles and filaments, negatively impacted pollen tube elongation, and plants produced a fewer number of abnormal pollen grains (Gou et al., 2012). High *PAE10* expression in apple was associated with shorter fruit

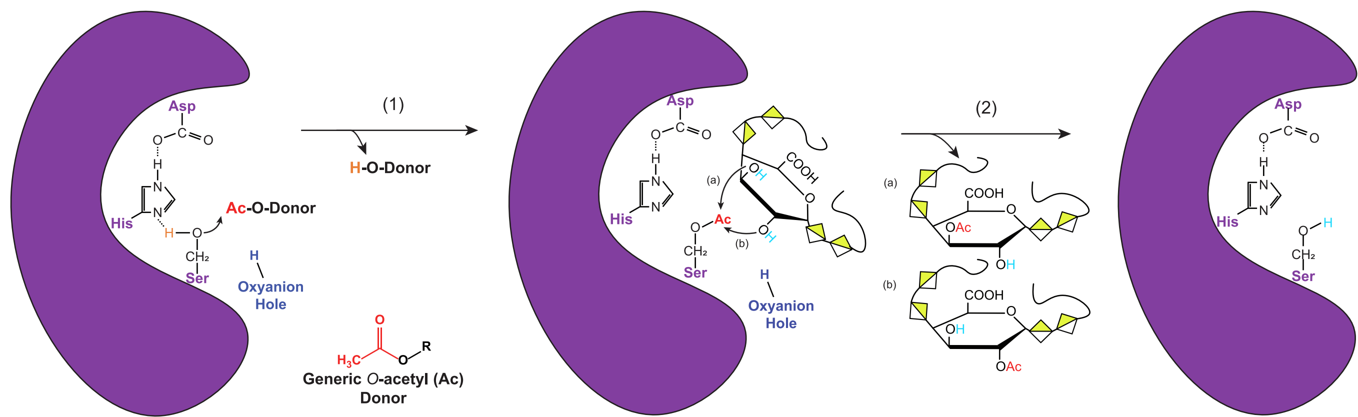

Fig. 2. A plausible mechanism for pectin *O*-acetyl transferases (POATs) is proposed. A catalytic triad consisting of Asp, His, and Ser is present in the highly conserved **D**xx**H** and GD**S**L motifs in plant TBL proteins. In step (1), the Ser residue of the POAT acts as a nucleophile and attacks the acetyl donor substrate, resulting in the formation of an acyl-enzyme (Acetyl-Ser) intermediate. The resulting oxyanion intermediate is stabilized by a positively charged pocket of the POAT enzyme, referred to as the oxyanion hole, coupled with the release of an alcohol coproduct (HO-Donor). In step (2), the C-3 (a) or C-2 (b) hydroxyl group of an acceptor GalA residue attacks the Acetyl-Ser, resulting in formation of a GalA containing acceptor acetylated at *O*-3 or *O*-2, depending on the regiospecificity of the individual enzyme. During these two steps, the Asp residue forms a hydrogen bond with the His residue, making the His residue excessively negative. Thus, the His residue then forms a hydrogen bond with the hydroxyl group on Ser in step (1) and on GalA in step (2), which assists their hydroxyl group to attack the acetyl group on the donor substrate and on the acetyl-enzyme intermediate, respectively. A generic acetyl donor and an HG acceptor are shown for simplicity.

shelf life (Wu et al., 2021). Studies of apple and citrus showed that the expression of *PAE* genes was upregulated in disease-susceptible varieties and downregulated in disease-tolerant varieties (Li et al., 2020; Reim et al., 2022). Aphids established phloem feeding earlier on the *Arabidopsis pae9* knock-out mutant. The expression of genes responding to biotic stress was downregulated in the mutant, as were the levels of defense-related hormones and metabolites (Kloth et al., 2019). *Arabidopsis PAE2* was induced in response to osmotic stress. *PAE2*, *PAE4*, and *PAE8* showed higher expression under salt stress (Philippe et al., 2017). Plant PAEs also appear to have roles in photosynthesis as the *pae11* knock-out mutant showed strong reductions in net CO_2 assimilation (Roig-Oliver et al., 2021).

The substrate specificity of plant PAEs has remained elusive. Mung bean PAE was shown to release acetate from sugar beet, apple and flax pectins (Bordenave et al., 1995). Pectins derived from *Arabidopsis pae8* and *pae9* mutants showed elevated acetate content (de Souza et al., 2014). Sugar composition analysis revealed reduced rhamnose content in pectins derived from *pae8* and *pae8 pae9* mutants, suggesting that RG-I in these mutants could be less extractable and that RG-I might be the substrate of PAE8 and PAE9 in *Arabidopsis*. Overexpressing *PAE9* in the *pae9* mutant reduced cell wall acetate to WT levels, but not lower (de Souza et al., 2014). Taken together, data suggests that pectin deacetylation may be highly regulated and substrate specific, but more work is needed to elucidate substrate specificity.

Open questions in pectin *O*-acetylation

Pectin *O*-acetylation is considered crucial for plant growth and development, but little is known about how it regulates vital processes in the plant cell wall. It is not known why pectin *O*-acetylation differs among various plants, tissues, and developmental stages of the same plant. It is also currently unclear how pectin *O*-acetylation contributes to the plant's defense mechanisms against biotic and abiotic stresses. TBLs may play a role in the *O*-acetylation of pectin; however, the need for biochemical confirmation of enzyme activity remains to be determined and the exact mechanism(s) for catalysis are unknown. Mutant studies suggest that TBLs and PAEs catalyze pectin *O*-acetylation and deacetylation, respectively. In the future, more research is required to establish the precise function, substrate selectivity and regiospecificity of all plant proteins involved in catalyzing the addition and modification of the *O*-acetyl substituents of HG, XGA, RG-I, and RG-II.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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